GSTP1 AND *NQO1* POLYMORPHISMS AS RISK INDICATORS OF LUNG CANCER IN LEBANON

By

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ABSTRACT

GSTP1 AND *NQO1* POLYMORPHISMS AS RISK INDICATORS OF LUNG CANCER IN LEBANON

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Abstract

GSTP1 and *NQO1* gene polymorphisms were considered to be risk indicators of tobacco induced lung cancer. *GSTP1* gene expresses a detoxifying enzyme and its polymorphism leads to a change in the amino acid Ile to Val at position 105 in the amino acid sequence of the enzyme. Thus changing its specificity and activity towards potent carcinogens, like the active benzo(a) pyrene-diol-epoxide (BPDE) present in tobacco. *NQO1* gene expresses a detoxifying enzyme, it protect cells and DNA from both natural and chemical quinones. *NQO1* gene polymorphism at position 609 in DNA sequence is a C-T transition which leads to a change in enzymatic activity to almost null activity. Both polymorphisms have been characterized by restriction fragment length polymorphism (RFLP). The sampled population comprised 195 individuals from a controlled population at both LAU Beirut and Byblos campuses. All

individuals voluntarily gave sputum sample and answered a respiratory health questionnaire. DNA was extracted from each sputum sample and polymerase chain reaction (PCR) and RFLP were performed for each of the genes respectively. Sampled individuals were of two age groups: 107 were under 25 and 88 were over 40. They were equally divided between smokers and non-smokers. For *GSTP1* the genotypic frequency distribution in the total population was: (II) 26.15%, (IV) 60.51% and (VV) 13.33%. While for *NQO1* the genotypic frequency was (CC) 31.28%, (CT) 55.38%, (TT) 14.87%. In the overall population a higher *GSTP1* (II) genotype frequency was determined in smokers while (IV) and (VV) were more frequent in non-smokers. The higher (II) frequency would suggest a lower risk of lung cancer in smokers. The *NQO1* (CC) genotype frequency was highest in smokers while that of (CT) and (TT) was highest in non-smokers, thus indicating a lower risk in smokers. The stratified analysis according to age and smoking showed unreliable and controversial spectrum of susceptibility between age groups which could be due to decrease of sample size with subgrouping

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Lena P. Ahmarani

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LIST OF ABBREVIATIONS

BAP Benzo(*a*) pyrene

BPDE Benzo(a) pyrene-diol-epoxide

COPD Chronic obstructive pulmonary disease

GST Glutathione-S-Transferase

Lung ca or LC Lung cancer

LOH Loss of heterozygosity

NQO1 NAD(P)H: Quinone Oxidoreductase or DT- diaphorase

NSCLC Non-small cell lung carcinoma

PAH Polycyclic aromatic hydrocarbons

PCR Polymerase Chain Reaction

RFLP Restriction Fragment Length Polymorphism

RT-PCR Reverse Transcriptase PCR

SCLC Small-cell lung carcinoma

SNP Single nucleotide polymorphism

"Adopt the pace of nature: her secret is patience".

Ralph Waldo Emerson

I dedicate this work to my loving parents: Pierre and Georgette.

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Chapter 1

INTRODUCTION

The global incidence of cancer is alarming due to rapidly aging population, as cancer is a disease mainly related to aging. In the year 2000 cancer accounted for 10 million new cases and 6 million deaths and 22 million patients. After the year 2020, it is estimated that each year 20 million people will develop cancer and three quarters of those would be living in a region where cancer prevention is available to less than 5%. Lung cancer was the most common cancer in year 2000, 1.2 million new cases (12%) and 1.1 million deaths (18%). Lung cancer is most frequent in men; women incidence rates are generally lower. It is mainly predominant in North America and Eastern Europe and also has moderate high rates in South America, Oceania and in parts of Asia. The major cause of lung cancer is tobacco smoking as incidence rates reflect past history of smoking. The risk of getting lung cancer is 30-fold higher in heavy smokers and it is the cause of 80% of lung cancers in Western countries (Vainio & Haitanen, 2003). Cancer prevention can be made available for most populations, in an approach similar to the attempt in this study to detect population at risk.

GLOBOCAN database contains huge amount of data available in the Descriptive Epidemiology Group of the International Agency for Research on Cancer. According to this database lung cancer in 2002 accounted for highest incidence and death rates among men in Lebanon (Globocan IARC, 2002).

One in ten smokers eventually will have lung cancer (Spivack et al., 2003). Victims of nicotine dependence smokers are constantly exposed to carcinogenic and genotoxic elements associated with tobacco consumption. In

the case of lung cancer, smokers are exposed in a dose-dependent mode to the major carcinogens causing the disease (Hecht, 1999).

The identification of chemical, physical, and biological agents and factors with potential for causing cancer, the potential of gene-environment interaction, increasing knowledge of carcinogenesis pathways and knowledge from human genome projects- all of these enter in the cancer prevention strategy see Appendix I (Figure. A) (Vaino & Haitanen, 2003). The promising strategy is studying the existing differences among individual in detoxification metabolism activity. The detoxification process is divided into three phases and for each phase different enzymes are important in reducing and conjugating and finally producing a form of the toxin, drug or carcinogens that are hydrophilic and ready to be excreted. Enzymes in the detoxification phases can either directly neutralize or activate toxins or carcinogens, activation usually happens in phase I by transforming the toxin into an active intermediate this latter can react with other compound through phase II enzymes action at this level it is conjugated or reduced to less active intermediates, finally either after phase II or phase III enzymes action render the toxin/ carcinogen in a readily hydrophilic, excreted form (Holland & Frei, 2003).

Knowing that not all smokers get cancer it is possible to look for differing characteristics in their genetic make up and try to point out if it could in future prevent some cancers just by studying differences in the ability of individuals to detoxify tobacco or other carcinogens. Alterations in any of these three phases can influence the sensitivity or resistance to a particular drug or xenobiotic toxin see Appendix I (Figure. B) (Holland & Frei, 2003).

The families of Glutathione-S-Transeferase (GST) and NAD(P)H: Quinone Oxidoreductase(NQO) enzymes are phase II detoxification enzymes that catalyze the conjugation of reactive intermediates to less reactive, more

hydrophilic compounds. They are expressed in human lung; this was verified by RT-PCR, immunoblot or immunohistochemistry, and enzyme activity (Spivack et al., 2003).

An extensive amount of polymorphic genes exist with million of nucleotide differences among random haploid genomes including nonexpressed sequence. Multiple alleles of a gene in a population, usually expressing different phenotypes, are called polymorphism. Such polymorphisms are not all associated with different phenotypes, but a fraction of genomic polymorphism could be responsible for subtle and complex effects on disease susceptibility. These polymorphisms could be responsible for susceptibility to hypertension, atherosclerosis, malignancy, psychiatric illness, and infection (Beaudet et al., 1998).

Glutathione-S-Transferase (GST) genes are polymorphic and are considered now to be risk modifiers for cancer (To-Figueras et al., 1999). These genes encodes enzymes highly expressed in lungs and has highest specific enzyme activity towards epoxides like the active benzo(a) pyrene-diol-epoxide (BPDE), a metabolite of tobacco smoke and also towards other reactive intermediates (Nazar-Stewart et al., 2003; To Figueras et al., 1999).

The two commonly expressed *GSTP1* variants showing an altered specific activity and affinities for electrophilic substrates are *GSTP1-a* and *GSTP1-b*. They differ by a single base pair difference A/G transition at nucleotide 313; causing an amino acid substitution from Ile to Val at position 105 nearby the hydrophobic binding site for electrophilic substrates. The Val variant was seen to have a lower activity for BPDE present in tobacco which could, if not detoxified, bind to DNA and maybe enhance some changes that could eventually be a carcinogenesis enhancer (Wang, et al. 2003-b; Watson et al., 1998). Benzo[*a*]pyrene (BaP) is the most extensively studied compound, and its ability to induce lung tumors upon local administration or inhalation is well

documented, its conversion to the active BPDE can cause mutations. It is present in tobacco smoke in high quantity as 20-40 ng /cigarette. As it is present in such high quantity in tobacco the main preventive measure the body can have is to try to get rid of that compound and this is done through detoxification mechanism (Hecht, 1999). However, as some of the detoxifying enzymes mentioned earlier were seen to be polymorphic with differences in their expression, specificity, and activity to detoxify such carcinogens, it is possible to study the risk of being susceptible to such carcinogens by looking for that kind of polymorphism (Wang et al., 2003-b; Watson et al., 1998; Bauer et al., 2003; Nioi & Hayes, 2004).

NAD(P)H: quinone oxidoreductase1 (NQO1) is an obligate two-electron reductase enzyme involved in chemoprotection and also can bioactivate some antitumor quinines. Considerable reports indicate its importance in protecting from both natural and chemical quinones (Ross et al., 2000; Nioi & Hayes, 2004). The major free radical species present in cigarettes was assumed to be a quinone–hydroquinone complex "held in a tar matrix" (Hecht, 1999). However, some quinone are not redox-stable this is why this enzyme has a ambivalent role as it can lead to bioactivation of some quinones and detoxification of others thus facilitating also excretion (Ross et al., 2000). The *NQO1* is polymorphic its variant form is characterized by a C-T base transition mutation at position 609 of the *NQO1* cDNA and this polymorphism is associated with reduced enzymatic activity. This allelic variant referred to as *NQO1-2* was shown to be present in 13–25% of the white population (Chen et al., 1999). As it is a null-phenotype due to reduced enzyme activity in vitro and to no expression of the protein that was seen to be rapidly degraded, it is possible to assess decreased chemoprotective role in-vivo. Thus *NQO1* play a role as possible susceptibility indicator of tobacco induced lung cancer (Ross et al., 2000).

Lung cancer risk exists in a population of healthy individuals due to the fact that a particular genotype will alter the detoxification ability of cells predisposing to lung carcinogenesis. Therefore, studying genetic polymorphisms in tobacco detoxification enzymes/ genes serve different purposes: prevention and treatment (genetic or drug-targeted therapy) (Wang et al., 2003-b; Watson et al., 1998; Bauer et al., 2003; Nioi & Hayes, 2004).

The goal of this study is to screen a controlled LAU Beirut and Byblos campuses population of healthy individuals (non cancer individuals) under two groups'; smokers and non-smokers at two age intervals, less than 25 and over 40, for *GSTP1* and *NQO1* polymorphisms. The study constituted of sample collection, filling out a respiratory disorder questionnaire, extraction of DNA from sputum (phenol/chloroform extraction), Polymerase Chain Reaction (PCR) for *GSTP1* and *NQO1* genes, and then Restriction Fragment Length Polymorphism (RFLP) assay specific for each gene. A descriptive statistical analysis is done by stratified analysis of results obtained from RFLP assay, smoking behavior and different age groups. Finally, results will be compared to those obtained by other researches to obtain different schemes of susceptibility by interpretation of the distributions of those polymorphisms inside the population.

Chapter 2

LITERATURE REVIEW

2.1. Clinical and biological aspects of lung cancer:

In the USA, lung cancer is the leading cause of cancer deaths and in Lebanon it has highest death rates among men when compared with other cancers (Mabry et al., 1998; Globocan IARC, 2002). The impact of disease is paradoxical: it is the most preventable solid tumor, as 90% of patients having the disease have been exposed to tobacco products and almost all of them through cigarette smoking (Mabry et al., 1998). The increase in smoking rates has been mounting dramatically among men since World War I, and followed later by a similar increase in women (Spivack et al., 2003).

The difficulties of defining genetic predisposition to lung cancer, and for making the diagnosis of this disease as well as in treating it, stems from the existence of four histological types of tumors that evolve from bronchial epithelium of smokers. The precise cellular relationships within tumor types remain unknown (Mattson et al., 1987). A large group of lung tumors comprise 75% of tumor carcinomas referred to as non-small cell lung carcinoma (NSCLC). The second group is referred to as small-cell lung carcinoma (SCLC) and comprises the remaining 25% of tumor neoplasms. Each of these forms of cancers is closely related to tobacco smoking (Mattson et al., 1987).

However it is important to note the valuable data from studies of genetic predisposition: only 10% of smokers at risk get lung cancer; eventhough most smokers have in their bronchial epithelium some degree of preneoplastic changes, which are mutations or aberrations that can lead to cancer (Fontana et al., 1984). Diagnosis at age 60 years implies that lung cancer evolves over a

prolonged period of time and involves multiple changes at genetic level. The death rates show that in all types of lung ca. therapeutic approaches are not at their best and this is one of the reasons to follow preventive measures (Mabry et al., 1998). Lung ca progress significantly before symptoms manifest however the common symptoms are there and increase over time: like expectoration and cough (Rom et al., 2000).

The initial diagnosis for each type always starts with clinical evaluation of chronic cough, weight loss, dyspnea, hemoptysis, hoarseness, or chest pain in patients with long history of smoking. A more complex evolution of lung cancer is seen in individual carrying biochemical features of both SCLC and NSCLC (Mabry et al., 1998). In order to study gene defects responsible for the evolution of sporadic and inherited disease it is important that biology and clinical aspects are taken into account. The patterns of disease evolution have been divided into genetic loci abnormalities, altered function of tumor suppressor genes and of oncogenes see Appendix I (Table 1) (Mabry et al., 1998).

2.2. Genetic aspects of lung cancer:

No familial forms of the common types of the disease have been determined. In fact specific genetic loci can be responsible for predisposition to lung tumor and genetic predisposition has a role in determining the risk of lung cancer among smokers (Holland & Frei, 2003).

The inherited pattern of lung cancer is even within a given family complex and many loci could be involved. The incidence of disease of a younger age could be a clue for inherited risk particularly in women as in general its incidence is lowers (Mabry et al., 1998).

The major problem is the huge and complex collection of lung cancer histologies that requires the study of the hereditary factors contribution to each

major subtype see Appendix I (Figure C & D) (Bourke et al., 1992; Larrieu et al., 1985 ; Capewell et al., 1992).

2.2.1. Genetic loci for sporadic forms of lung cancer:

Cytogenetic changes occur both in NSCLC and SCLC karyotypes deletion on chromosome 3 is seen in most SCLC cell lines, and in NSCLC primary tumors 31 clonal karyotypic aberrations are seen like loss of chromosome 9 and 13. Deletion of genetic material is a very common event in human cancers (solid tumors) (Mabry et al., 1998).

These deletions involve loss of heterozygosity (LOH) of the expression of either the maternal or paternal alleles of a gene. If this is accompanied by mutation of the remaining allele, as in the tumor-suppressor gene such as *p53*, an important regulatory mechanism of cell proliferation and differentiation is lost. Loss of heterozygocity is an early event and may occur at the stage of dysplasia or carcinoma in situ. It occurs at different positions within the genome and has certain "hot spots." LOH is detected using molecular genetic techniques such as restriction fragment length polymorphism (RFLP) or polymerase chain reaction (PCR). However the same genes that have undergone LOH in hereditary cancers frequently also undergo LOH in "spontaneous" cancers (Holland & Frei, 2003; Jassem & Jassem, 1998). There's a general pattern for LOH events during LC progression: parental alleles are found at early stages of cancer and in normal proliferating tissue (hyperplasia) but their loss progresses gradually from partial to complete loss with later cancer stages (Rom et al., 2000).

Instability changes in microsatellite repeat sequences have been studied in lung cancers and showed diverse and conflicting results; such changes are more characteristic of NSCLC rather than SCLC although earlier studies indicated the opposite (Jassem & Jassem, 1998).

To date no distinct genetic syndromes for lung cancer have helped identify the genes mutations that specify predisposition but few genes exists for which inherited mutations patterns or inherited polymorphisms could have a role (Mabry et al., 1998).

In genes involved in sporadic lung cancer mutations in the *Ha-ras* gene (rare polymorphism) was seen in sporadic forms of NSCLC and with increased frequency in patients with lung cancer but more evidence is needed (Sugimura et al., 1990; Vineis et al., 1991).

Genetic syndromes for other types of cancer were associated with the occurrence of lung cancer. Li-Fraumeni syndrome and Bloom syndrome, which are both autosomal diseases and known to predispose to different cancers, while bilateral retinoblastoma is a childhood ocular malignancy (Holland & Frei, 2003).

2.2.2. Genes involved in sporadic lung cancer:

Some of the known dominant active tumor suppressor genes and oncogenes have alterations that occur in existing lung cancer and provide insight on the progression steps for these cancers. (Mabry et al., 1998)

Several genes have been identified as being genetically and/or epigenetically altered in lung cancer cells and the timing has been assessed by molecular analyses of cancerous cells at the different stages of cancer (Ohgaki et al., 2003)

2.3. Molecular Epidemiology:

Molecular epidemiology is the result of the confluence of several disciplines. It includes the detection of carcinogen-macromolecular adducts (DNA-addducts as a direct genotoxic measure, and protein-adducts as a substitute), as well as normal DNA sequence variants (heritable variations), and mutations in target genes (somatic changes) (Holland & Frei, 2003).

The first aspect of molecular epidemiology is to investigate gene-environment interactions: chemical carcinogens (from environment or tobacco) once internalized are metabolized to reactive species that cause DNA damage (through carcinogen DNA adducts). Then to study innate ability of DNA repair enzymes which may reduce or ablate the overall damage caused by DNA - bound carcinogens if not repaired genetic changes (mutations, clastogenesis) may occur. The two categorizable genetic traits that are studied: carcinogen metabolism and DNA repair (host factors) (Holland & Frei, 2003).

2.3.1. Mechanism of Tobacco Carcinogenesis:

Nicotine dependence provides the link through which smokers are repeatedly exposed to carcinogenic and genotoxic elements associated with tobacco consumption. In LC, smokers are exposed in dose-dependent fashion to the major carcinogens causing the disease see Appendix I (Figure E) (Hecht et al., 1999).

All known carcinogens in tobacco products require metabolic activation for binding to DNA. There are competing detoxification reactions and the balance between metabolic activation and detoxification in an individual will, to some extent, determine that person's risk when exposed to carcinogens. This balance is determined by individual levels and activities of carcinogen metabolizing enzymes, such as cytochromes P450, glutathione S-transferases, N-acetyltransferases, and uridine diphosphoglucuronosyl transferases. To date the exogenous mechanism of DNA damage remains the valid mechanism for carcinogenesis but there is recognition for both endogenous and exogenous factors in carcinogenesis (Perera & Weinstein, 2000). Multiple enzymes participate in many steps see Appendix I (Figure F).

The major metabolic activation pathway of Benzo(a) Pyrene (BaP) present in tobacco at high concentrations is its conversion to its 7,8-diol-9,10-epoxides (BPDE); one of the four enantiomers is highly carcinogenic and reacts with

DNA to form adducts with N2 of deoxyguanosine. The major metabolic activation pathways of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and its main metabolite,), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL occur by hydroxylation of the carbons adjacent to the N-nitroso group (a-hydroxylation), which leads to the formation of two types of DNA adducts: methyl adducts, such as 7-methyguanine or O6-methylguanine, and pyridyloxobutyl adducts (Hecht, 1999).

Cancer formation is a complex process that involves multiple mutations in different genes. It is against this principle to give a biological or mechanistic role for xenobiotic metabolizing genes in cancer development. As described above most chemicals require activation, which has been referred to as initiation phase the earliest phase in carcinogenesis. To date all research has indicated a very limited role to association between polymorphisms in xenobiotic metabolizing genes (Pelkonen et al., 2003). Benzo(a)pyrene metabolism, which is well characterized represents about 1% of total metabolism of tobacco, it has a major carcinogenic effect (Pelkonen et al., 2003). DNA adducts can also be formed from metabolism of endogenous chemicals such as lipid peroxidation products that can also forms DNA adducts (Guengueric, 2000).

Tobacco smoking causes not only lung cancer but also other cancers of the oral and nasal cavities, oesophagus, larynx, pharynx, pancreas, liver, kidney, stomach, urinary tract and cervix. As tobacco carcinogens exert their effects by interaction of their reactive intermediates with DNA to form DNA adducts and these active intermediates react also with cellular proteins (Philips, 2002). The effects of smoking are evident by the detection of elevated levels of carcinogen–DNA adducts in many human tissues (not directly exposed to tobacco smoke) and of carcinogen–protein adducts in blood. These chemicals

can cause oxidative DNA damage. Smoking is a causative factor in cancers of these sites. The effects of passive smoking, which also causes lung cancer in nonsmokers, is also evident in elevated levels of protein adducts in exposed non-smokers so exposed, relative to non-exposed non-smokers (Philips, 2002). For exposure to tobacco smoke, coupling gas chromatography to mass spectrometry (GC/MS) has provided a tool to measure aromatic amine protein adducts such as 4-aminobiphenyl hemoglobin. These studies have shown a dose-response relationship between the extent of smoking, type of tobacco used, and the adduct levels (Holland & Frei, 2003).

Most often, metabolic polymorphisms were determined by the use of indicator drugs to study alterned expression or activity (eg, caffeine, debrisoquine, dextromethorphan, dapsone, and isoniazid). However, these assays are being replaced by direct genetic assays that allowed the investigation of diverse host factors for which indicator drugs were not available, and it has been applied to a wide variety of cancers, including lung, head, and neck (Holland & Frei, 2003). The 30 years of battle against cancer is partially won as more insight about carcinogenesis and the etiology and protective factors is available. In a way cancer can be preventable nowadays. In this new era of cancer prevention which includes modulation of DNA damage and repair mechanisms: DNA methylation, antioxidant rearranging and oxidative stress modulation, target receptors and signaling pathways, cell cycle controls and checkpoint, and antiangiogenic properties. Increased knowledge will allow using biomarkers having an early role in carcinogenesis and more research on the mechanisms of putative cancer relationship. "The key to the success of this strategy is careful targeting" (Vainio & Hietanen, 2003).

The main goal is to identify risk factors for the disease and its outcome. The variation among humans in carcinogen biodistribution, metabolism, DNA

adducts formation, DNA repair, and potential responses to tumor promoters have important implications in determining cancer risk (Holland & Frei, 2003). When combined with carcinogen bioassays, molecular epidemiology can contribute to cancer risk assessment by: hazard identification, dose-response assessment, exposure assessment and risk characterization see Appendix I (Figure G) (Holland & Frei, 2003).

The interindividual differences in human responses to carcinogens have been illustrated constantly with attention given mainly to heritable polymorphisms in genes involved in carcinogen metabolism. Another potentially important source of interindividual variability in relation to carcinogenesis is DNA repair capacity. Polymorphisms in DNA repair genes have also been extensively studied in order to assess their association with cancer risk (Kiyohara et al., 2002). Tobacco-associated lung cancer has been observed to cluster in some families, prompting further work on genetic determinants of toxin metabolism (Mabry et al.; 1998).

2.4. Genetic Epidemiology:

Cytochrome P450 polymorphisms (CYP), involved in carcinogen activation, and polymorphisms in glutathione-S- transferases (GST), uridine diphosphate (UDP) glucuronosyltransferases, sulfotransferases, and N-acyltransferases (NAT), involved in both carcinogen activation and detoxication, could explain variations in cancer susceptibility among the human population. Evidence that absent protection of a functionally intact *GSTM1* gene correlates with an increased risk of tobacco induced lung cancer. Currently, there is a need for improved epidemiologic study design that integrates DNA adducts measures with indicators of metabolic capacity (Kiyohara et al., 2002). Many DNA-repair genes have been described recently, but relatively few polymorphisms

have been identified. Nevertheless, molecular epidemiologic studies have provided evidence that genetic variation in these attributes can be a human cancer risk factor (Kiyohara et.al, 2002).

2.5. Studies on environmental genotoxic exposure and metabolic genotypes:

Schoket et al. (2001) studied the impact of metabolic genotypes on levels of genotoxic exposure markers. The impact of single metabolic genotypes and their combinations on biomarkers of exposure to carcinogens (DNA adducts) was usually weak if any reported in literature. In this research both chemical exposure and level of DNA adducts, and genetic polymorphisms were studied. In two populations: the first population was Hungarian potroom workers in Aluminum plants and the second population Hungarian patients undergoing pulmonary surgery for lung cancer or other lung disease. Among them were smokers and ex-smokers. Meaning that one population of healthy workers was exposed to polycyclic aromatic hydrocarbons (PAH) in Industrial exposure the second which consisted of lung patients was only exposed to smoking. Interaction between *GSTM1* and *GSTP1* Ile 105 Val genotypes was investigated in association with selected gene polymorphisms of phase I and II detoxification enzymes: *CYP1A1* polymorphism at *MspI* restriction site, *CYP1A1* Ile 462 Val, *CYP1B1* Leu 432 Val, *CYP2C9* Arg 144 Cys, *CYP2C9* Ile 359 Leu and *NQO1* Pro187Ser genotypes. Result of this study showed that DNA adducts levels were high in *GSTP1* Ile Ile position 105 homozygous individuals who had *GSTM1* (null) genotype compared to those who did not have it. The interactions between *GSTM1* and *GSTP1* alleles, in association with particular genotype combinations of *CYPs* also, correlated with increased levels of bronchial aromatic DNA adducts in smoking lung patients.

Georgiadis et al. (2004) made a very important research by studying phase I and phase II enzyme polymorphisms on Lymphocyte DNA adducts in subjects exposed to both air pollution and environmental tobacco smoke. In their results they found out that only *CYP1A1*2A* polymorphism when examined alone had significant effect on bulky DNA adducts during a certain period of time. In *GST* family different alleles combination with *CYP1A1* polymorphisms also showed different amounts of DNA adducts. Seasonal differences were reported in the amount of DNA adducts when having these *GST* variant alleles (V). This could mean that the expression of *GST* genes is related to some qualitative environmental conditions.

Another study showed that genetic polymorphism can modify bladder cancer risk especially with increased tobacco exposure. Also results showed that smoking is more important than genotype as risk factor (Moore et al., 2004).

Esophageal cancer (EC) is the sixth most common cause of cancer mortality worldwide. The incidence of EC is variable among populations and this variation attains 50-fold risk differences between different groups (Sepehr et al., 2004). In a study comparing different Iranian populations they hypothesized that polymorphic allele genes having an etiology in EC could have a role in three populations one found to be at high risk one medium risk and one low risk. They studied allelic differences in Phase I and phase II (*GST*s) drug metabolizing genes and alcohol and aldehyde dehydrogenase and also the DNA repair enzyme. Mostly polymorphisms in *CYP1A1* and in O^6-*MGMT* (DNA repair enzyme gene polymorphism) were found to increase risk (Sepehr et al., 2004).

Liang et al. (2005) studied the association of lung cancer susceptibility with the following single nucleotide polymorphisms (SNPs): in *CYP1B1*, *GSTP1*,

and *hOGG1*. The study was done on the Chinese population of Nanjing, a matched case-control study of 227 patients with lung cancer: studying the variant alleles association with lung cancer susceptibility. The SNPs studied were: *CYP1B1* showing a C-to-G transition at nucleotide 1294 (change of leu to val in codon 432). The *CYP1B1* polymorphism causes an increase in *P53* mutation seen in multiple cancers. And the second SNP studied was *GSTP1* showing A to G -transition at codon 105 (with an amino acid substitution Ile to Val). And another SNP in *hOGG1* (causing Ser 326 Cys) was studied. *CYP1B1* polymorphism studied was found to modulate effect of individual susceptibility of lung cancer among smokers. But for *GSTP1* and *hOGG1* no association with lung cancer risk in the Chinese population studied (Liang et al., 2005).

2.6. GSTP1 Gene Polymorphism:

2.6.1. Glutathione S-Transferases:

Glutathione S-Transferases are multi-gene family of enzymes involved in detoxification by reducing potent drugs or carcinogens through conjugation and in few instances they activate a large variety of chemicals by forming a more active intermediate that can be toxic either directly or indirectly (Hayes & Pulford, 1995). Since their discovery in 1970's many articles concerning them have been published. GSTs catalyze the nucleophilic attack of of reduced glutathione (GSH) with toxic substances forming a less reactive form of these toxic substances and decrease their reactivity with cellular macromolecules. These proteins (GST) are found in almost all living species (Eaton & Bammler, 1999).

The glutathione S-Transferase family of enzyme includes seven GSTs. These enzymes are cytosolic enzymes. In the biochemical classification the cytosolic GST isozymes 5 classes α, μ, σ, π, z are recognized (Strange et al., 2000; Watson et al., 1998). Those enzymes have a dimeric structure, they catalyse

the conjugation of reduced glutathione (GSH) to a variety of electrophiles like arene oxides, unsaturated carbonyls, organic halides and other substrates products of oxidative stress as well as DNA –reactive intermediates (Strange et al., 2000; To-Figueras et al., 1999; Watson et al., 1998).

2.6.2. Glutathione Molecule:

Glutathione is a natural tripeptide found in the body and highly active. It has vital roles in cells: antioxidation, maintenance of redox state and the modulation of immune response and detoxification of xenobiotics. In the cancer case it metabolism can play both pathogenic and protective role. It also has an important role in conferring resistance to chemotherapeutic drugs; thus can protect cells in bone marrow, breast, colon, larynx and lung cancers (Balendiran et al., 2004).

2.6.3. Tobacco, carcinogens and GST enzymes activity:

Tobacco smoke constituents polycyclic aromatic hydrocarbons (PAH), aromatic amines and nitroso compounds contribute to tobacco potency and carcinogenicity (Strange et al., 2000). But these compounds have to become active to cause damage or mutagenesis for this they have to be metabolized to form DNA bound adduct. GST enzymes are phase II detoxifying enzymes that protect cellular macromolecules from damage caused by cytotoxic and carcinogenic agents (Wang et al., 2003; Watson et al., 1998).

Chemical activation to highly reactive species or detoxification reactions could be responsible for individual susceptibility to cancers (Strange et al., 2000). The finding that *GSTP* gene deletion can really alter susceptibility to carcinogenesis in the mouse indicates that individual variability in the expression of this gene also may be an important factor in cancer susceptibility in humans (Henderson et al., 1998).

Berhane et al. (1994) have reported that GSTP1 enzyme is more effective in the detoxification of electrophilic -unsaturated carbonyl compounds produced

by radical reactions, lipid peroxidation, ionizing radiation, and drug metabolism than other GSTs.

2.6.4. *GST* genes:

The association of *GSTP1-B* (Val/Val) allele with cancer risk would be expected since it has lower activity due change in one amino acid at codon 105 from isoleucine to valine. From molecular modeling information it is known that this amino acid site lie in hydrophobic binding site for electrophilic substrates and therefore it affects substrate binding (Harries et al, 1997).

Also results showed that there's more accumulating evidence that polymorphism in glutathione S-transferases associated with cancer susceptibility and *GSTP1* polymorphism maybe of particular importance as it is highly expressed in tissues such as lung, bladder, colon, testicular and prostate tissue (Harries et al., 1997). The null alleles (double mutant) of a number of GST genes result in an increase in the risk of various cancers: lung, bladder, gastric, colorectal, breast, liver and kidney cancer (Balemdiran, 2004). A deletion of the *GSTP1* gene from mice has been reported to result in increased skin tumorigenesis. These findings suggest that *GSTP1* is an important determinant in cancer susceptibility and that its increased synthesis or expression prevent toxic compounds from accumulating in the cells (Usami et al., 2005).

GST genes are polymorphic and are thought now to be risk modifiers for cancer. The two expressed forms: GSTP1 (Ile- 105) and GSTP1 (Val- 105) enzymes have different catalytic activities (To-Figueras et al., 1999). This type of enzyme is highly expressed in lungs and has highest activity and specificity towards epoxides like the active benzo(a) pyrene-diol-epoxide (BPDE) a metabolite of tobacco smoke and also towards other reactive intermediates (Nazar-Stewart et al., 2003; To-Figueras et al.,1999). The properties of the class π-GST isozyme (GST P1) have been studied due to considerable interest

in their relationship to carcinogenesis and association with human cancers (Usami et al.; 2005). GSTP1 is almost undetectable in normal rat hepatocytes and is markedly overexpressed in hepatic foci spontaneously or after treatment with carcinogens. It is the predominant GST isozyme found in human cancer tissue and sometimes used as a putative tumor marker of both rodent and human disease. It is also, the enzyme with the highest activity in the detoxification of carcinogens such as the final carcinogenic metabolites of benzo[a]pyrene (Usami et al.; 2005).

The two commonly expressed GSTP1 variants showing an altered specific activity and affinities for electrophilic substrates are GSTP1-a and GSTP1-b. They differ by a single base pair difference A/G transition at nucleotide 313; causing an amino acid substitution from Ile to Val at position 105 nearby the hydrophobic binding site for electrophilic substrates. The Val variant was seen to have a lower activity for BPDE and considered as mutant form of enzyme (Wang et al., 2003; Watson et al., 1998).

GSTP1 is also over-expressed in tumors and elevated levels are found in tumors of stomach, colon, bladder, oral, breast, and skin. Some cancer models use GSTP1 protein as marker for pre-neoplasia. In addition an increased activity at tumor site has been related to inherent drug resistance however this mechanism has not been elucidated (Harries et al., 1997; Watson et al., 1998).

A 10-fold-inter-individual variation in GST activity has been reported when comparing normal and tumor tissues (Watson et al., 1998).

2.6.5. Studies on *GSTP1* polymorphism association to lung cancer risk:

The *GSTP1* position 105 polymorphic alleles studied are: the wild type is Isoleucine (Ile) - 105 is referred as I and the variant allele is Valine (Val)-105 and is referred to as V. The following table summarizes the different results obtained by studies comparing the genotypes frequency in cancer cases to control healthy population.

Table 2.1: *GSTP1* genotypic frequency distribution in different case-control studies.

	Genotype	Cases (%)	Controls(%)
Harries et.al 1997	*GSTP1* II	43.5%	51%
	GSTP1 IV	45.2%	42.5%
	GSTP1 VV	11.3%	6.5%
Kihara et.al 1999	*GSTP1* II	73.5%	71.6%
	GSTP1 IV	21.8%	25.3%
	GSTP1 VV	4.7%	3.1%
Miller et.al 2002	*GSTP1* II	46%	44%
	GSTP1 IV	42%	46%
	GSTP1 VV	12%	10%
Wang et.al 2003	*GSTP1* II	41.1%	43.4%
	GSTP1 IV	49.2%	46.1%
	GSTP1 VV	9.7%	10.5%
Nazar-Stewart et.al 2003	*GSTP1* II	42%	40.9%
	GSTP1 IV	46.6%	48%
	GSTP1 VV	11.4%	11.1%
Schneider et.al 2004	*GSTP1* II	44.4%	47.9%
	GSTP1 IV	41.7%	40.8%
	GSTP1 VV	13.9%	11.3
Chan-Yeung et.al 2004	*GSTP1* VV	34.6%	33.3%
Skuladottir et.al 2005	*GSTP1* II	47.9%	46.25%
	GSTP1 IV+VV	52.1%	53.74%

As seen in table 2.1 studies of different populations showed that the %(II) genotype frequency decreases in cases as opposed to controls whereas the % (VV) frequency increases in lung cancer cases and the opposite is seen in controls; the percent increase of (VV) frequency in cases is around 1% in most studies. The prevalence of *GSTP1* genotypes is (II): 34%-73.5% and (IV): 21%-46% and (VV): 3%-13% (Harries et.al, 1997; Wang et al., 2003 Schneider et al., 2004).

The *GSTP1* (VV) and *GSTM1* null genotypes have been linked to bronchitis and asthma and these genotypes were related to slower growth of children. Especially when associated with asthma meaning that children having one of the above genotype had lower lung function and lower ability to protect themselves from harming substances and from oxidative stress (Gilliland et al., 2002). The Respiratory questionnaire will aid in studying any link between genotype studied and general respiratory health.

Polymorphisms in some detoxification enzymes are thought to increase the risk of developing chronic obstructive pulmonary disease (COPD), but the ultimate role of genetic variability in antioxidant and/or detoxification enzymes in COPD remains obscure (Kinnula, 2005).

All of these facts suggest that genetic polymorphism of *GSTP1* is important in cancer etiology and therapy.

2.7. *NQO1* polymorphism:

2.7.1. NQO1 enzyme:

NQO1 is a 2- or 4-electron reductase that maintains quinones and their derivatives in a reduced state where they can more readily be conjugated and then excreted. An *NQO1* polymorphism at position 609 in exon 6 (C609T) in

the human *NQO1* gene results in a proline to serine substitution at position 187 in the amino acid sequence of the NQO1 protein, resulting in loss of enzyme activity. The *NQO1* polymorphism frequency varies among ethnic groups, for example, 4% in Caucasians versus 22% in Chinese (Bauer et al., 2003).

The NQO1 enzyme formerly referred to as DT-diaphorase, is an important enzyme in the metabolism of xenobiotics. NQO1 can be a detoxification or an activation enzyme, depending on the substrate. It catalyzes the two-electron reduction of quinoid compounds to the readily excreted hydroquinones, preventing the generation of free radicals and reactive oxygen, thus protecting cells from oxidative damage (Chen et al., 1999; Xu et al., 2001).

2.7.2. NQO1 and P53:

According to a recent finding on mechanism of genetic susceptibility it has been demonstrated that NQO1 happens to stabilize p53 protein (*P53* tumor suppressor gene expression) especially in response to oxidative stress (Asher et al., 2001).

Given the wide range of cancers that have been associated with a lack of NQO1 due to the *NQO1* polymorphism, it is conceivable that NQO1 is functioning via a non-catalytic mechanism to protect against neoplasia (Anwar et al., 2003).

2.7.3. *NQO1* polymorphism and activity:

NQO1 enzyme has an important role in detoxification of benzene metabolite qunones. A polymorphism in *NQO1*, a C609T substitution, has been identified, and individuals homozygous for this change (TT) have no detectable NQO1 which for that purpose was considered as the mutant form or null allele. In addition exposed workers to benzene with a (TT) genotype could have an increased risk of benzene hematotoxicity leading to leukemia (Bauer

et al., 2003). In a study on exposure to a noncytotoxic concentration of benzene metabolite hydroquinone (HQ), it was found that such exposure: induce both NQO1 and soluble thiols, and protected against HQ-induced apoptosis. Failure to induce functional NQO1 in mutant (TT) bone marrow cells when compared to induction of normal NQO1 protein in both heterozygous (CT) and homozygous (CC) bone marrow cells; has suggested an increased risk of benzene poisoning in individuals homozygous for the *NQO1* (T) allele (Moran et al., 1999). Multiple detoxification systems, including NQO1 and glutathione protect against benzene metabolite-induced toxicity (Wolf, 2001).

2.7.4. *NQO1* polymorphism and Cancer:

Considerable evidence is accumulating from epidemiological studies showing that NQO1 protects against tumorigenesis (Nioi & Hayes, 2004). The variant form of NQO1 examined in vitro has less than 4% the catalytic activity of wild-type. In vivo loss of activity increases as protein is less stable and studies on degradation showed that the mutant degradation is faster and happens through ubiquitin/proteasome pathway (Siegel et al., 2001). The variations of the mutant allele in a population differ with ethnic origins from 4-20%. *NQO1(T)* mutant allele has been linked to increased urothelial tumours, cutaneous basal cell carcinoma, pediatric leukaemia, colorectal cancer, oesophageal squamous cell carcinoma, gastric cardiac carcinoma (Nioi & Hayes, 2004). *NQO1* wild type are those having C-609 in DNA sequence the variant or mutant are those having T-609 in DNA sequence.The *NQO1* genotypic frequency prevalence in most populations studied: (CC) and (CT) vary from 35%-65% and (TT) from 4%-25% this last varies with ethnic origin. The actual relation to cancer predisposition has been questioned as some

studies found that (TT) genotype has a protective role (Chen et al., 1999; Lin et al., 2003).

Another study conducted by Xu et al. (2001), showed that (TT) is a predisposing factor for lung cancer especially when associated with smoking (heavy smokers) and former heavy smokers (smoked intensively for a short period of time). This study was pioneering study due to its large sample size 1900 individuals and because it took in account smoking behaviors (Xu et al., 2001). Some studies even showed no relationship between NQO1 polymorphism and lung cancer (Lewis et al., 2001; Yin et al., 2001).

From the available studies NQO1 is thought to have an important role in protecting or activating carcinogens present in tobacco and studying the genotypic distribution can possibly give an idea about its activity and eventually can help in risk analysis (Bauer et al., 2003; Moran et al., 1999; Nioi & Hayes, 2004; Xu et al., 2001).

After taking into account most of the research studies made on the *GSTP1* polymorphism and *NQO1* polymorphism: revealed that the varying genotypes in a particular healthy population can be a possible indication towards assessing the risk of lung cancer and the actual variation of these genotypes in that population. Individuals having these variant *GSTP1* or *NQO1* genotypes have reduced GSTP1 and NQO1 respective activities (Siegel et al., 2001; Harries et al., 1997). Therefore they would be at higher risk due to lower detoxification of carcinogens and mutagenic compounds of tobacco metabolism such as BPDE detoxified by GSTP1 and B(a)P -3,6- quinone detoxified by NQO1 and these chemicals can cause mutations by binding directly to DNA (Wang et al., 2003-b; Ross et al., 2000).

Chapter 3

MATERIALS AND METHODS

3.1. Sample collection:

The research project began in February 2004 and sputum samples were collected between April and July 2004. The sampling was done according to a statistical design that recommended collecting 200 samples from a controlled population present at the LAU campuses (Byblos and Beirut) and individuals within two age groups under 25 and over 40.

According to the statistical design 8 groups of individuals were distributed into 25 per group as illustrated in Table 3.1.

Table 3.1: Categories of individuals sampled for the study

Smokers N=100	f<25yrs n=25	f>40yrs n=25	m<25yrs n=25	m>40yrs n=25
Non-smokers N=100	f<25yrs n=25	f>40yrs n=25	m<25yrs n=25	m>40yrs n=25

(f:females, m:males, N: total number of individuals & n:number of individuals per group)

Samples of phlegm were collected after forced expectoration in labeled sterile urine containers and were stored at -20 °C until extraction.

Following the theoretical sampling design described in table 3.1 the sampling was done randomly and required the signed consent of individuals to participate in the study by answering a specially designed questionnaire about the donor's respiratory health (Xu et al., 2001). A sample copy is shown in Appendix III.

The questionnaire required answering 10 sections related to age, gender then symptoms related to respiratory problems e.g. cough and phlegm or episodes of wheezing, breathlessness, chest colds, chest illnesses, specific lung infection (bronchitis, pneumonia, hay fever, chronic bronchitis, emphysema or asthma). After completion and signing of the questionnaire individuals were asked to give a sputum sample. The process of sputum release is difficult for a healthy individual and requires specific instructions.

3.2. DNA extraction from sputum (adapted from cell culture extraction):

According to method outlined in ES cell DNA extraction: tube method (Hedrick Lab protocols).

Samples were thawed in duplicate (250 µl of aliquots) were put in an eppendorf tube and then 250 µl of lysis buffer added to each tube containing the sample. The lysis Buffer stock solution contained 10 mM Tris-HCl pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5% (w/v) sarkosyl (N-laurylsarcosine, Sigma #L-9150), and 1 mg/ml Proteinase K (AB gene, AB-0504, United Kingdom) that is added to lysis buffer fresh each time.

The mixtures were then incubated at 60°C for 2 hours in eppendorf Thermomixer comfort, Eppendorf Germany after which tubes were removed and then were added 250 µl of the Phenol/Chloroform/Amyl solution (in a ratio of 25:24:1) to each tube and mixed by tapping gently the tubes for around 1min until an emulsion was formed. Centrifugation (Heraeus Sepatech 13R Biofuge, Germany) was done at room temperature until phases were well separated then the aqueous phase was transferred to a new tube. And

phenol/chloroform/amyl extraction was repeated until no more protein phase (white thick phase between organic and aqueous phases) was seen at interface of aqueous and organic phases.

Then to the aqueous phase obtained 250 µl of chloroform were added and mixed then centrifuged, afterward the aqueous phase was removed to a new tube and the extraction with chloroform was repeated two or three times to remove phenol traces.

Then 10 µl of 5 M NaCl were added to each of aqueous samples obtained and mixed well followed by addition of 500 µl of ice cold ethanol and additional mixing. The ethanolic solution obtained is placed on ice for 30 min. Then the solutions were centrifuged at 12,000 x g for 10min at 0°C - 4°C. The supernatant was discarded carefully in order not to disturb the DNA-pellet. Finally tubes were half filled with 70% Ethanol and were spun for 2 min at 4°C. The supernatant was pipetted and discarded and tube was left for fifteen minute to air dry. The DNA was finally dissolved in 30 µl of TE buffer (10 mM Tris, 10 mM EDTA, pH=8.0): by pipetting the TE buffer on the walls of tube in order not to disturb the pellet then the samples were left to dissolve at least 1 hour at 37 °C.

3.3. *GSTP1* polymorphism analysis:

The polymorphism analysis was done according to the method described by To-Figueras et al. (1999).

After DNA extraction each sample was assayed for *GSTP1* polymorphism and PCR mix preparation was done for a 20 µl volume: 50-60 ng of DNA sample to which were added: PCR Buffer (AB gene) 1 X, $MgCl_2$ (AB gene) 1.5 mM, dNTPs (AB gene) each 200 mM, primers GSTP1-F 8 pmol, and GSTP1-B 8 pmol (Thermo electron Corporation, USA), DNase free water was added to obtain 20 µl total volume, Taq polymerase (AB gene) 0.5 u.

3.3.1. PCR cycling condition:

The PCR reactions were performed in a thermal cycler (Perkin Elmer 2400, CT, USA) under the following cycling conditions for each reaction: first denaturing step 11 min at 95°C; pause add Taq polymerase then resume, followed by 32 cycles of three temperature each cycle: denaturation 95°C for 50 sec, annealing 54°C for 50 sec, elongation 72°C for 50 sec, ended by additional final elongation step at 72°C for 5 min.

The PCR primers sequences:

Table 3.2: *GSTP1*, PCR primers sequences

GSTP1 forward	5'-ACC CCA GGG CTC TAT GCG AA-3'
GSTP1 reverse	5'-TGA GGG CAC AAG AAG CCC CT-3'

3.3.2. Restriction analysis for *GSTP1*:

Done according to method outlined in To-Figueras et al. (1999). BsmAI (10 u/ µl) cuts at site of *GSTP1* gene in Exon 5 at A to G transition of position 313 of nucleotide sequence resulting in Ile to Val at position 105 in amino acid sequence.

Sequence: GTCTC is recognized by restriction enzyme when there is a G transition at position 313 in *GSTP1* gene sequence.

Restriction site: GTCTCN↑NNNN

CAGAGNNNNN↑

The PCR product (10 µl) were incubated at 37°C for 16 hours with 10 u of BsmAI (Fermentas, Life Sciences, USA) and 1 X enzyme buffer and 7 µl water (DNase free).

Restriction analysis for *GSTP1* yields in (II) genotype (uncut 176 bp fragment) for (IV) genotype (176 bp, 91 bp, and 85 bp fragments) and (VV) genotype (91 bp, 85 bp fragments) (Harries et al., 1997) .

3.4. *NQO1* polymorphism analysis:

NQO1 polymorphism analysis was done according to method done by Sarbia et al. (2003). PCR mix preparation for a 30 µl volume: Sample DNA 50ng, DNase free water 17.6 µl, PCR Buffer (AB gene) 1X, MgCl2 (AB gene) 1.5 mM, dNTPs (AB gene) each 10 mM, and primers NQO1- F 10 pmol and NQO1- B 10 pmol (Thermo electron corporation), Taq polymerase (AB gene) 1U.

3.4.1. PCR cycling condition:

The PCR reactions were performed in a thermal cycler (Perkin Elmer 2400, CT, USA) under the following cycling conditions for each reaction:

first a denaturation step for 14 min at 95°C then pause, hot start add Taq Polymerase, resume followed by 40 cycles of 3 temperatures each: 94°C for 1 min, 56°C for 1 min, 72°C for 2 min.

Table 3.3: *NQO1*, PCR primers sequences

NQO1 forward	5'-AAG CCC AGA CCA ACT TCT -3'
NQO1 reverse	5'-ATT TGA ATT CGG GCG TCT GCT G-3'

3.4.2. Restriction Analysis *NQO1:* Done according to method outlined in Sarbia et al. (2003). HinfI (10U/µl) cuts at restriction site of *NQO1* gene C609T: recognizes sequence GANTC and cut at if T is present instead of C at position 609 in the *NQO1* gene sequence.

Restriction site: G↑ANTC

CTNA↑G

PCR product (10 µl) was incubated for 16 hours at 37°C with 40 U of HinfI (Fermentas, LIFE SCIENCES).

NQO1 gene product uncut fragment is 172bp fragment, the heterozygote (CT) possess two bands one corresponding to the uncut (C) at 172 bp and one to the cut (T) alleles at 100 bp. The homozygote having the two variants (T) allele will have only one band at around 100 bp.

3.5. Gel Electrophoresis:

After restriction digestion was performed for each sample 3 µl of restriction digest product were mixed with 3 µl of gel loading buffer and loaded in 2.5% Midi ABgarose (AB gene, AG-0300/b) with EtBr (0.05%) added to gel and run in a gel submarine (Pharmacia Biotech GNA 200) at the conditions 80mA, 20 watts for 40 min and then gels were viewed in UV transilluminator (UVP) and documented with Olympus digital camera.

3.6. Statistical Analysis:

After performing restriction fragment length polymorphism (RFLP) assay, data relative to each sample (genotypes and questionnaire data) were recorded in Excel spreadsheets. The "simple descriptive statistical analysis" was done using Excel program on all results obtained from genotypic analysis and questionnaire. Results were then subject to stratified analysis of different variables (genotypes) to smoking and then results were plotted for comparison. A normal distribution plot for variant genotype was done to provide an estimate about the deviation from mean of expected results (SPSS 12 program). A chi-square test (Excel program) was done to study if allele distributions were in Hardy-Weinberg equilibrium (Russel, 1998).

Chapter 4

RESULTS

4.1. Sampling:

The samples were collected at LAU campuses: from April to June in Byblos campus and from June to July at Beirut campus. Around 20-40 samples were collected each week. Some samples were not used (discarded) because the sputum was insufficient for extraction and some did not yield the necessary DNA concentration to obtain a result in both gene polymorphisms assays. Those that gave no results were around 5% of the total 213 samples collected and the others were the discarded portion to finally obtain results for 195 samples.

4.2. Questionnaire:

Smokers reported more symptoms of coughing and phlegm or episodes of both (related or not to having a cold) especially in the over 40 age group (50% more than non-smokers). In the overall population (195 individuals) 10 non-smokers and 12 smokers' students reported: they have had an unusual bronchitis or respiratory infection even requiring hospitalization in their childhood or adolescence.

Two individuals reported that their mothers have had lung cancer and one of them died of lung cancer the other has been operated for lung cancer. The one that died from lung cancer was a non-smoker, as voluntarily declared by the

sampled individual, whereas the other, no information was obtained about her smoking history as no such question is found in the questionnaire.

4.3. Categories of sampled individuals:

The different categories of the overall 195 individuals studied are illustrated in figure 4.1. In this population 107(54.87%) persons were under 25 and 88 (45.13%) were over 40. Of these 101(51.79%) were males and 94 (48.21%) were females. The number of smokers was 101(51.79%) and 94(48.21%) non smokers. The smoker groups consisted of 43 (42.57%) females and 58 (57.43%) males. The non-smoker group consistéd of 51(54.26%) females and 43(45.74%) males.

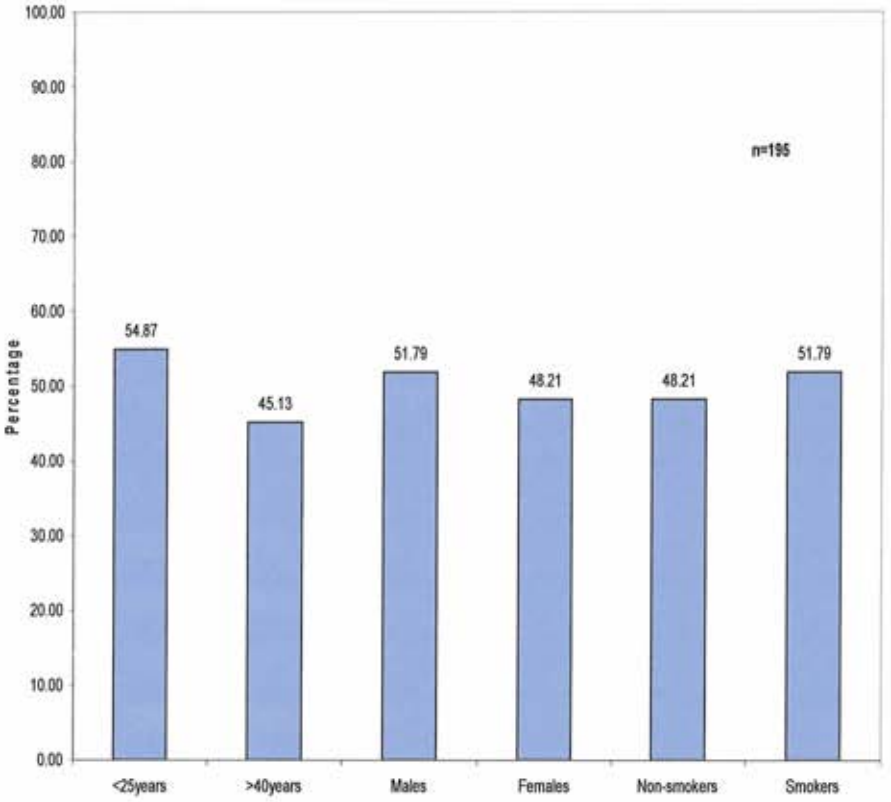


Figure.4.1: Categories (%) of sampled individuals.

4.4. Determination of genotypic frequency:

The DNA was extracted from samples and restriction fragment length polymorphism (RFLP) assay was done for each gene on each of the samples obtained. The *GSTP1* polymorphism yield an amino acid change from Isoleucine to Valine so the possible genotypes are referred to as (II), (IV) and (VV) (Harries et al., 1997). The *NQO1* polymorphism is a C-T transition and genotypes can be designated to as (CC), (CT) or (TT) (Chen et al., 1999).

A sample of gel illustrations for *GSTP1* polymorphism is shown in figure.4.2.

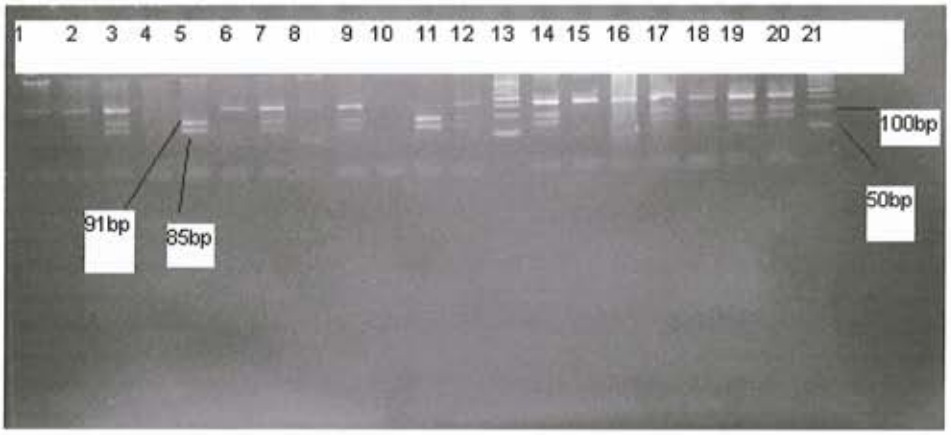


Figure.4.2: *GSTP1* polymorphism after RFLP assay results Gel- Lane1 genotype (II), lane 3 genotype (IV), lane 5 genotype (VV), Ladder 50bp in Lane13 and 21.

The following figure is a sample of *NQO1* gel electrophoresis done after Restriction Digestion with HinfI .

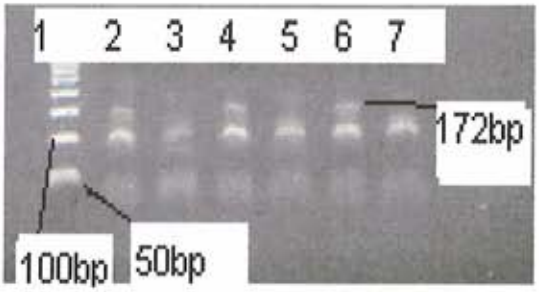


Figure.4.3: *NQO1* RFLP gel: Lane 1 Ladder 50bp, Lane (2, 4, 5, 6) have (CT) genotype, Lane (3) (TT) genotype.

The RFLP results gave the distribution of these respective genotypes in the population studied as illustrated in figure 4.4 For *GSTP1* gene the percentage frequency of each genotype in the overall population was the following (II) 26.15%, (IV) 60.51% and (VV) 13.33%. For *NQO1* gene the percentage frequency of each genotype in overall population was (CC) 31.28%, (CT) 55.38%, (TT) 14.87%. See Figure. 4.4.

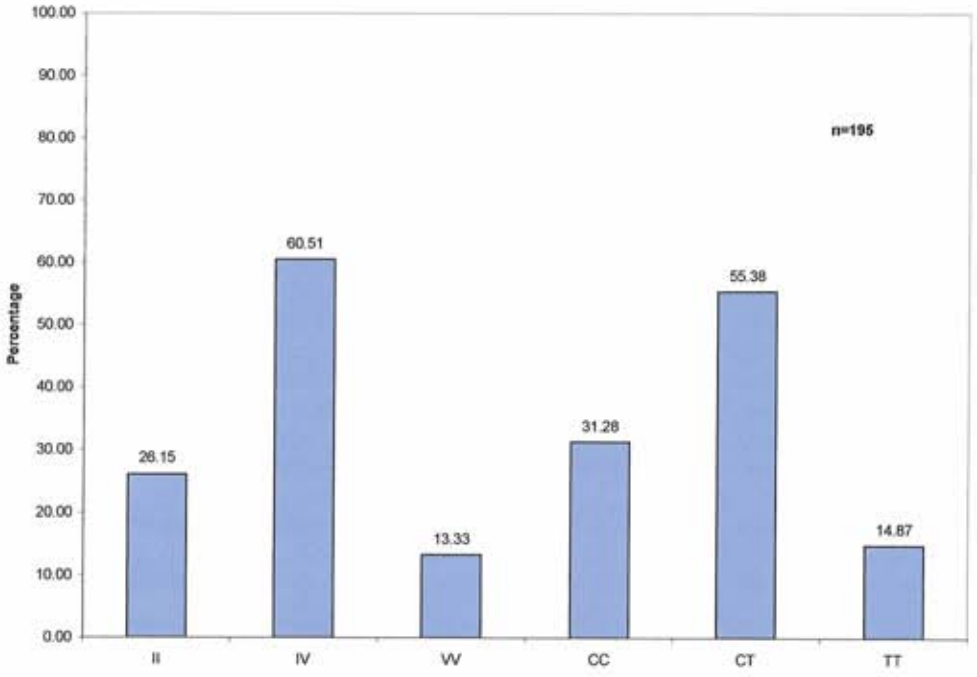


Figure.4.4: Genotypic frequency determination in the overall population. (*GSTP1* genotypic frequencies indicated as % (II), and % (IV) and % (VV); and *NQO1*genotypic frequencies referred to as % (CC), % (CT) and % (TT).

4.5. Genotypic distribution comparison between smokers and non smokers in the overall population studied:

As illustrated in figure 4.5 the *GSTP1* genotypic distribution comparison between smokers and non smokers showed: the frequency of (II) 29.7% of smokers versus 22.34% of non-smokers, and that of (IV) in 57.43% of smokers and 63.83% of non-smokers, as for (VV) found in 12.87% of smokers and 13.87% nonsmokers. The *NQO1* polymorphisms distributions comparison: frequency (CC) was found in 28.71% of smokers and 32.98% of non-smokers, frequency of (CT) in 55.45% of smokers and 53.19% of non-smokers whereas for (TT) in 15.84% of smokers versus 13.83% of non-smokers.

In the overall population *GSTP1* (II) genotype frequency was slightly higher in smokers versus non-smokers whereas *GSTP1* (IV) and (VV) frequencies were higher in non-smokers versus smokers. In *NQO1* genotypic distribution comparison showed that (CT) and (TT) genotypes' percentages of distribution were slightly higher in non-smokers versus smokers whereas (CC) higher in smokers. It is important to note that there is a higher frequency of male smokers versus female smokers in the sampled population.

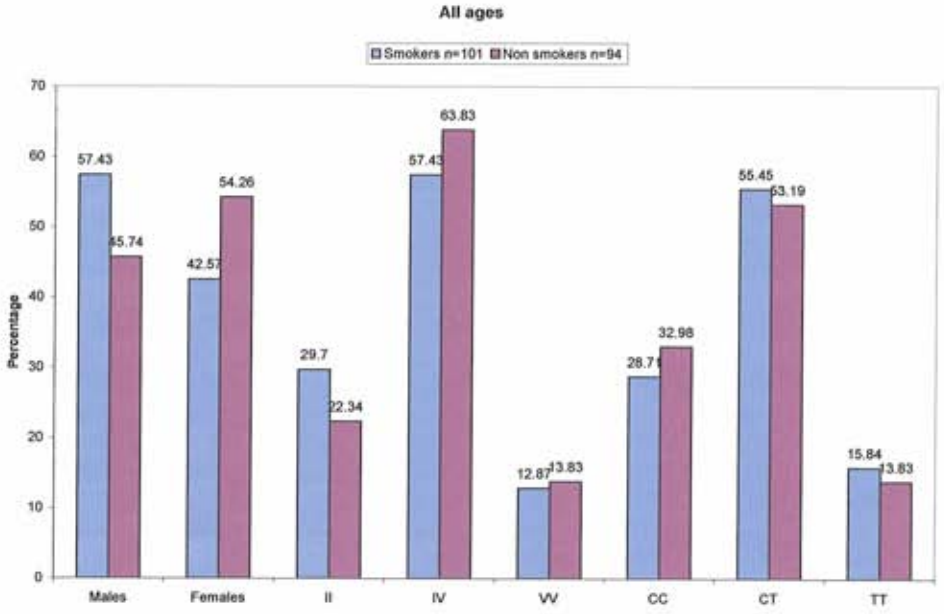


Figure.4.5: Genotypic frequency distribution comparison between smokers and non smokers in the overall population studied. (Symbols as in Figure. 4.4).

4.5.1. Comparison of genotypic distribution between smokers and non-smokers in the age group under 25 years:

As illustrated in Figure. 4.6 the comparison of *GSTP1* and *NQO1* genotypic distribution between smokers and non-smokers in this age group were as follows for the *GSTP1* genotypic distribution: (II) genotype frequency was 31.58 % of smokers versus 30% of non-smokers, and (IV) frequency was 50.88% in smokers and 52 % in non-smokers; also 17.54% of smokers had (VV) genotype versus 18% of non-smokers. The *NQO1* genotypic distribution comparison between smokers and non smokers showed: 40.35% of smokers having (CC) genotype versus the 38 % of non-smokers, and 49.12% of

smokers having (CT) genotype versus 48% of non-smokers; also in that group 10.53% of smokers had (TT) genotype versus 14% of non-smokers.

In the under 25 years old age group, the comparison between smokers and non-smokers showed for *GSTP1* that the (II) genotype frequency similarly to what was observed in the overall population was slightly higher in smokers versus non-smokers; however the (IV) and (VV) frequencies in the non-smokers were slightly higher than what was seen in smokers. Also for *NQO1* in this group the (CC) and (CT) genotypic frequencies were slightly higher in smokers versus non-smokers and (TT) genotype frequency was higher in non-smokers versus smokers.

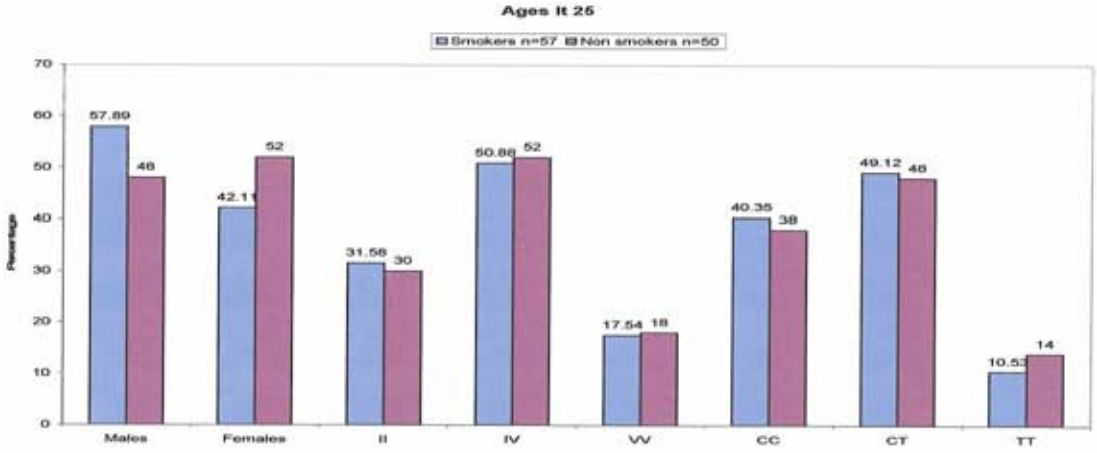


Figure.4.6: Genotypic distribution comparisons between smokers and non smokers in the under 25 age group. (Symbols as in figure 4.4)

4.5.2. Comparison of genotypic distributions between smokers and non smokers in the over 40 age group:

The comparison of the *GSTP1* and *NQO1* genotypic distribution between smokers and non-smokers was as presented in figure 4.7. The *GSTP1* genotypic frequency distributions in the over 40 age group were: 27.27% of smokers had (II) genotype as opposed to 13.64% of non-smokers, for (IV) genotype 65.91% of smokers had it versus 77.27% of non-smokers and the (VV) genotype was observed in 6.82% of smokers versus 9.09% of non smokers. The comparison between *NQO1* genotypic distribution in this group showed for (CC) genotype 13.64% of smokers versus 27.27% of non-smokers, and for the (CT) genotype 63.64% of smokers in comparison to the 59.09% of non- smokers while (TT) genotype was seen in 22.73% of smokers 1.6-fold higher than the 13.64% of non-smokers.

Summarizing the *GSTP*1 genotypic distribution comparison in this age group it is established that (II) genotype frequency is higher in smokers versus non-smokers; the (IV) genotype frequency higher in non-smokers versus smokers while (VV) frequency slightly higher in non-smokers versus smokers. And for *NQO1* genotypes in this age group the (CC) genotype frequency was higher in non-smokers versus smokers and (CT) and (TT) genotypes frequencies were higher in smokers versus non-smokers.

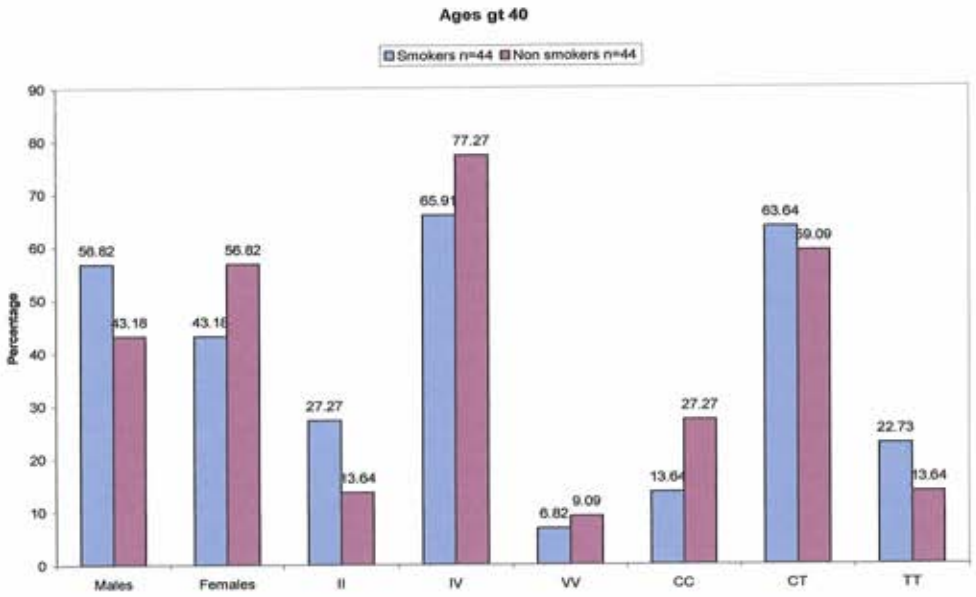
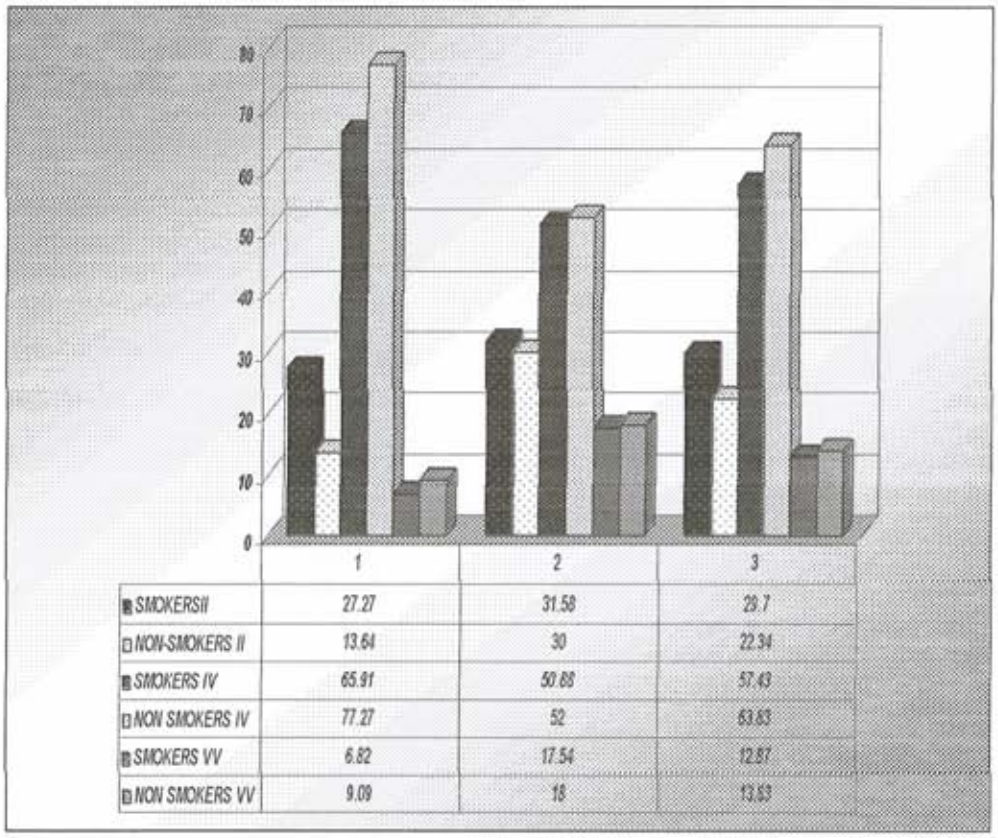


Figure.4.7: Genotypic distribution comparisons between smokers and non smokers in the over 40 age group.(Symbols as indicated figure 4 .4)

4.6. Comparison of *GSTP1* genotypic frequencies between age groups and according to smoking behavior:

As seen in figure 4.8 there is a large difference in genotypic distribution between the two age groups 1 (over 40) and 2 (under 25). The frequencies of *GSTP1* genotypes (II) and (IV) are highest in the over 40 age group whereas (VV) is highest in the under 25 age group and was almost two fold higher than the frequency of (VV) genotype in the over 40 age group. However, inside the same group there was no such difference in the frequencies except for (IV) frequency in the over 40 age group of smokers when compared to non-smokers. Finally the overall population (group 3) showed a striking difference between (II) genotype frequency of smokers as compared to that of non-smokers.



	1	2	3
SMOKERSII	27.27	31.58	29.7
NON-SMOKERS II	13.64	30	22.34
SMOKERS IV	65.91	50.88	57.43
NON SMOKERS IV	77.27	52	63.63
SMOKERS VV	6.82	17.54	12.87
NON SMOKERS VV	9.09	18	13.53

Figure.4.8: Comparison of *GSTP1* genotypic frequencies between groups according to smoking behavior. (Groups are referred to as 1 over 40 age group, 2 under 25 age group and 3 the overall population)

4.7. Comparison of *NQO1* genotypic frequencies between age groups and according to smoking behavior:

Figure. 4 .9 shows the significant difference in genotypic frequencies when comparing between group 1 and group 2. The *NQO1* genotypic distributions comparison between age groups showed that (CC) frequency was highest in group 2 in both smokers and non-smokers. While the highest frequencies of (CT) and (TT) genotypes were observed in group1 smokers and non-smokers. And almost equal results for (TT) genotype frequency in both age groups.

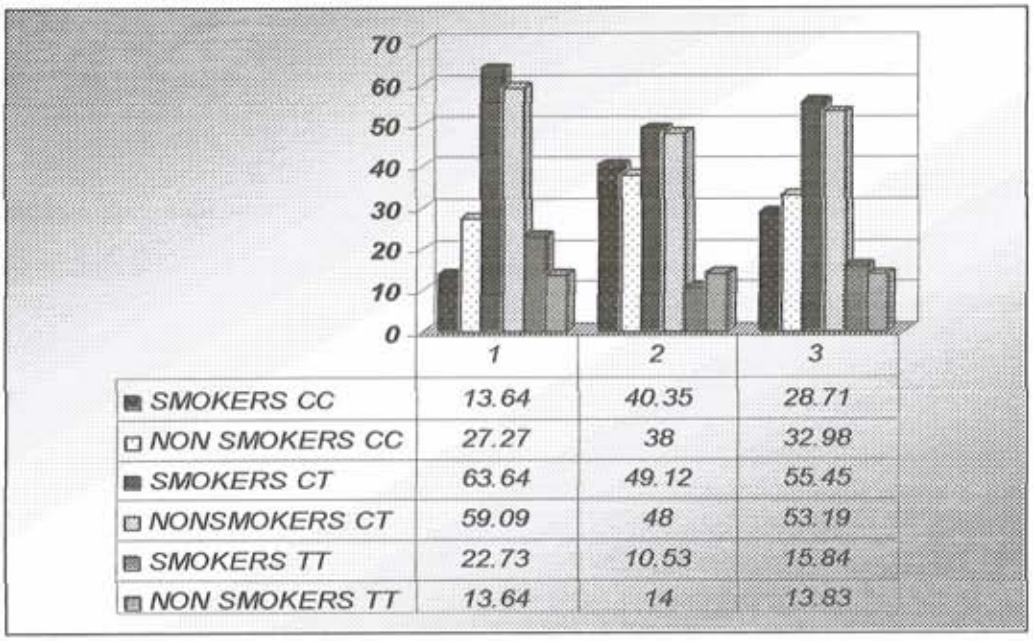


Figure.4.9: Comparison of *NQO1* (%) genotypic frequencies between groups according to smoking behavior. (Groups are referred to as in figure 4.8)

4.8. Respiratory Health Results and *GSTP1* polymorphism:

In individuals who had respiratory troubles and were smokers studying *GSTP1* genotypic association showed that only 1 had (VV) genotype and the actual healing from that person's bronchitis required one month. The distribution of *GSTP1* genotypes in this smokers group was: (II) 6 /12 and (IV) 5/12 and (VV) 1/12. Similar results were seen in the non-smoker group were only 1/10 had the (VV) genotype and that individual had asthma. Among these pulmonary disease cases one reported having had pneumonia. This 22 years-old individual who had (IV) genotype was a heavy smoker who started smoking at 13 yrs old and smoked 2 packs per day.

4.9.1. Normal Distribution of Variant genotypes:

The normal distribution of (VV) and (TT) genotypes frequencies in the different groups showed in the following histograms with their actual deviation from mean of expected values and this is represented in figures 4.10 &4. 11.

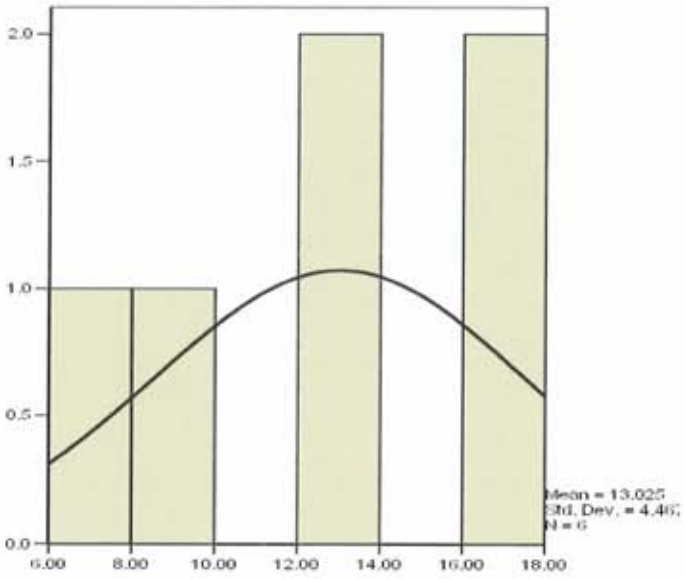


Figure. 4.10: Variation of frequency of (VV) genotype from mean of expected values.

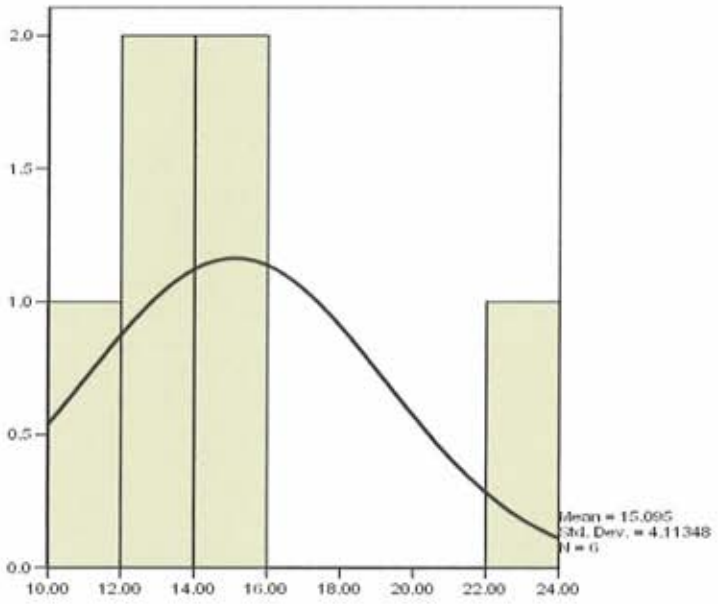


Figure.4.11: Normal distribution of frequency of (TT) genotype and deviation from mean of expected values.

4.9.2. Hardy-Weinberg Equilibrium fit- test:

Hardy-Weinberg law can be used as null hypothesis to which the genetic structure of any particular population can be compared. The expected genotypes are calculated using Hardy –Weinberg proportions (p^2, 2 pq and q^2) where p = [2 x (number of homozygotes) + (number of heterozygotes)]/ (2x total number of individuals) and q =1-p. Eg. The *GSTP1* genotypes' expected frequencies of (II), (IV) and (VV) are respectively f(II)= p^2x N, f(IV)=2pqxN, f(VV)= q^2xN (Russel, 1998).

Table 4.1: Chi–square test results for overall population.

Genotype	N(observed) in N=195	n(expected) in N=195
GSTP1(II)	51	62
GSTP1(IV)	118	96
GSTP1(VV)	26	37
Chi-test Value=0.005906 Pvalue=0.94		
NQO1(CC)	61	68
NQO1(CT)	108	94
NQO1(TT)	29	33
Chi-test Value=0.211965 Pvalue=0.9		

The genotype distributions of these two genes respectively fitted the Hardy-Weinberg equilibrium with $p>0.05$ at one degree of freedom. This means that the probability of the differences between observed and expected values is due to chance is high and the observed numbers of genotypes fit the expected numbers under Hardy-Weinberg law (Russel, 1998).

The Hardy- Weinberg equilibrium Fit test for the two age groups separately:

Table 4.2: Chi-square test for under age 25 group

Genotype	N(observed) in N=104	n(expected) in N=104
GSTP1(II)	32.94	35
GSTP1(IV)	55.04	50.67
GSTP1(VV)	19	18.34
Chi-test Value=0.77 Pvalue=0.40		
NQO1(CC)	40.77	41.8
NQO1(CT)	50.5	48.13
NQO1(TT)	12.75	13.84
Chi-test Value=0.89 Pvalue=0.35		

The distribution of *GSTP1* and *NQO1* genotypes fitted the Hardy-Weinberg equilibrium in this age group similarly to the overall population although in the overall population, the P value was higher and this could be due to the sample size difference 104 compared to 195. The Hardy-Weinberg law to be tested one necessary condition is that population should be large (Russel, 1998).

Table 4.3: Chi-square test for over 40 age group

Genotype	N(observed) in N=91	n(expected) in N=91
GSTP1 (II)	37.22	53.24
GSTP1(IV)	64.77	33
GSTP1(VV)	7.955	5.24
Chi-test Value=1.01 E-08 Pvalue=?		
NQO1(CC)	18.61	23.79
NQO1(CT)	55.84	45.48
NQO1(TT)	16.55	21.76
Chi-test Value=0.0937 Pvalue=0.8		

For the age 40 *GSTP1* genotypic variation p value is over the 0.9>0.05 and therefore there is no argument against it, and the represented alleles are in Hardy Weinberg equilibrium although this age group is less representative of the population due to its small size (91 individuals).

Chapter 5

DISCUSSION

Smoking prevalence in Lebanese adult population is 53.6% whereas in USA this prevalence is around 25.7 % and is diminishing at a steady rate (Baddoura & Wehbe-Chidiac, 2001, Globocan IARC, 2002). This high prevalence in adult population is critical as lung cancer risk increase with smoking, and more than 90% of lung cancers are caused by cigarette smoking (Mabry et al., 1998; Saldivar et al., 2005).

The fact that cancer is a disease mainly related to aging and involve multiple mutations can mask the existence of detoxification mechanism and repair mechanism (Holland & Frei, 2003). Lung cancer is caused by exogenous factors such as tobacco carcinogens studying differences in capability of detoxification metabolism can give an estimate of probable risk (Mabry et al., 1998). *GSTP1* and *NQO1* gene/enzyme variants have been chosen as risk indicators as both have a role in tobacco detoxification and their variant forms were found to have diminished activity (Siegel et al., 2001; Watson et al., 1998). GSTP1 is one of major detoxification enzymes highly present in lung tumors and it has a role in detoxification of tobacco procarcinogens by catalyzing gluthatione conjugation to electrophilic substances (Balendiran et al, 2004). NQO1 is a two-electron reductase and metabolizes different xenobiotics (drugs) and is important in chemoprotection (Ross et al., 2000). Therefore, since the identification of polymorphisms in *GSTP1* gene and *NQO1* gene and knowing that these polymorphisms showed different levels of efficiency and activity in xenobiotic metabolism and tobacco carcinogenics detoxification, there have been considerable researches done on this topic (Ingelman-

Sundberg, 1998). Gilliland et al., (2002-a) study provided the design for this project whose goal was to try to determine if *GSTP1* and *NQO1* polymorphisms are associated with smoking induced methylations of *p16* and O(6)-methylguanine-DNA methyltransferase (*MGMT)* in order to understand their role in inducing cancers by inducing mutations.

GST genes polymorphisms effect on cancer susceptibility were the subject of many studies both one gene at a time and combinations of different polymorphisms in this large family of genes. Results obtained by the different studies showed that there is a need to really do more research on *GSTP1* polymorphisms as it has been mostly studied in combination with other *GST* gene polymorphisms like *GSTM1* and *GSTT1* (Kihara et al., 1999; Wang et al., 2003-a).

For *GSTP1* some studies have linked its polymorphism with general lung function and lung diseases (Gilliland et al., 2002-b; He et al., 2004). In our study 10% of sampled individual have had lung illness during their lifetime (according to questionnaire), one case of pneumonia having *GSTP1*- (IV) genotype and one case of asthma with *GSTP1*-(VV) genotype. In this latter case the (VV) cannot be correlated to decreased lung function because of only one case but other studies showed that *GSTP1* variants were associated with decreased lung function (Gilliland et al., 2002; He et al., 2004).

Most of studies done so far implied that there is a relationship between cumulative dose of smoking and *GSTP1* polymorphism on lung cancer susceptibility. Results of a study done in China showed that *GSTP1* increases cancer risk when combined with *GSTM1* and smoking (Wang, et al., 2003-a). Also Kihara et al. (1999) studying the frequency of *GSTP1* polymorphisms in Japanese population found a significant ethnic origin of distribution of the frequency of alterations when compared with African-Americans and white Caucasians. The *GSTM1* null genotype was seen to increase risk when

associated with *GSTP1* variant genotypes (IV or VV) in smokers group age 50-59. In comparison to above studies the results of our study showed a prevalence of *GSTP1* genotypes: (II) 26.15%, (IV) 60.51% and (VV) 13.33% in the overall population (see Table 2.1). The percentage frequency obtained for each genotype fit in the intervals relative for this genotype. Comparing the results that we obtained for *GSTP1*-(II) genotype frequency results agree with those obtained by Kihara et al. (1999) on Japanese Population.

However, other studies showed that there was no association between *GSTP1* polymorphism alone and elevated risk of lung cancer (Schneider et al., 2004; Wang Y et al, 2003; Liang et al, 2005). The genetic susceptibility attributed to this family of enzymes is expected to be dose dependent with smoking exposure, but the extent of association of its different polymorphisms remain controversial (Schneider et al., 2004). The study done by Schneider et al. (2004) on the *GSTM1*, *GSTT1* and *GSTP1* polymorphisms found no association between lung cancer risk even with increasing cumulative smoking dose. In a case- control study of *GSTP1* polymorphism and lung cancer risk in white Caucasians it was seen lung cancer risk is higher when having another *GSTP1* polymorphism (Ala 114Ala) genotypes but no association of (Ile105Val) with lung cancer risk (Wang et al., 2003-b). Recently Liang et al. (2005) in China using an advanced polymorphism assay using (one step PCR method) found no relationship between *GSTP1* variants alone and lung ca risk. Their results were consistent with studies of Schneider et al. (2004), Lewis et al. (2001) and Wang et al. (2003-a). Similarly, a study associating the lung cancer risk to the combined effect of (*GSTP1*, *GSTM1-null* and *GSTP1* and *p53* variants) was done on a large Caucasian population showed that additional investigations are needed to study complementary biomarkers associated with lung cancer risk in order to clarify the possible mechanism of carcinogenesis (Miller et al., 2002).

Nazar- Stewart et al. (2003) study showed that presence of *GSTM1* (null) allele had a modest increase on lung cancer risk especially in heavy smokers. They also found out no role for *GSTT1* polymorphism and recommended that more study on *GSTP1* polymorphism risk increases are needed.

A study found that the association of passive smoking and lung cancer could be enhanced by *GSTP1* (I105V) polymorphism: in smokers and in non-smokers having *GSTP1* (VV) genotype exposed to environmental tobacco (Miller et al., 2003). In addition a recent study by Wenzlaf et al. (2005), showed that in non-smokers exposed to 20 or more years of household environmental tobacco smoke exposure (ETS), carrying the *GSTM1* null genotype alone increases risk to 2.3-fold while in this same category of high ETS those carrying the *GSTM1* null and the *GSTP1* (V) allele were at 4-fold higher lung cancer risk. The frequency of *GSTP1*-(VV) in our study differed by around 1% from those of Miller et al. (2003), Nazar-Stewart et al. (2003) and Schneider et al. (2004). The risk of lung cancer related to *GSTP1* genotypes was further examined by stratified anaylsis with smoking behavior in many studies (Schneider et al., 2004; Nazar-Stewart et al., 2003; Miller et al., 2002). In our study the genotypic frequency and smoking were analyzed in order to see any correlation that could imply a potential risk. About 13.3% of the whole population studied carrying the *GSTP1*-(VV) genotype would be at higher risk for lung cancer if exposed to environmental tobacco as implied by Miller et al. (2003). However, Wang et al. (2003-a) found no correlation between smoking and *GSTP1*-(VV) genotype and cancer risk. Whereas Nazar Stewart et al. (2003) found a non-significant decreased risk associated with *GSTP1* (VV) genotype when no other variables were studied. But, this latter study also found that the length of smoking history when associated with a *GSTP1* (VV) genotype has increased risk in light smokers and decreased risk in heavy smokers. This implies that amount of smoking: can confer different

risk when associated to a specific genotype and can affect the detoxification process. (Nazar- Stewart et al., 2003). Possibly, *GSTP1* is more important in lower phases of detoxification and then other enzymes are responsible for detoxification at higher concentration of carcinogens (Nazar- Stewart et al., 2003). The fact that differences exist is mainly due to the etiological factors of lung cancer as far as we know *GSTP1* (II) and *GSTP1* (VV) differ in activity and specificity (Harries et al, 1997). Schneider et al. (2004) reported that for heavy smokers with a minimum of one copy of *GSTP1* the variant allele (V) the risk was higher. Kihara et al. (1999) compared both non-smokers cancer patients and non-smoker healthy individuals. As the healthy individuals had higher frequency of (IV) and (VV) genotype this suggested that having one (V) allele correlate with decreasde risk as its frequency was higher in healthy rather than lung cancer patients. The results we obtained in our smoking stratified analysis with genotype (grouping variables analysis) showed that smokers group had a higher (II) frequency and lower frequencies of both (IV) and (VV). Are those individuals at higher risk or at lower risk? If we rely on previous studies it would mean that the risk is higher in the population that was screened during this study.

Little information is available about young individuals and lung cancer risk. Most of the previous research included lung ca cases, done on age group 60± 15 years, and as mentioned earlier lung cancer happens over a prolonged period of time. Our study age groups were under 25 and over 40 and marked differences were obtained in their genotypic frequencies distribution, and this could contribute to differential susceptibility potentials between those groups. Skuladottir et al. (2005) studied young cancer patients age 59 and younger in Denmark and Norway (pooled study). The study population consisted of 320 lung cancer cases. Taking into account smoking exposure the following variant alleles were found to increase risk: *GSTM1-H, GSTM3-H, GSTP1-H,*

GSTT1-H, GPX-H, NAT2-H, MPO-H, NQO1-H. These latter genotypes when combined were associated with high risk, also no effect for individual genotype on lung cancer risk was determined. However, *GSTP1* genotypes (IV or VV) were found to have a greater lung cancer risk in former smokers who smoked for a short period of time. The odd ratio: is the ratio of same genotype frequencies between matched individuals(age, and smoking). The odd ratio was found to be equal 1.3 when comparing lung cancer cases to healthy controls (Skuladottir et al., 2005) Similar odd ratio values were obtained by six other studies e.g. Lewis et al. (2002), Perera et al. (2002).

It is important to note that we had a small number of former smokers in our study in order to rely on *GSTP1* – (VV) genotype and short period of smoking for risk assessment as such relation was found in above studies. Relying on results of Skuladottir (2005) and Miller (2003) we could postulate that with the very high percentage of carrier (IV) and double mutants (VV) obtained in our study, in comparison to those studies we may have a population at risk as having those genotypes was found to increase the risk. However, with a larger population size and matching age and smoking variables, could have given different results.

The second gene *NQO1* has been studied as it encodes an important enzyme in detoxification of xenobiotics (Lin et al., 2003). Its variant form *NQO1* (TT) genotype has reduced to null activity in the expressed enzyme suggesting it has involvement in lung cancer disease (Siegel et al., 2001). The *NQO1* association with lung cancer predisposition has been studied extensively. Chen et al.(1999) study established that mutant *NQO1*-C609T was found less in lung cancer cases than in controls and taking in consideration previous study done on lung cancer epidemiology in the same population and that there was no difference in smoking exposure in this population. It was thought that genetic susceptibility plays a role in that differential risk. In this Hawaian

population studied by Chen et al. (1999) there is three ethnic origins: Japanese, Caucasians and native Hawaians. The (TT) genotype was twice higher in Japanese versus Caucasians and lower risk of lung ca was observed when comparing Japanese smokers with Caucasians smokers. This result could substantiate *NQO1* role in activating some potent lung carcinogens and its absence would decrease lung cancer risk (Chen et.al; 1999). For the overall population screened in our study in comparison to Chen et al. (1999) study we had (TT) genotype frequency not differing a lot from that of Japanese (origin) population with low risk associated to this genotype. However, the (CT) frequency is similar to that of Caucasians population. Could this suggest that in our population those having (TT) genotype frequency (14.87%) of the population are at lower risk?

NQO1 was shown also to activate dinitropyrenes, formed from incomplete combustion from urban areas (diesel engines, kerosene heaters and gas burners.), which are proven potent lung carcinogens in animal (Lin et al., 2003). They are present as airborne particles in Japanese and Taiwan urban areas whereas they are absent in Nanjing China (Yin et al., 2001). That finding could explain that no relation between *NQO1* genotypes and lung cancer was seen in that region (Yin et al., 2001). In a study done by Lin et al. (2003), results showed that smokers with wild-type *NQO1* genotype (CC) had higher risk of lung adenocarcinoma. NQO1 seems to either activate some potent carcinogens present in tobacco where the wild-type form of the enzyme has highest activity, or that cigarette smoke could affect NQO1 activity by making it more active towards other environmental carcinogens, meaning that smokers having (CC) genotype were at higher risk (Lin et al., 2003). In our study, we had higher (CT) and (TT) frequencies in smokers of all ages so the analogy with the study by Lin et al. (2003) results on increased risk of lung adenocarcinoma in smokers with (CC) genotype, could contribute to

estimating a lower risk in the overall LAU studied population. It is however important to note that we have no evidence about exposure or non exposure of LAU individuals to dinitropyrenes. Some areas are very dense and polluted and could hold more carcinogens concentration like these dinitropyrenes that are capable of binding to DNA.

In a UK population Lewis et al. (2001) study results showed no apparent relationship between *NQO1* polymorphism and SCLC. However, *NQO1* (T) allele present in at least one copy would cause a 4-fold increased risk of SCLC in Caucasians. This high risk which was only seen in heavy smokers, suggests that NQO1 enzyme has an important action on carcinogenic effect of tobacco smoke (Lewis et al., 2001). In our population study a higher percentage of (TT) genotype was seen in smokers and mainly in group age over 40 and the (CT) genotype is higher in smokers in both age groups thus there could be a higher risk in the over 40 age group having (TT) genotype which were around 22.7% of the population.

The largest population study on *NQO1* polymorphism and lung cancer risk was conducted on a Caucasian population of 1900 individuals (Xu et al., 2001). This study showed the (TT) genotype fraction in the population is slightly higher in SCLC cases when compared with cases of adenocarcinoma and other subtypes of lung cancer. They also found a linear association between lung cancer and smoking intensity in all genotypes, and no direct association of *NQO1* polymorphism alone with cancer risk (Xu et al., 2001). But, (CT) and (TT) genotypes correlated with higher risk than wild type (CC), in former smokers (smoked intensely over a short lapse) as well as in current smokers (Xu et al., 2001). This latter study was underlining the importance of NQO1 activity on carcinogenesis in the early age after an individual starts to smoke because even former smokers who have (T) allele were at higher risk. Does that mean that phase II metabolism represented by having functional

wild type (CC) have a role in early stages of carcinogenesis (Xu et al., 2001). In our case although the population was smaller but we had a higher percentage of (TT) genotype and (CT) genotype in smokers especially if associated with high intensity of smoking, could indicate a higher risk if we rely on the study done by Xu et al (2001).

Saldivar et al. (2005) who recently did a stratified analyses on age, gender and smoking status found that the risk was higher for lung cancer when associated *NQO1* (T) allele in individuals younger than 62, and in females and in non-smokers but with caution that such analyses would decrease the size of the sample due to subgrouping variables. Whereas, Bock et al. (2005), found out that the *NQO1* (T) allele is somehow protective as it correlated with decreased risk of later age of diagnosis for lung cancer. Those diagnosed aged over 50 years, having (CT) and (TT) genotypes had 0.48 times lower lung cancer risk than individuals with (CC) genotype (Bock et al., 2005). Whereas Lawson et al. (2005) study reported higher risk for lung cancer was in male smokers having *NQO1* (T) allele. This finding could explain the suspicion that in Lebanon the relatively high number of male casualties from lung cancer could have a higher (T) ratio in smokers. Our population of smokers had higher number of males but still our population is small to make such conclusion.

Although, some evidence is accumulating on the role of *NQO1* and tobacco induced cancer some studies found no link (Liang et al., 2005). It has been established that heavy smokers are more at risk when (T) allele is found (Xu et al., 2001). This assumption is based on both population studies and the fact that (T) allele is a null allele (Saldivar et al., 2005). What remain to be validated are the susceptibility variables: is it related to ethnicity, gender, smoking exposure or it is just the combination of environmental factors associated to having or not a (T) allele (Yin et al., 2001; Lawson et al., 2005; Saldivar et al., 2005).

A third group of researchers investigated both *NQO1* and *GSTP1* contribution to lung cancer risk (Lin et al., 2003; Kiyohara et al., 2002; Skuladottir et al, 2005). A Taiwanese population study found that *GSTP1* polymorphism is a risk factor for lung squamous cell carcinoma and that cigarette smoking was necessary for associating *NQO1* polymorphism with risk of lung adenocarcinoma (Lin et al., 2003).

In our study allele frequencies of both *GSTP1* and *NQO1* were in Hardy-Weinberg equilibrium, frequencies of variant alleles were similar to those observed by other studies (Xu et al., 2001; Miller et al., 2002) e.g (TT) genotype frequency should be between 4-20% and (VV) genotype frequency between 3-13%.

The importance of *NQO1* and *GSTP1* variants in acting in concert was studied by Gilliland et al (2002). They found out that both variants' are related to DNA damage by association of *NQO1* and *GSTP1* with *MGMT* and *p16* methylation. The association of both variants that have decreased activity towards some tobacco carcinogens may be the cause of tobacco induced damage that disrupts the chromatin structure and such change in the integrity of genome would permit some inappropriate methylations of normally protected *p16* and *MGMT* promoters regions (Gilliland et al., 2002-a). To date studies on this topic are controversial and the main issue is to find the proper mechanism. The studies of most detoxifying enzymes polymorphisms of are based on lung cancer cases studies and are not always convincing because different susceptibility schemes are made by each researcher (Gilliland et al., 2002-a). In order to provide the link between carcinogenesis and tobacco detoxifying enzyme polymorphisms results obtained from this study should be combined with the study on *p16* and *MGMT* methylations done by Daoud (2005). Knowing that *p16* methylations is one of the primary mutations in NSCLC carcinogenesis progression (Rom et al., 1999). As far as we know that

NQO1 and *GSTP1* have an important activity in detoxifying carcinogens present in tobacco and their polymorphisms studies had underlined some important aspects of disease susceptibility, as both polymorphisms lead to diminished enzyme activity (Siegel et al., 2001; To-Figueras et al., 1999). GSTP1 and NQO1 enzymes are important in the first steps of carcinogenesis (Hecht et al., 1999; Saldivar et al., 2005). Their presence or allelic variation would imply a change in the microenvironment of cells leading to more DNA damage (Gilliland et al., 2002-a) or in some cases protecting from their own activating effects on some chemicals (Yin et al., 2001; Chen et al., 1999).

Variations existed in our population between groups (with groups' size ~50 individual per group) of smokers and non-smokers and among the different age groups. These differences may not be conclusive but they are a good indicative estimate. Results obtained in this study were based on a controlled population of healthy individuals and striking differences were seen when comparing genotypes of smokers and non-smokers when considering both polymorphisms. We obtained a high frequency in (TT) genotype in the over 40- age group smokers. This establishes a high lung cancer risk as results from studies done on this polymorphism showed higher risk existing in Caucasians when *NQO1* variant (TT) genotype is associated with smoking regardless of smoking intensity. The LAU smokers group had higher *GSTP1* (II / IV) and (II/ VV) genotypes frequencies ratio compared to some studies referred that *GSTP1* (VV) alone increases risk even with environmental exposure to tobacco smoke (ETS). Based on this finding it is possible to determine high risks even in non-smokers having (VV) genotype exposed to tobacco or other source of carcinogens.

Chapter 6

CONCLUSION

This study was done on a controlled healthy population and on xenobiotic metabolism enzyme/gene polymorphisms as risk indicators of tobacco induced lung cancer in Lebanon. The study has determined genotypic frequency variation of the two detoxifying genes *GSTP1* and *NQO1* polymorphims in order to assess lung cancer risk in LAU population. The DNA was extracted from sputum samples obtained after forced expectoration and PCR and RFLP were done respectively for each gene.

- It is important to note that for genotypic distribution differences were marked between age groups.
- Our population had a larger number of male smokers, this could explain why lung cancer has more male victims in Lebanon. This assumption cannot be made however with a small sample size as ours.
- In analogy to studies that indicated a higher risk could exist for small cell carcinoma of esophagus (SCCE) or prostate cancer in smokers when having (II) genotype which were 27.27% of population.
- The LAU smokers group had higher *GSTP1* (II / IV) and (II/ VV) genotypes frequencies ratio and other studies has shown that *GSTP1* (VV) alone increases risk even with environmental exposure to tobacco smoke (ETS). Based on this finding it is possible to determine high risks even in non-smokers having (VV) genotype exposed to tobacco or other source of carcinogens.
- A high frequency in (TT) genotype was obtained in the over 40- age which could be at higher risk. As the results from different studies implied that a

high risk exists when *NQO1* variant (TT) genotype is associated with smoking regardless of smoking intensity.

- The allelic frequencies of both *GSTP1* and *NQO1* were found to be in Hardy-Weinberg.

Smoking association with LC is a risk factor and many studies showed that variable genotypes *GSTP1* - (VV) and *NQO1*- (TT) can increase this risk up to 4- fold. Therefore the importance of studying the genotypic frequency of *NQO1* and *GSTP1* in Lebanese population will give insight for future preventive or therapeutic strategies.

Recommendations:

- In order to study genotypic frequencies and do comparison between subgroups a larger population size should be considered.
- The marked differences should be emphasized between genotypic frequencies between age groups rather than between smokers and non-smokers.
- There is an urgent need for improved epidemiologic study design that integrates DNA adducts measures with indicators of metabolic capacity.
- Compare genotypic frequencies of *NQO1* and *GSTP1* in lung cancer patients to those obtained in this study in addition to comparing smokers patients and non-smokers patients genotypic frequencies.
- Sequence these genes to look for new polymorphism at same of different locus to relate to smoking and cancer. Study other Phase I and Phase III gene polymorphisms.
- Studying genotypes of *GSTP1* and *NQO1* in cancer patients can be essential before starting any kind of chemotherapy as these are important xenobiotic / drug metabolizing enzyme and happens to be overexpressed in specific tumors conferring drug resistance or drug targeting potentials.

Chapter 7

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Appendix I

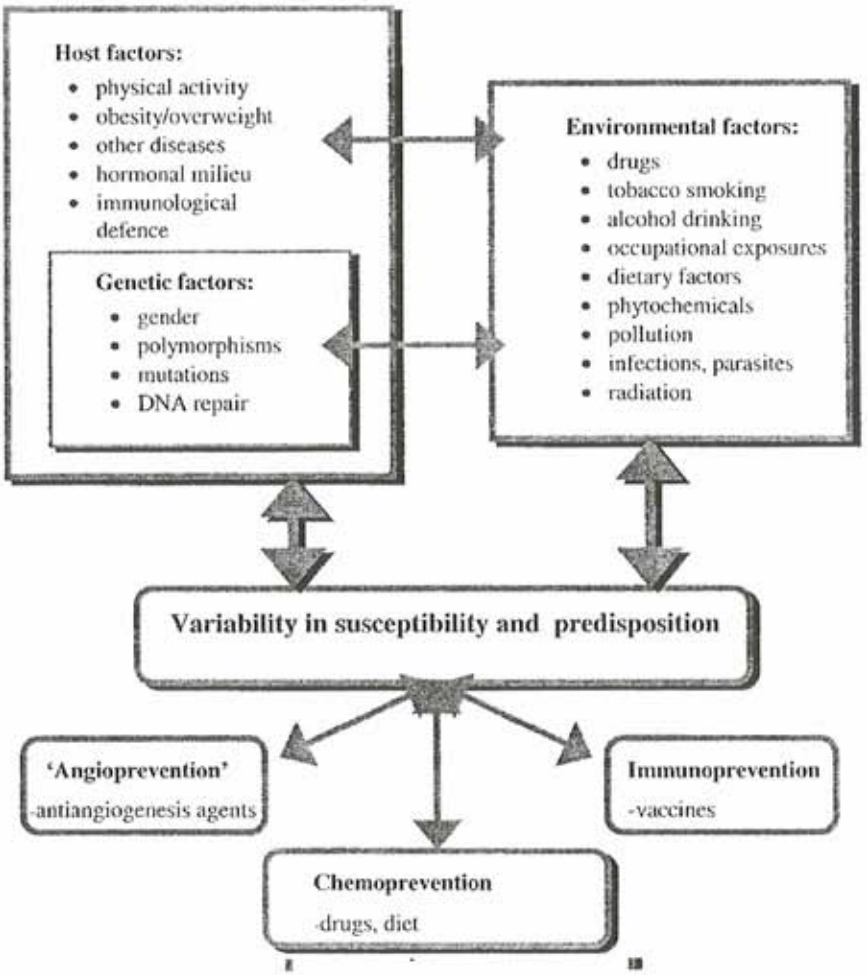

Figure. A: Cancer Prevention Strategy (Vaino & Haitanen, 2003).

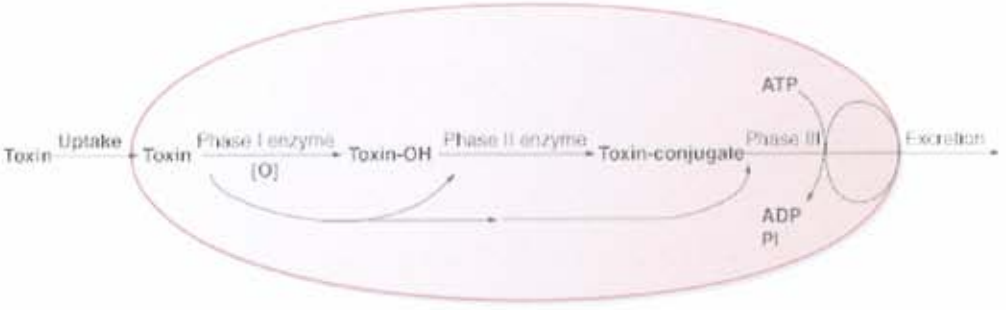

Figure. B: Detoxification Phases ((Holland & Frei, 2003)

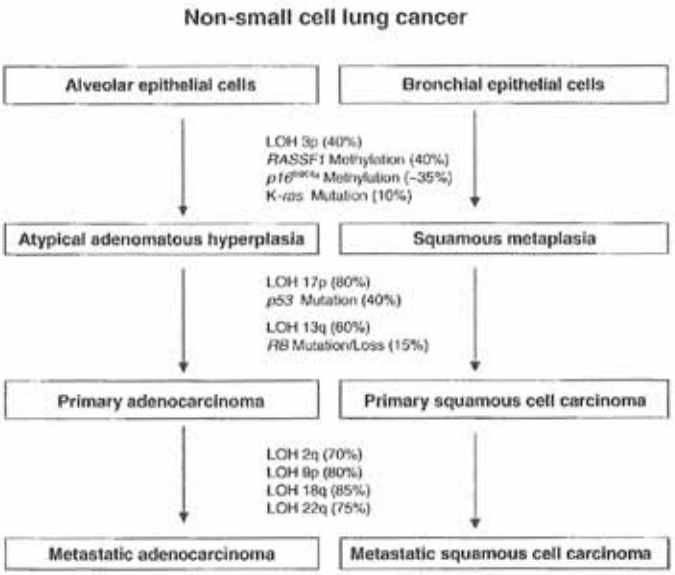

Fig. 5. Non-small cell lung cancer

Figure.C: Mutations occuring during NSCLC progression (Ohgaki et al., 2003).

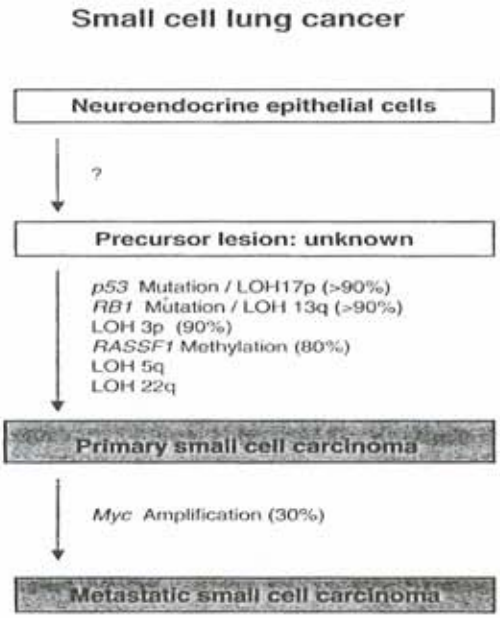

Fig. 6. Small cell lung cancer

Figure. D: Mutations occurring during SCLC progression (Ohgaki et al., 2003).

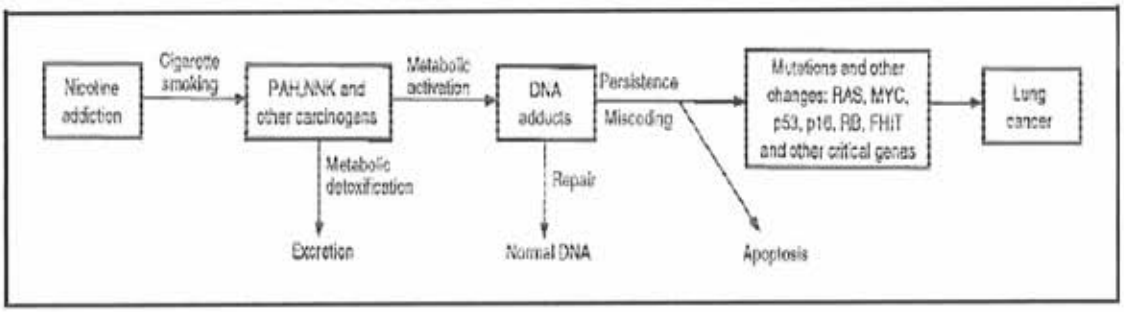

Fig. 1. Scheme linking nicotine addiction and lung cancer via tobacco smoke carcinogens and their induction of multiple mutations in critical genes. PAH = polycyclic aromatic hydrocarbons; NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

Figure. E: Nicotine dependence and lung cancer carcinogenesis (Hecht, 1999).

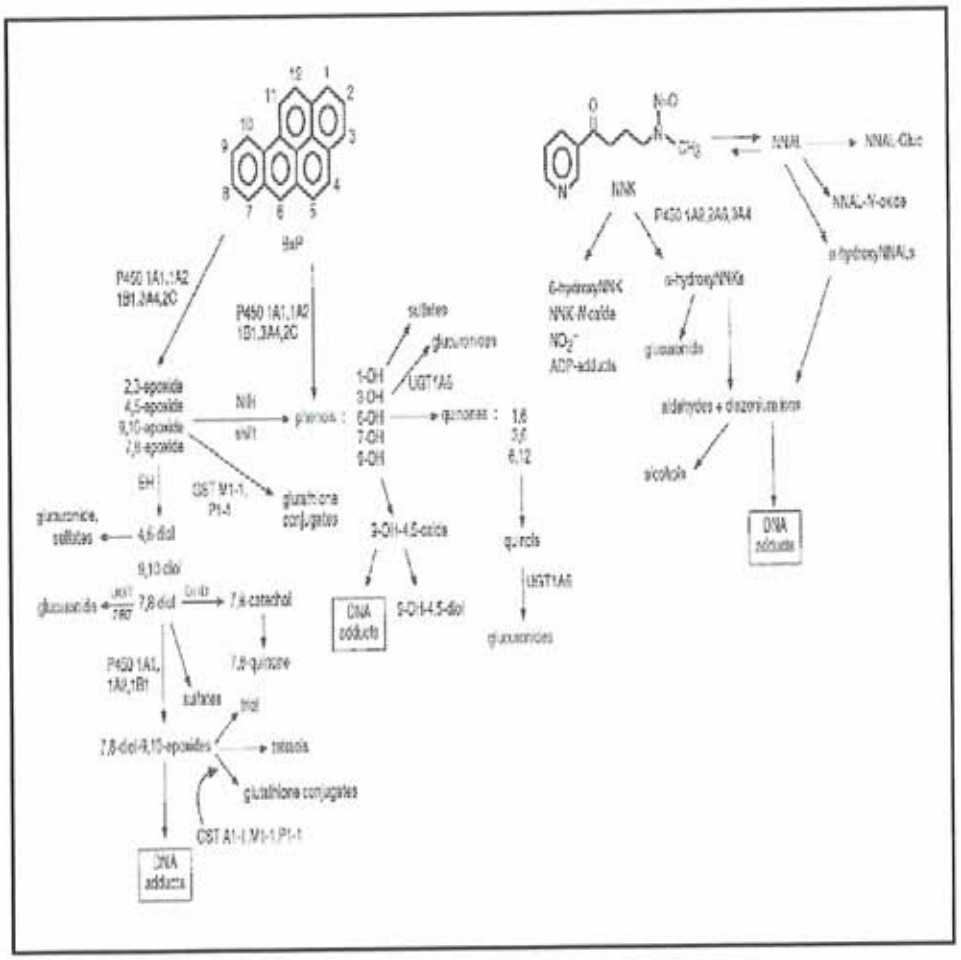

Fig. 4. Metabolic pathways of benzo[a]pyrene (BaP) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), modified from (22,30,33). Some human enzymes involved in the various reactions are indicated (79–109). EH = epoxide hydrolase; DHD = dihydrodiol dehydrogenase; UGT = UDP-glucuronosyl transferase; GST = glutathione S-transferase; P450 = cytochrome P450; NNAL = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; 1-OH, 3-OH = 1-hydroxy BaP, 3-hydroxy BaP, etc. Other abbreviations are defined in the text.

Figure. F: Detoxification role of enzymes and role of tobacco carcinogens in the formation of DNA adducts (Hecht; 1999).

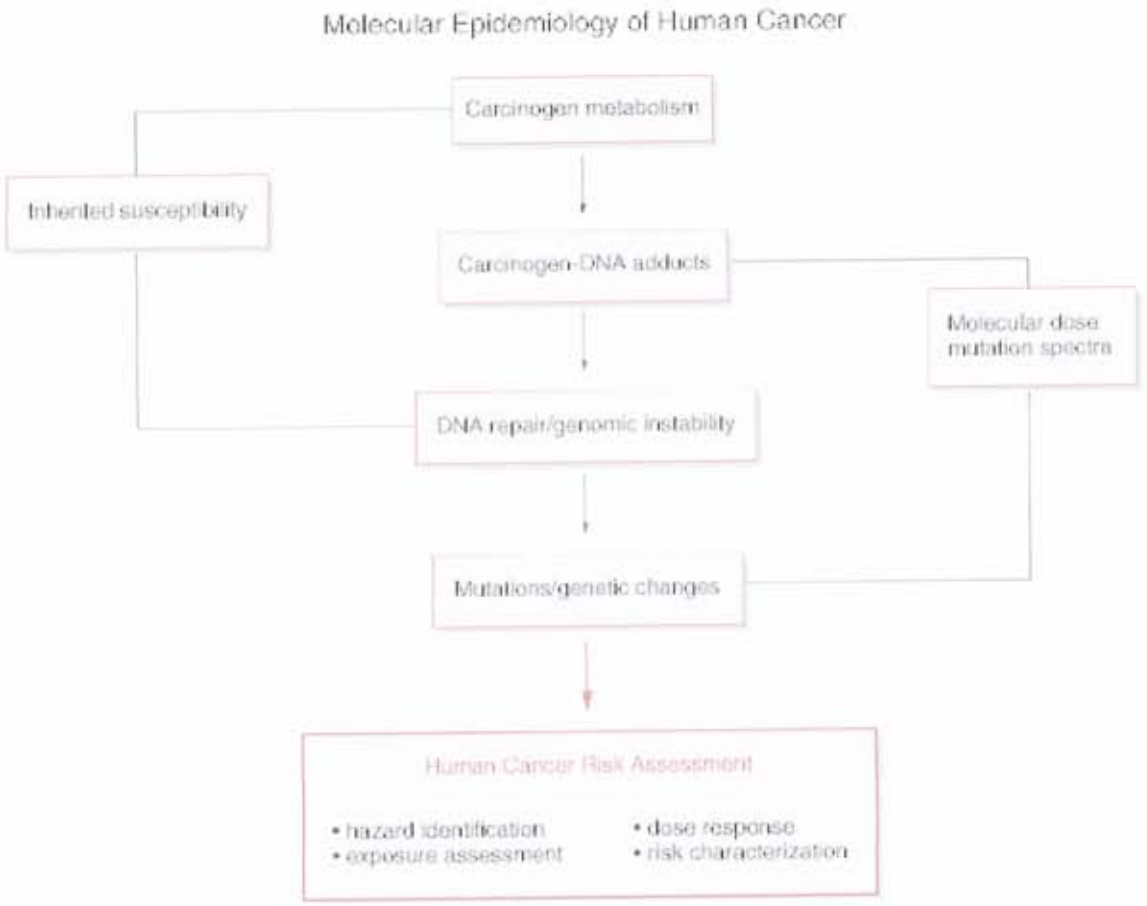

Figure.G: Molecular epidemiology goals (Holland & Frei, 2003)

Table 1

Summary of Oncogenes Changes in Lung Cancer (Mabry et al., 1998).

Ras Family Genes	Like other solid tumors mutations occur frequently in LC. K-ras in NSCLC at frequencies approaching 50%. H-ras mutations frequencies are very low for NSCLC and N-ras are rare. K-ras mutations common in adenocarcinoma very rare in bronchoalveolar carcinomas and not described in SCLC. Ras mutations are generally negative prognostic factor in patients with adenocarcinoma.
Myc Family Genes	Members of this family are dominant oncogenes that can be activated in LC usually by gene amplification. C-myc amplification in SCLC is a negative prognostic factor and is three times more common in treated patients more than untreated patients. Amplification of C-myc correlated with twofold reduction in median survival. The C-myc protein alters the drug resistance to treatment.. L-myc and N-myc are also found in SCLC and SCLC cell lines but prognostic implications are not certain.
Tumor Suppressor genes involved in sporadic lung cancer	
P53 Gene	Best defined tumor suppressor gene change is mutation of the P53 gene in LC. Loss of function correlates most with very frequent LOH that occurs on chromosome 17 region p13.1 in all LC types. P53 changes happens in all human cancers and 50% in NSCLC and 90%in SCLC. Most frequent mutations are observed in G-T transversions and may reflect bulky DNA adducts resulting from carcinogens found in tobacco smoke. BPDE binds directly to hot spots for mutations of p53 found in LC.
P16	Alterations in the cyclin dependent kinase inhibitor encoding gene occur frequently in LC. Both DNA Methylation of the transcription site and homozygous deletions of P16 occur always in NSCLC rather than SCLC tumors. Taking in consideration that Rb is more frequently mutated in SCLC and tumor cells appear to need only the inactivation of one gene in cell propliferation pathway. The exact mechanism of carcinogenesis is still not known but it is important to study the P16 role as it would be valuable in assessi genetic susceptibility for LC.

Rb	Rb gene plays a critical role in cyclin D pathway which controls cell cycle. Rb gene is present on chr13 region q14 and is altered in all SCLC and in 30-40% of NSCLC. In NSCLC it becomes more common at late clinical stage.
FHIT-1	Altered gene on chromosome 3p region which undergoes LOH in both SCLC and NSCLC. Altered transcription splice products for FHIT-1 gene is located in region 3p14 and in fragile site region FRA3B found in some familia renal tumors were also frequently characterized in lung cancers. However future research will actually elucidate the importance of this gene in Lung Tumorigenesis.

Appendix II
Results Tables

RESULTS TABLES:

Table1: Overall Population Groups' Size

	population n=195	%
Group		
<25	107	54.87
>40	88	45.13
M	101	51.79
F	94	48.21
NS	94	48.21
S	101	51.79

Table 2: Contribution of each genotype in Overall Population

Genotype	number	%
II	51	26.15
IV	118	60.51
VV	26	13.33
CC	61	31.28
CT	108	55.38
TT	29	14.87

Table 3: Smoking/Non smoking Stratification with genotypes In Overall population

	Males	Females	II	IV	VV	CC	CT	TT
Smokers n=101	57.43	42.57	29.7	57.43	12.87	28.71	55.45	15.84
Non smokers n=94	45.74	54.26	22.34	63.83	13.83	32.98	53.19	13.83

Table 4: Smoking/Non smoking Stratification with genotypes in Group Age <25

	Males	Females	II	IV	VV	CC	CT	TT
Smokers n=57	57.89	42.11	31.58	50.88	17.54	40.35	49.12	10.53
Non smokers n=50	48	52	30	52	18	38	48	14

Table 5: Smoking/Non smoking Stratification with genotypes in Group Age >40

	Males	Females	II	IV	VV	CC	CT	TT
Smokers n=101	57.43	42.57	29.7	57.43	12.87	28.71	55.45	15.84
Non smokers n=94	45.74	54.26	22.34	63.83	13.83	32.98	53.19	13.83

Appendix III Questionnaire

LEBANESE AMERICAN UNIVERSITY
FACULTY of ARTS and SCIENCES
GRADUATE STUDIES

Please answer this questionnaire if you are willing to participate. You were selected by a scientific sampling procedure, and your cooperation is very important to the success of this study.

ALL INFORMATION OBTAINED IN THE STUDY WILL BE KEPT CONFIDENTIAL AND USED FOR MEDICAL RESEARCH ONLY.

The purpose of this research is to study changes in the lungs resulting from smoking habits, in comparison to lungs of non-smokers.

I approve to participate in this research and I agree that at the end of this questionnaire I will give a phlegm/sputum sample.

Signature:

Thank you for your willingness to participate.
This is a questionnaire you are asked to fill out.
Please answer the questions as frankly and accurately as possible.

ATS-DLD-78-A

ADULT QUESTIONNAIRE - SELF COMPLETION
(for those 13 years of age and older)

IDENTIFICATION

IDENTIFICATION NUMBER: #####

NAME: _____ _____ ___
(Last) (First) (MI)

STREET _____

CITY _____ STATE ____ ZIP _____

PHONE NUMBER: ( ) _____-_____

INTERVIEWER: ###

DATE: _____
MO DAY YR

==========

1. Age _____

2. Place of Birth: _____

3. Sex:
   1. Male ____
   2. Female ____

4. What is your marital status?
   1. Single ____
   2. Married ____
   3. Widowed ____
   4. Separated/Divorced ____

6. What is the highest grade completed in school? _____
   (For example: 12 years is completion of high school)

SYMPTOMS

These questions pertain mainly to your chest. Please answer yes or no if possible. If a question does not appear to be applicable to you, check the does not apply space. If you are in doubt about whether your answer is yes or no, record no.

COUGH

7A. Do you usually have a cough? (Count a cough with first smoke or on first going out-of-doors. Exclude clearing of throat.)[If no, skip to question 7C.] 1. Yes ___ 2. No ___

B. Do you usually cough as much as 4 to 6 times a day, 4 or more days out of the week? 1. Yes ___ 2. No ___

C. Do you usually cough at all on getting up, or first thing in the morning? 1. Yes ___ 2. No ___

D. Do you usually cough at all during the rest of the day or at night? 1. Yes ___ 2. No ___

IF YES TO ANY OF THE ABOVE(7A,7B,7C, OR 7D), ANSWER THE FOLLOWING:
IF NO TO ALL, CHECK DOES NOT APPLY AND SKIP TO 8A.

E. Do you usually cough like this on most days for 5 consecutive months or more during the year? 1. Yes ___ 2. No ___

8. Does not apply __

F. For how many years have you had this cough? _____
   Number of years
   Does not apply __

PHLEGM

8A. Do you usually bring up phlegm from your chest? (Count phlegm with the first smoke or on first going out-of-doors. Exclude phlegm from the nose. Count swallowed phlegm) 1. Yes ___ 2. No ___

[If no, ski

B. Do you usual like this as much as twic days out of the week? 1. Yes ___ 2. No ___

C. Do you usual at all on getting up or f orning? 1. Yes ___ 2. No ___

D. Do you usual at all during the rest of t ? 1. Yes ___ 2. No ___

IF YES TO ANY OF T , OR D), ANSWER THE FOLLO
IF NO TO ALL, CH AND SKIP TO 9A.

E. Do you bring s on most days for 3 consecut e during the year? 1. Yes ___ 2. No ___
Does not apply __

F. For how many y trouble with phlegm? _____ Number of years
Does not apply __

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EPISODES OF COUGH

9A. Have you had pe s of (increased*) cough ing for 3 weeks or more e
*(For individual ave cough and/or phlegm) 1. Yes ___ 2. No ___

IF YES TO 9A:

B. For how long hav ast 1 such episode per year _____ Number of years
Does not apply __

==========

WHEEZING

10A. Does your chest e zy or whistling:

1. When you hav 1. Yes ___ 2. No ___
2. Occaisonally olds? 1. Yes ___ 2. No ___
3. Most days or 1. Yes ___ 2. No ___

IF YES TO 1, 2,

B. For how many years n present? _____ Number of years
Does not apply __

11A. Have you ever had a wheezing that has made you feel s th? 1. Yes ___ 2. No ___

IF YES TO 11A:

B. How old were you wh our first such attack? _____ Age in years
Does not apply __

C. Have you had 2 or more such episodes? 1. Yes ___ 2. No ___ Does not apply __

D. Have you ever required medicine or treatment for the(se) attack(s)? 1. Yes ___ 2. No ___ Does not apply __

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BREATHLESSNESS

12. If disabled from walking by any condition other than heart or lung disease, please describe and proceed to Question 14A.

Nature of condition(s):_____

13A. Are you troubled by shortness of breath when hurrying on the level or walking up a slight hill? 1. Yes ___ 2. No ___

IF YES TO 13A:

B. Do you have to walk slower than people of your age on level because of breathlessness? 1. Yes ___ 2. No ___ Does not apply __

C. Do you ever have to stop for breath when walking at your own pace on the level? 1. Yes ___ 2. No ___ Does not apply __

D. Do you ever have to stop for breath after walking about 100 yards(or after a few minutes) on the level? 1. Yes ___ 2. No ___ Does not apply __

E. Are you too breathless to leave the house or breathless on dressing or undressing? 1. Yes ___ 2. No ___ Does not apply __

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CHEST COLDS AND CHEST ILLNESSES

14A. If you get a cold, does it usually go to your chest? (Usually means more than 1/2 the time) 1. Yes ___ 2. No ___ Don't get colds__

15A. During the past 3 years, have you had any chest illnesses that have kept you off work, indoors at home, or in bed? 1. Yes ___ 2. No ___

IF YES TO 15A:

B. Did you produce phlegm with any of these chest illnesses? 1. Yes ___ 2. No ___ Does not apply __

C. In the last 3 years, how many such illnesses, with (increased) phlegm, did you have which lasted a week or more? _____Number of illnesses _____No such illnesses _____Does not apply

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PAST ILLNESSES

16. Did you have any lung trouble before the age of 16? 1. Yes ___ 2. No ___

17. Have you ever had any of the following:

1A. Attacks of Bronchitis? 1. Yes ___ 2. No ___

IF YES TO 1A:

B. Was it confirmed by a doctor? 1. Yes ___ 2. No ___ Does not apply __

C. At what age was your first attack? _____ Age in years Does not apply __

2A. Pneumonia (include bronchopneumonia)? 1. Yes ___ 2. No ___

IF YES TO 2A:

B. Was it confirmed by a doctor? 1. Yes ___ 2. No ___ Does not apply __

C. At what age did you first have it? _____ Age in years Does not apply __

3A. Hayfever? 1. Yes ___ 2. No ___

IF YES TO 3A:

B. Was it confirmed by a doctor? 1. Yes ___ 2. No ___ Does not apply __

C. At what age did it start? _____ Age in years Does not apply __

18A. Have you ever had chronic bronchitis? 1. Yes ___ 2. No ___

IF YES TO 18A:

B. Do you still have it? 1. Yes ___ 2. No ___ Does not apply __

C. Was it confirmed by a doctor? 1. Yes ___ 2. No ___ Does not apply __

D. At what age did it start? _____ Age in years Does not apply __

19A. Have you ever had emphysema? 1. Yes ___ 2. No ___

IF YES TO 19A:

B. Do you still have it? 1. Yes ___ 2. No ___ Does not apply __

C. Was it confirmed by a doctor? 1. Yes ___ 2. No ___ Does not apply __

D. At what age did it start? _____ Age in years Does not apply __

20A. Have you ever had asthma? 1. Yes ___ 2. No ___

IF YES TO 20A:

B. Do you still have it? 1. Yes ___ 2. No ___ Does not apply __

C. Was it confirmed by a doctor? 1. Yes ___ 2. No ___ Does not apply __

D. At what age did it start? _____ Age in years
Does not apply __

E. If you no longer have it, at what age did it stop? _____ Age stopped
Does not apply __

21. Have you ever had:

A. Any other chest illnesses? 1. Yes ___ 2. No ___
If yes, please specify _____

B. Any chest operations? 1. Yes ___ 2. No ___
If yes, please specify _____

C. Any chest injuries? 1. Yes ___ 2. No ___
If yes, please specify _____

22A. Has doctor ever told you that you had heart trouble? 1. Yes ___ 2. No ___

IF YES to 22A:

B. Have you ever had treatment for heart trouble in the past 10 years? 1. Yes ___ 2. No ___
Does not apply __

23A. Has a doctor ever told you that you have high blood pressure? 1. Yes ___ 2. No ___

IF YES to 23A:

B. Have you had any treatment for high blood pressure (hypertension) in the past 10 years? 1. Yes ___ 2. No ___
Does not apply __

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OCCUPATIONAL HISTORY

24A. Have you ever worked full time (30 hours per week or more) for 6 months or more? 1. Yes ___ 2. No ___

IF YES to 24A:

B. Have you ever worked for a year or more in any dusty job? 1. Yes ___ 2. No ___
Does not apply __

Specify job/industry: _____ Total years worked __
Was dust exposure 1. Mild ___ 2. Moderate ___ 3. Severe ___ ?

C. Have you ever been exposed to gas or chemical fumes in your work? 1. Yes ___ 2. No ___
Does not apply __

Specify job/industry: _____ Total years worked __
Was dust exposure 1. Mild ___ 2. Moderate ___ 3. Severe ___ ?

D. What has been your usual occupation or job -- the one you have worked at the longest?

1. Job-occupation: _____
2. Number of years employed in this occupation: _____
3. Position-job title: _____
4. Business, field, or industry: _____

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MOKING

25A. Have you ev? (NO means less than 2 s or 12 oz. of tobacco in han 1 cigarette a day for 1 y

1. Yes ___ 2. No ___

IF YES t(

B. Do you nc (as of 1 month ago)?

1. Yes ___ 2. No ___
Does not apply __

C. How old w rst started reg- cigarette

____ Age in Years
Does not apply __

D. If you ha cigarettes com- pletely, hen you stopped?

____ Age stopped
Check if still smoking ___
Does not apply __

E. How many csmoke per day now?

___ Cigarettes/day
Does not apply __

F. On the ave e time you smoked, how many c: smoke per day?

___ Cigarettes/day
Does not apply __

G. Do or did garette smoke?

1. Does not apply __
2. Not at all _____
3. Slightly _____
4. Moderately _____
5. Deeply _____

26A. Have you ever gularly? (YES means mor acco in a lifetime.)

1. Yes ___ 2. No ___

IF YES to 2(

BI. How old wer started to smoke a pip

____ Age

2. If you have g a pipe com- pletely, hov when you stopped?

____ Age stopped
Check if still smoking pipe ____
Does not apply __

C. On the average over ie you smoked a pipe, how much pipe tobacco did u smoke
____ oz per week lard pouch of tobacco contains 1 1/2 oz)
Does not apply __

D. How much pipe tobacc ing now? ___ oz per week
Not currently smoking a pipe ___

E. Do or did you inhale e?

1. Never smoked ____
2. Not at all _____
3. Slightly _____
4. Moderately _____
5. Deeply _____

27A. Have you ever smoked cigars regularly? (Yes means more than 1 cigar a week for a year). 1. Yes ___ 2. No ___

IF YES to 27A:

B1. How old were you when you started smoking cigars regularly? ____ Age

2. If you have stopped smoking cigars completely, how old were you when you stopped? ____ Age stopped
Check if still smoking cigars___
Does not apply __

C. On the average over the entire time you smoked cigars, how many cigars did you smoke per week ? ___ Cigars per week
Does not apply __

D. How many cigars are you smoking per week now? ___ Cigars per week
Check if not smoking cigars currently __

E. Do or did you inhale the cigar smoke?
1. Never smoked ____
2. Not at all _____
3. Slightly _____
4. Moderately _____
5. Deeply _____

FAMILY HISTO

28. Were either nts ever told by a doctor that they had a chron h as:

	MOTHER			
	. DON'T KNOW	1. YES	2. NO	3. DON'T KNOW
A. Chronic brochitis?	_____	_____	_____	_____
B. Emphysema?	_____	_____	_____	_____
C. Asthma?	_____	_____	_____	_____
D. Lung cancer?	_____	_____	_____	_____
E. Other chest conditions?	_____	_____	_____	_____

29A. Is parent current

_____ _____ _____ _____

B. Please Specify:

—ıg _____ Age if living

—ı _____ Age at death

Don't Don't know _____

C. Please specify c

_____ _____