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THE ROLE OF GENETIC POLYMORPHISMS OF N-ACETYLTRANSFERASE 2 (NAT2) ENZYME IN THE RISK OF BLADDER CANCER IN THE LEBANESE POPULATION

BY
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AN ABSTRACT OF THE THESIS OF

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Title: The role of genetic polymorphisms of N-acetyltransferase 2 (NAT2) enzyme in the risk of bladder cancer in the Lebanese population

Introduction In Lebanon, bladder cancer has an unusually high prevalence. The correlation between N-acetyltransferase 2 (*NAT2)* polymorphisms and carcinogenesis is established in the literature for different types of cancers. Individuals who smoke and carry the slow acetylator phenotype may be at a higher risk of developing bladder cancer as NAT2 is unable to deactivate the carcinogenic aromatic amines found in cigarette smoke.

Methods Data and DNA from 86 controls and 50 bladder cancer cases were analyzed. Seven *NAT2* SNPs were genotyped using Restriction fragment length polymorphism technique (RFLP): *C282T, G191A, C481T, G590A, G857A, A803G,* and *T341C*. Haplotypes were constructed, and 2 phenotype groups were defined as follows: carriers of one or more *NAT2*4* alleles were considered fast acetylators, and the rest were considered slow acetylators.

Results A significantly higher percentage of patients with bladder cancer were males (79.6% vs. 53.1%) and smokers (67.3% vs. 50.6%). There was no difference in mean age between cases and controls (70.14+/- 11.87 vs. 71.95 +/- 17.98 respectively). The allele frequencies of the genotyped SNPs were comparable to Caucasian populations, and they were all in HWE. Sixty five % were slow acetylators among controls, and 62% were slow acetylators among cases, and this was not statistically different. No significant association was found between NAT2 phenotype and bladder cancer risk, even after adjusting for sex and smoking.

Conclusion This is the first study on the association of *NAT2* polymorphisms with bladder cancer risk in a Lebanese sample. Further recruitment is ongoing to increase the power of the study.

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LIST OF ABBREVIATIONS

NAT	N-acetyltransferase family
PAHS	Polycyclic aromatic hydrocarbons
RA	Reactive atypia
AUS	Atypia of unknown significance
HA	Urothelial hyperplasia
PUNLMP	Papillary urothelial neoplasia of low malignant potential
CYP	Cytochrome P450
DME	Drug Metabolizing Enzyme
UD	Urothelial dysplasia
FDA	Food and drug administration
CIS	Carcinoma In situ
NIUC	Noninvasive papillary urothelial carcinoma
GST	Glutathione-S-transferase family
TURBT	Transurethral resection of bladder tumor
TCC	Transitional cell carcinoma
SCC	Squamous cell carcinoma
PCR	Polymerase chain reaction
HCA	Heterocyclic amines
SNP	Single nucleotide polymorphism
MeIQx	2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline
PhIP	2-amino-1-methyl-phenylimidazo[4,5-b]pyridine

Ppm	Parts per million
BNA	B-naphthylamine
WHO	World health organization
UGT	Glucoronosyl-transferases
SULT	Sulfotransferases
AQP3	Aquaporin gene family
AS3MT	Arsenite methyltransferase family
MAF	Minimal allele frequency
DNA	Deoxyribonucleic acid
Bp	Base pair
HWE	Hardy Weinberg Equilibrium
LD	Linkage Disequilibrium
IARC	International Agency for Research on Cancer

CHAPTER I

INTRODUCTION

A. Bladder cancer

1. Epidemiology of bladder cancer

Bladder cancer is a serious problem spreading throughout the world (Kirkali Z. et al., 2005). Bladder cancer is the 7th most prevalent cancer in men and the 17th most prevalent in women worldwide (Kakehi Y. et al., 2010). Epidemiological studies in Lebanon have shown that bladder cancer has an unusually high prevalence. In 2004, bladder cancer in Lebanon was the second most prevalent cancer in males, and the third among both sexes (Shamseddine A.I., Musallam K.M., 2010).

Risk factors for bladder cancer are diverse but most importantly include smoking history and occupational exposure to urothelial carcinogens, namely polycyclic aromatic hydrocarbons (PAHS), aromatic amines, and N-nitroso compounds (Kirkali Z. et al., 2005). About 30% of bladder cancer in females, and 65% of bladder cancer in males are due to cigarette smoking (Baris D. et al., 2009).

The incidence of bladder cancer in Lebanese patients, particularly males, is higher than figures from neighboring countries in the region and parallels those of France, UK and the US (Shamseddine A. et al., 1998).

2. Bladder cancer grading

A bladder tumor is classified as low grade when having little cytologic abnormality, few mitoses, and the histological architecture is well maintained. However, when the urothelium contains a multitude of cytologic abnormalities along with many mitoses, with too many nuclei of different shapes and large nucleoli, and the histological architecture is disturbed with the disappearance of stratification, then this tumor is classified as high grade (Hartmann A., Bertz S., 2011).

3. Histologic types of urothelial tumors

Urothelial tumors are divided into 2 categories: benign urothelial changes and malignant urothelial lesions.

a. Benign Urothelial changes

i. Reactive atypia (RA) in the urothelium and atypia of unknown significance (AUS)

RA is identified when different cellular abnormalities occur with changes in the architecture of the urothelium. These are also accompanied by extensive inflammation involving lymphocytes and granulocytes. These reactive urothelial changes can be secondary to urocytitis, calculi, instrumentation, or intravesical treatment. However, if no inducing factor is clearly identified, then the tissue abnormality is classified as AUS (Hartmann A., Bertz S., 2011).

ii. Urothelial hyperplasia (HA)

HA is characterized by a thick urothelium due to stratification of a number of cell layers with no cytological abnormalities. When this type of abnormality occurs solely, then it is not considered premalignant; nevertheless, molecular analyses showed that many abnormalities of bladder cancer are linked (Hartmann A., Bertz S., 2011).

iii. Urothelial papilloma

A urothelial papilloma occurs when a fragile fibrovascular core is enclosed by urothelium, and cannot be differentiated from the normal urothelium. It is characterized by distinct papillary leaves sometimes branching but without combining. The urothelium has no abnormality and the superficial cells are most of the time particularly noticeable. Mitosis rarely occurs, and if it does, then it is in the basal cell layer. Urothelial papillomas are usually found in younger patients and children; however, the incidence of the lesions is low (1 to 2% of all bladder tumors) (Hartmann A., Bertz S., 2011).

iv. Inverted urothelial papilloma

As the name indicates, this lesion has an inverted growth, but with a rare incidence of cytological abnormalities. It has a smooth surface enclosed by a normal histological and cytological urothelium. The cords and nests migrate in a great manner

from the surface urothelium into the lamina propria but not into the muscle of the bladder wall. The islands and cords attached by anastomosis have similar width of papillary abnormalities that are migrated into the lamina propria. The central part of the cords has urothelial cells, and the periphery has a fence-like shape of basal lamina, stromal cells, and vessels. An abnormal change in the squamous cells and pseudoglandular or glandular differentiation can happen at times. There are no papillary constituents growing out of the organ (Hartmann A., Bertz S., 2011).

v. Papillary urothelial neoplasia of low malignant potential (PUNLMP)

This type of lesion is similar to urothelial papilloma but with more cellular proliferation making the urothelial wall thicker than normal. PUNLMP papillary structures are distinct, delicate, and in line with the urothelium with maintained polarity and little or no cytological abnormality (Hartmann A., Bertz S., 2011).

b. Malignant urothelial lesions

i. Urothelial dysplasia (UD low grade)

UD contains cytological and structural changes with no polarity or nuclear crowding. The ratio of nucleus to cytoplasm is augmented, and the nuclei have abnormal borders with moderate hyperchromatic chromatin and barely noticeable

nuclei. The multiplying cells exist in the basal fraction of the dysplastic urothelium and not in the whole thickened part of the urothelium (Hartmann A., Bertz S., 2011).

ii. Carcinoma In situ (CIS high grade)

Invasive urothelial carcinoma originates from CIS. CIS has a flat urothelium associated with a lot of cytological abnormalities and big hyperchromatic nuclei and extensive mitoses (Hartmann A., Bertz S., 2011).

iii. Noninvasive papillary urothelial carcinoma (NIUC), low and high grade

Low grade NIUC is associated with some modifications in the cytology and structure of the urothelium. High grade NIUC is associated with cytological abnormalities and a distinct difference in the architecture of the urothelium. Recurrent mitoses occur and the connections among the urothelial cells are no longer found (Hartmann A., Bertz S., 2011).

iv. Invasive bladder cancer

Urothelial carcinoma is classified as an invasive tumor when it aggressively intrudes further than the basement membrane. Invasive bladder cancer is categorized according to the TNM classification system (Hartmann A., Bertz S., 2011).

c. Transitional cell carcinoma (TCC) and squamous cell carcinoma (SCC)

Transitional cell carcinoma is the most common type of the urinary bladder cancer (90% of bladder malignancy). It is divided into 2 groups: superficial and muscle invasive transitional cell carcinoma. Superficial tumors constitute 80% of transitional tumors that can be managed by TURBT technique; however 15-20 % of TCC tumors are invasive and are controlled by more aggressive techniques (Lapham R.L. et al., 1997).

Squamous cell carcinoma of the urinary bladder occurs at a higher frequency in the Middle East and Africa than in the USA. Usually, this type is found in advanced stage, and is more common in males and elderly people. This type is characterized by clinically evident symptoms such as cystitis, abdominal and pelvic pain. Hematuria may also be a sign of squamous cell carcinoma. Also, a palpable pelvic or abdominal mass is obvious in these patients accompanied with a painful sensation (Donna E.H., Fergany A., 2012)

4. Bladder cancer staging

A healthy bladder epithelium is formed from a transitional cell lining known as urothelium (25). Under the urothelium lies the subepithelial connective tissue named the lamina propria which includes few smooth muscle fibers. TNM system is used to classify various bladder tumors. This system describes how far is the tumor invasion (T), the extension into local or regional lymph nodes (N), and the availability of distant

metastasis (M). T, N, and M categories are further divided into subcategories. Next to the lamina propria lies the detrusor muscle which is bordered by perivesical fat tissues. T classifications vary according to the tumor place existing in the urinary bladder tissues. T4a entails a tumor affecting neighboring organs such as the prostate, uterus, abdominal wall, etc… (Ploeg M., Witjes J.A., 2011). Table1 illustrates TNM classification of bladder cancer staging.

5. Diagnosis of bladder cancer

Some of the techniques that are commonly used to detect bladder cancer pathology and stage are urinary cytology and transurethral resection of bladder tumor.

a. Urinary cytology

Urinary cytology detects high grade urothelial carcinoma and carcinoma in situ (Ploeg M., Witjes J.A., 2011). In these cases, tumors and urine are in direct contact, and tumor cells tend to escape through the urine because of the absence of cell-cell interaction of tumor cells (Ploeg M., Witjes J.A., 2011). This test is highly specific, highly sensitive, and costs little (Kirkali Z. et al., 2005). However, this test is less sensitive for low- grade bladder cancer (Kirkali Z. et al., 2005).

b. Transurethral resection of bladder tumor (TURBT)

TURBT is a technique that is used to both test and treat superficial bladder cancer. Under general or local aneasthesia, visible tumors are resected for pathological testing, and to specify staging and grading (Allaparthi S., Balaji K.C., 2011).

TURBT can be done in two ways: staged and en bloc. Staged TURBT consists of 3 phases. Briefly, phase 1 and 2 are done when the tumor is resected gradually, going from one side to the other, starting superficially reaching the base. Even the tissues lying under the tumor are resected. The third phase is the removal of the tissue around the tumor base. All parts that are resected are analyzed together by a pathologist, or they can be sent separately so that each phase is analyzed solely. As for the en bloc technique, resection loops are used with a diameter of 1cm. So tumors less than 1cm can be removed as one sample. Because no fragmentations are done, protection of the tumor position is maintained in relation to bladder epithelium, and less cautery artifact at the tumor base is found; this technique is thought to provide more accurate pathological evaluation (Kirkali Z. et al., 2005).

6. Pathogenesis of bladder cancer

Until now, two main pathways describe the potential pathogenesis of bladder cancer, and a third pathway is still being studied (Hartmann A., Bertz S., 2011).

The first pathway describes the formation of noninvasive papillary tumors as originating from urothelial hyperplasia. The second pathway describes the origin of urothelial dysplasia and carcinoma in situ that constitute 20 to 30% of bladder tumors. The second pathway is characterized to be biologically aggressive whereby tumors can invade neighboring tissues such as the muscle (Hartmann A., Bertz S., 2011).

Many molecular changes were found for each pathway. The most common molecular changes are the loss of heterozygosity and the loss of chromosome 9 arms. The deleted arms of chromosome 9 contain 4 commonly deleted areas that contain crucial genes for cell-cell regulation which may be responsible for tumorigenesis (Hartmann A., Bertz S., 2011).

Also the most recurrent oncogene mutation in urothelial carcinoma is in the fibroblast growth factor receptor 3 gene(FGFR3) on chromosome 4p16.3, encoding a classical tyrosine kinase receptor making the enzyme subunit constantly active (Hartmann A., Bertz S., 2011).

7. Carcinogenesis

There is strong evidence that a mutation is the first important step in carcinogenesis. In 1983, Moolgavkar S.H. talked about the two-stage model of carcinogenesis. The first event is the change of a stem cell into intermediate cell by a rate of μ1, and the second event is the transformation of an intermediate cell into a malignant one by a rate of μ2 (Figure 1). An initiator is a molecule that increases μ1,

and a promoter molecule is the one that functions on intermediate cells to increase α2, the rate of division of intermediate cells, or decrease β2, the rate whereby intermediate cells are differentiated and dead, or does both. These events lead to the increase in the number of intermediate cells causing intermediate lesions. Moreover, the large number of intermediate cells will give the chance to one of the intermediate cells to change into a malignant one through the second stage of carcinogenesis. It is postulated that a carcinogen that acts on stage one of carcinogenesis is more dangerous than that working on stage two because, even if the carcinogen of stage one is ever stopped, the cancer incidence rate will not return to baseline due to the fact that the intermediate cells are already loaded in number. However, stopping individual exposure to phase two carcinogens reverses the cancer incidence rate back to pre-exposure levels (Moolgavkar S.H. 1983).

8. Bladder cancer and the environment

The environment in both developed and developing countries is embracing increasing levels of carcinogens that can cause bladder cancer (Ribbal M.J., 2011). Bladder cancer is a disease that can be expressed differently due to a different natural history (Kirkali Z. et al., 2005). Ribal M. states, "Bladder cancer is a disease of the environment and age" (Ribbal M.J., 2011). People are growing more in age, and this tends to increase the risk of cancer generation (Ribbal M.J., 2011).The exposure of individuals to toxic metabolites comes in many forms such as occupational exposure to

aromatic amines and dyes (Negri E., La Vecchia C., 2007). Other toxic compounds arise from artificial sweeteners, carcinogens in drinking water such as arsenics and chlorination byproducts, etc. (Negri E., La Vecchia C., (2007).

9. Risk factors associated with bladder cancer

Risk factors for bladder cancer are diverse but most importantly include smoking history and occupational exposure to toxins. The risk factors for SCC include Schistosomiasis (S. Haematobium), chronic bacterial infection, renal calculi, long term catheterization, as well as low fluid intake (Michaud D.D. et al., 1999), and possible risk factors include arsenic contamination of drinking water (Marshall G. et l., 2007). While the risk factors for TCC include occupational exposure to aromatic amines (Olfert S.M. et al., 2006).

a. Family history of bladder cancer

Familial bladder cancer is related to the genetic inheritance of cancer causing mutations causing the spread of cancer among relatives and family members. In bladder cancer, this fact is still considered rare when compared to other cancer types (Kirkali Z. et al., 2005). First- degree relatives who have bladder cancer individuals in their family have two times the risk of getting this disease in comparison with individuals with no family history of bladder cancer (Negri E., La Vecchia C., 2007). Moreover, the risk

increases when bladder cancer patients are young in age (Negri E., La Vecchia C., 2007).

b. Arsenic

Arsenic arises from nature. It is found in air, soil and water as organic and inorganic forms (Letasiova S. et al., 2012). Inorganic form of arsenic is the harmful type (Letasiova S. et al., (2012). Arsenate is the common form of arsenic existing in drinking water (Chung C.J. et al., 2013). In the body, arsenate is transformed to arsenite by reduction reaction (Chung C.J. et al., 2013). Then, arsenite is transformed to monomethylarsonic acid and dimethethylarsinic acid by a one carbon metabolism pathway and S-adenosylmethionine being the methyl donor (Chung C.J., Huang C.Y. et al., 2013). The whole process occurs in the liver, and these metabolites are cleared out from the body through urine (Chung C.J. et al., 2013).

It was previously thought that biotransformation of arsenic was a detoxification process. However, proofs are getting more in number stating that these metabolites may be more harmful than the inorganic arsenic types (Chung C.J. et al., 2013). Epidemiological studies have shown that individuals with poor arsenic methylation have higher risk of urothelial carcinoma, skin lesions and vascular diseases (Chung C.J., Huang C.Y. et al., 2013). Also, bladder cancer was associated with drinking water containing concentrations of arsenic exceeding 300-500ug/ml (Letasiova S. et al., 2012).

c. Aromatic amines

Aromatic amines are the only agents whose association with bladder cancer has been clearly defined but other agents also include polycyclic hydrocarbons and N-nitroso compounds. These amines include benzidine that was quickly banned from the industrial world and its derivative B-naphthylamine (BNA) which is associated with a 200-fold increase in the risk of bladder cancer. It is an aromatic amine resulting from the distillation of coal and coal tar that has been banned from Switzerland, Italy, Great Britain and Japan. In the United States, it is not banned but there is strict regulation of its usage (Olfert S.M. et al., 2006).

Certain metals (aluminum) as well as paints, dyes and industrial oils/cutting fluids that may contain aromatic amines and/or polycyclic hydrocarbons have thus been associated with bladder cancer (Olfert S.M. et al., 2006).

Most occupational exposures to the agents mentioned above occur among smokers and workers employed in aromatic amine manufacture, dyestuff manufacture and use, rubber and cable manufacture, textile and leather works, driving occupations, coal tar, aluminum and gas industries. These occupations include but are not limited to gas workers, roofers, chimney sweeps, aluminum smelter workers, coal miners, blacksmiths, truck drivers or individuals exposed to exhaust fumes, glass processors, brickyard workers, tar and asphalt workers (Olfert S.M. et al., 2006).

i. Dyes

Until the end of 1970, a number of aromatic amines that are known as urothelial carcinogens such as 4-aminobiphenyl (4ABP), BNA, and 4-chloro-o-toluidine were utilized in dyes such as hair dyes, paint and printing ink (Harling M. et al., 2010). 4-ABP and other molecules of related family are known to cause mutagenicity in vitro and carcinogenicity in animals. Accordingly, 4-ABP was prohibited from the US market in mid 1950s and from the European market in 1998 (Ros M.M. et al., 2012). Oxidation of the aromatic amines to N-hydroxyarylamines makes them carcinogenic. The quantity of 4-ABP differs in hair dyes, and the significant exposure of this compound is caused by the repetitive usage of hair dyes. According to the International Agency for Research on Cancer (IARC), hairdressers and barbers might be at larger risk of developing bladder cancer from their exposure to hair dyes (Ros M.M. et al., 2012).

The aromatic amine carcinogenic effect may show many decades later. So although several aromatic amines were removed, others were kept in the hair dye, hence hairdressers working before 1980 may need to be closely followed up for potential bladder cancer development. Many epidemiological studies have been done on bladder cancer in association to hair dresser occupation. However no uniformity and many times no statistically significant results were shown among them due to the lack of statistical power. A meta-analysis done by Harling et al included 42 studies and showed statistically significant results regarding the increase of bladder cancer risk associated

with hairdresser occupation when all studies are analyzed together as one study. Also the summary risk ratio (SRR) increased with the duration of working as a hairdresser (more than 10 years).There was no difference in the risk after adjustment for smoking. Furthermore, studies categorized as high quality and others as moderate quality resulted into same SRR (Harling M. et al., 2010).

ii. Rubber and cable manufacture

BNA exposure also occurs from rubber and cable manufacture, and at least 3 very large studies showed an association with bladder cancer and mortality (Monson R.R., Fine L.J., 1978; Sorahan T. et al., 2000; Veys C.A. 2004).

iii. Diesel exhaust exposure

Exposure to diesel exhaust is also a risk factor for bladder cancer especially in truck and bus drivers as well as gas station workers (Biofetta P., Silverman D.T., 2001; Schoenberg J.B. et al., 1984; Silverman D.T. et al., 1983).

iv. Heterocyclic amines

In 1977, Dr. Sugimura was the first to observe that heating protein containing food (such as meat) produces mutagens. These compounds are aromatic amines

containing an exocyclic amino group and nitrogen atoms inside the aromatic skeletal structure; they were hence called heterocyclic amines (HCA). HCAs are produced from amino acids, creatinine, and sugar molecules (Nagao M., 1999). 2-amino-3,8-dimethylimidazol[4,5-f] quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) are the most common types of HCAs and structurally similar to cancer forming aromatic amines(Jones R.F. et al., 1992). As a matter of fact, at least ten different HCAs were proven to stimulate the formation of tumors in the rat and the mouse (Nagao M., 1999). For instance, when mice ingested 50-800ppm of HCA, there was formation of tumors in several organs including the urinary bladder (Nagao M., 1999).

v. Cigarette smoking

"Cigarette smoking is a major cause of cancer of the urinary bladder" (Bryant M.S. et al., 1988). Also, it increases the risk 2 to 3 folds with a 20-30 year latency period (Baris D. et al., 2009). Cigarette smoke carries about 3800 chemicals which include several carcinogens such as polynuclear aromatic compounds, aromatic amines, and others (Bartsch H. et al., 1993). Smoking contains a mixture of carcinogens in the vapor phase attached to particulates (Talaska G. et al., 1991). The sources of smoke are three: mainstream, sidestream, and the exhaled smoke produced by the smoker (World Health Organization, 1986). Carcinogenic arylamines, such as 4-aminobiphenyl, β-naphthylamine and 2-amino-3-methyl-imidazol [4, 5-f] quinoline are also found in the

mainstream and side stream cigarette smoke (Hein D.W. et al., 1993). Also, polycyclic aromatic hydrocarbons, aromatic amines, and nitrosamines are several carcinogens in cigarette smoking (Talaska G. et al., 1991).

The association between bladder cancer and cigarette smoking has been obvious since many years, and was shown in many case-controls as well as cohort studies. Also, it is known that the risk increases by 2-4 times in smokers when compared to non-smokers; and the risk is also increased with the number of cigarettes and the duration of smoking (Negri E., La Vecchia C., 2007).

The story varies among different types of cigarettes. Black tobacco smoke is more carcinogenic than white tobacco smoke by a factor of two to three (Malaveille C. et al., 1989). This is due to the higher amounts of 4-aminobiphenyl and 2 naphthylamine found in the black tobacco smoke compared to white tobacco (Vineis P. et al., 1988). Furthermore, 4-aminobiphenyl hemoglobin adducts were 1.5 times greater in black tobacco smokers than white tobacco smokers and urinary mutagens were higher in black tobacco users compared to blond (Malaveille C. et al., 1989). Also, a study done by Samanic et al proved that the blond tobacco type is associated with late stage carcinogenesis (Samanic C. et al., 2006). This was apparent due to the direct decrease in the risk of bladder cancer in individuals who had quit smoking of blond tobacco for many years (about 25 years). This decline however stopped in three to four years and never reached baseline even after 25 years of stopping smoking. In contrast, a study done by Vineis P. illustrated that black tobacco has early and late-stage

carcinogenesis effect. This was obvious because black tobacco smokers showed increasing risk of bladder cancer when timing or age at starting to smoke was assessed, and a marked variation in risk when smoking was stopped, with a plateau that never reached the level of that of non-smokers (Vineis P. et al., 1988). Also, there was no difference with the intensity of smoke, and no safety was shown when filters were used.

d. Schistosomia Haematobium

The World Health Organization (WHO) reported that Shistosomia infection affects 200 million people. There are four types of schistosomes: S. *haematobium, Schistosoma Mansoni*, *Schistosoma Japonicum*, *Schistosoma Mekongi. S. Hematobium*. The latter is the one correlated with bladder cancer. This phenomenon was primarily found in Egypt by Fugresson in 1911. The eggs that are laid from these schistosomes in the urinary bladder form irritation and deposit fibrous tissue leading to the formation of carcinogenicity. Also, due to infection from schistosomes, the ova that are laid in the urinary bladder may aggravate a strong inflammatory reaction leading to the formation of oxygen-free radicals. These free radicals will either lead to genetic mutations or lead to the production of carcinogenic compounds; thus malignancy. Also, usually bacterial super infection escorts schistosomiasis leading to the formation of squamous cell neoplasia. Moreover, the bacteria escorting schistosomiasis lead to the production of N-nitrosamines (Zaghloul M.S., 2012).

e. Chlorinated water

Drinking water decontaminators contain a lot of highly reactive molecules that give rise to unwanted compounds when interacting with organic products. Chlorine is the most broadly used disinfectant in drinking water. It produces trihalomethanes, the most common disinfectant by-product (Villanueva C.M. et al., 2007).

A number of toxicological and epidemiological studies have shown an increase in bladder cancer risk associated with drinking chlorinated water. However, when the IARC tested the carcinogenicity of chlorine in drinking water on humans, there was no sufficient proof for this fact. This conclusion was based on ecological and death certificate studies. Moreover, a number of epidemiological studies done after 1991 which somehow filled the gap of limitations that were found in previous years and tested for chlorine consumption throughout human life, showed an increase in risk of bladder cancer. Nevertheless, and in 1999, IARC repeated the testing of several human disinfectant byproducts such as chloroform and other trihalomethanes and concluded that there was no enough proof for the carcinogenicity of these latter compounds (Villanueva C.M. et al., 2003). In conclusion, it is still inconclusive on whether disinfectant compounds possess a carcinogenic effect and whether they are associated with bladder cancer risk.

f. Coffee

Roasted coffee is a combination of more than a thousand chemicals among which many may modify cancer risk by numerous biological means. The main compound found in coffee is caffeine that was shown to both induce and depress tumors depending on the species and the phase of administration. In addition to caffeine, specific diterpenes found in coffee may have anticarcinogenic effects by stimulating phase II enzymes known to detoxify carcinogens, inhibiting phase I enzyme that lead to carcinogen activation, and inducing intracellular antioxidant defense mechanisms(Yu X. et al., 2011).

Several studies were done on coffee consumption and bladder cancer and results were conflicting whereby some showed protective effects and others showed a considerable increase in cancer risk. It is possible that potential increase in bladder cancer risk occurs when coffee is consumed in high amounts, i.e. more than 5 cups per day. It is important to note that it is difficult to test coffee alone in relation to bladder cancer as this habit is usually accompanied with smoking; a study done on nonsmokers to test for coffee consumption and bladder cancer risk was done and showed a little surplus of bladder cancer risk in nonsmokers who drank 10 or more cups of coffee per day (Sala M. et al., 2000).

g. Gender

Men are affected by this disease more than women by a factor of 3 or 4 (Kirkali Z. et al., 2005). This may be due to the fact that a higher percentage of males smoke when compared to females, and smoking is an important risk factor in bladder cancer (Negri E., La Vecchia C., 2007). Also, males tend to work in occupations whereby they are exposed to chemicals like aromatic amines that are also main risk factors for bladder cancer formation (Negri E., La Vecchia C., 2007). A very important factor that may explain the difference in bladder cancer risk between males and females is the hormonal effect. Hormonal receptors are found in the urinary bladder. In rodents, androgens stimulate bladder tumor growth while estrogens prevent tumor growth. Male animals develop more bladder cancer, and female parous animals develop bladder cancer more than nulliparous females. A study and metanalysis done by Carol A. et al showed that there is a lower risk of bladder cancer development in parous women and those who use postmenopausal hormonal therapy in comparison to nulliparous women and those who do not use hormonal therapy (Davis-Dao C.A. et al, 2011).

h. Age

Mainly, bladder cancer occurs in patients above 50 years of age. The analysis of California Cancer Registry revealed a high incidence of bladder cancer in elderly people (age 85 years or more). Aging, being a risk factor, is due to several reasons. First, age can cause the buildup of enough genetic and cellular incidents in the

neoplastic procedure. Also, growing in age exposes the individuals more intensely to possible carcinogens such as cigarette smoke especially that the clinical effect of these carcinogens appear at a later stage in life such as bladder cancer development. Moreover, the voiding abnormality found in old people increases the contact of the bladder to urinary toxins (Morgan T.M. et al., 2012).

B. Drug metabolism

Cellular homeostasis is maintained by numerous enzymes inside the human body (Kadlubar S., Kadlubar F.F., 2010). Drug metabolism permits the usage of nutrients as well as detoxification and clearance of dangerous metabolites (Kadlubar S., Kadlubar F.F., 2010). Drug metabolizing enzymes (DMEs) are categorized into two groups: phase I and phase II enzymes.

Phase I DMEs add a functional group to molecules rendering them more polar. Phase I reactions include reduction, hydrolysis, or cyclization/decyclization reactions. Cytochrome P450 (CYP) enzymes are one example of this group. Sixty per cent of biotransformation of FDA-approved drugs is utilized by this super family of metabolic enzymes. For some metabolites formed in this phase, their polarity is enough for their excretion. For others, their excretion requires phase II metabolism (Kadlubar S., Kadlubar F.F., 2010).

Phase II DMEs are known as detoxification enzymes as they aid in the excretion of metabolites. This happens by the addition of, for example, an acetyl group

to the functional group added by phase 1 DMEs. The most well-known families are the glutathione S-transferases (GST), UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT), and N-acetyltransferases (NAT) (Kadlubar S., Kadlubar F.F., 2010).

Significant inter-individual variability in the expression and activity of DMEs and transporters results in wide differences in the rate and extent of elimination of drugs among individuals. This variability is controlled by both genetic, disease states, as well as environmental factors. Accordingly for example, any genetic polymorphism that leads to a decreased activity of an enzyme that is involved in carcinogenic activation may be associated with decreased cancer risk, and the opposite is true.

C. Drug metabolizing enzymes and bladder cancer

In the past few decades, a rapid increase in cancer incidence was observed that has been attributed to the increased exposure to a variety of environmental carcinogens. It is known that many of the carcinogens require metabolic activation in order to realize their toxic effect, and that there exists wide inter-individual as well as inter-ethnic differences in susceptibility to cancer; therefore, it is supposed that genetic typing of the relevant DMEs may explain some of the variability in the cancer process, including bladder cancer (Ingelman S.M., 2001).

1. Metabolism of arsenic and bladder cancer risk

The metabolism of arsenic involves several enzymes such as CYP1A1, SULT1A1, GSTT1, and GSTM1. So any polymorphism in the mentioned enzyme coding genes leads to inter individual variability in the biotransformation of arsenic, hence an association with the risk of urothelial cancer (Chung C.J. et al., 2013).

A case-control study done by Lesseur et al in 2012 revealed the importance of genetic polymorphisms and arsenic exposure in association to bladder cancer risk (Lesseur C. et al., 2012). The study included 832 bladder cancer cases and 1191 controls. Levels of arsenic were measured using toenail clipping. All the subjects had the same water supply since 15 years. Also the exposure to arsenics was verified by gathering data on other sources containing arsenics used by the subjects. Thirteen SNPs were genotyped from candidate genes: *AQP3, AS3MT, GSTZ1, GSTP1, and GSTO2;* also deletions in *GSTM1 and GSTT1* were detected. Results showed that there was a significant interaction between arsenic and genetic variants in the high arsenic exposure group. One of the observations obtained was the interaction of the *GSTP1* gene with high levels of arsenic and their effect on bladder cancer risk with a *P*-value of 0.03. Also, there was a statistical significant result between homozygous mutant genotypes of *GSTP1* and bladder cancer risk: OR= 5.4 [95% CI: 1.5-20.2].

2. Metabolism of HCAs and bladder cancer risk

PhIP, a common HCA resulting from cooked food, is metabolized by CYP1A2, 1A1 and 1B1. The resultant hydroxy metabolites are then esterified and further activated by phase 2 enzymes such as NAT2 enzymes. Then this PhIP structure can bind to the guanine at carbon 8. It also reacts with cysteine residue of proteins (Nagao M., 1999). It is important to note that although PhIP and other HCAs are proven to be carcinogenic, little metabolic knowledge is found for their evaluation as potential urinary bladder carcinogens (Jones R.F. et al., 1992).

An interesting study done in 2012 by Lin et al proved that exposure to heterocyclic amines from meat intake is associated with bladder cancer risk (Lin J. et al., 2012). The study included 884 incident bladder cancer cases and 878 healthy controls. Results showed that groups who have been consuming the largest portion of red meat showed the most significant odds ratio for bladder cancer association compared to those who have been eating less red meat (lowest quartile of meat intake). Accordingly, the odds ratio of the 2^{nd} quartile is 1.17[95% CI; 0.87-1.58], followed by 3^{rd} quartile of odds ratio 1.47 [95% CI: 1.09-1.99)] followed by the 4^{th} quartile with odds ratio of 1.95 [95% CI: 1.41-2.68]. There was also a trend among the quantities of red meat consumption with a *P*-value<0.001.

3. Metabolism of arylamines from cigarette smoke and bladder cancer risk

a. Metabolism of arylamines

As shown in Figure 2, oxidation of arylamines first takes place in the liver by CYP1A2 producing N-hydroxyarylamine that is a carcinogenic metabolite. This metabolite can either enter in the blood circulation, be oxidized to nitrosarenes and form covalent adducts with hemoglobin, or enter the following pathway (Kadlubar F.F., Badawi A.F., 1995). Following CYP1A2 hydroxylation, the hydroxyarylamines can be excreted into the urine and then reabsorbed into the uroepithelium whereby O-acetylation occurs by NAT1 enzymes and leading to the formation of N-acetoxyarylamine metabolites. The latter metabolites are unstable and form spontaneous hydrolysis to arylnitrenium ions. These ions bind to DNA, forming covalent adducts that produce mutations in genes responsible for cellular proliferation, thus forming more malignant expression (Hein D.W. et al., 1993). It is important to note that N-hydroxy arylamine can solely form DNA adducts with an acidic urinary PH (Kadlubar F.F., Badawi A.F., 1995).

In addition to NAT1 acetylation, the hydroxylated arylamines can be metabolized by SULT and GST enzymes in the liver into products that are harmless and excreted in the urine (Kadlubar F.F., Badawi A.F., 1995). Furthermore, N-acetylation of arylamines with NAT2 tends to compete with N-oxidation; hence N-acetylation with NAT2 is considered a deactivation process of these carcinogenic arylamines, especially those that are linked to urinary bladder (Hein D.W. et al., 1993).

b. DMEs genetic polymorphisms and bladder cancer risk with aromatic amines

As shown above, cigarette smoke compounds are eliminated with NAT2 assistance; furthermore, hydroxylated aromatic amines are either deactivated by several DMEs including GSTs or activated into carcinogenic compounds by NAT1 enzymes in the uroepithelium (Moore L.E. et al., 2011).

It has been shown by a study of Yuan *et al* in 2008 on 731 bladder cancer cases and 740 controls that the risk of bladder cancer increased among individuals carrying the null or low activity genotypes of *GSTM1*, *GSTT1*, and *GSTP1*, and the strongest association was with the *GSTM1* null genotype (Moore L.E. et al., 2011). In addition, a study done by Cascorbi in 2001 demonstrated that the frequency of the combination of fast NAT1 and slow NAT2 acetylator was 5.96 times more in bladder cancer cases than controls with an occupational exposure history.

To date, only few genetic polymorphisms cancer association studies have been performed in the Middle East. Most of the research has been performed on Turkish people. The few studies done on the Middle Eastern population for *NAT2* polymorphism showed unusually high prevalence of *NAT2*5* and *NAT2*6* alleles in comparison to other ethnicities. Woolhouse *et al*. reported a frequency of 0.530 for *NAT2*5* and 0.210 for *NAT2*6* in the Emiratis (Woolhouse N.M. et al., 1997).

Therefore, an independent study for the Lebanese population is interesting and necessary, and in this research project, the focus was on *NAT2* genetic polymorphisms and the risk of bladder cancer.

D. N-acetyl transferases (NAT)

N-acetyl transferases are enzymes found in the cytosol of the human cell (Hein D.W. et al., 1995). They belong to the family of phase II drug metabolizing enzymes. Both genes, *NAT1* and *NAT2* are located on human chromosome 8p22 (Hein D.W. et al., 2000). The coding regions of *NAT1* and *NAT2* are similar by 87%. However, *NAT1* originates from one single exon, while *NAT2* results from the combination of the coding exon as well as a noncoding site of 100bp found at a distance of 8kb away from the translation start site (Hein D.W. et al., 2000).

1. Distribution of NAT enzymes

NAT1 and NAT2 differ in their distribution throughout the human body (Walker K. et al., 2009). While NAT1 is known to be distributed in fetal and adult cells, NAT2 is commonly distributed in liver cells and has a lesser activity in the small intestine, colon and other tissues (Walker K. et al., 2009). Also, *NAT1* is distributed in the urothelium (Cascorbi I. et al., 2001).

2. Acetylation process by NAT enzymes

NAT enzymes aid in the translocation of acetyl groups from different cofactors, such as acetyl-CoA, to the exocyclic amine group of a wide spectrum of arylamines and hydrazines (Hein D.W. et al., 1995). Acetylation is a two step-process:

first, the acetyl group from Acetyl-CoA is transferred to the cysteine residue at the active site of the NAT enzyme ending up with a free CoA (Walker K. et al., 2009). Then, the acetylated NAT donates the acetyl group to the amino group of the substrate leading at last to a free enzyme. NAT acetylates aromatic amines to amides and aromatic hydrazines to hydrazides (Walker K. et al., 2009).

There are no exclusive substrates for either NATs. However, table 2 illustrates some substrates that are preferably metabolized by NAT1 or NAT2 (Walker K. et al., 2009).

3. NAT2 genetic polymorphisms

NAT2 polymorphisms were discovered 40 years ago in patients taking isoniazid for the treatment of tuberculosis and who were responding to this drug differently in terms of toxicity. This difference was marked as "isoniazid acetylation polymorphism" for a long time until the effect of polymorphisms of *NAT2* also emerged with other drugs/carcinogens regarding their metabolism, toxicity and effectiveness (Hein D.W. et al., 2000).

To date, seven missense substitutions (*G191A, T341C, A434C, G590A, A803G, A845C, and G857A*) and four silent mutations (*T111C, C282T, C481T, C759T*) are recognized inside the coding region of *NAT2*. Different combinations of the mentioned substitutions lead to the generation of 26 *NAT2* alleles. *NAT2**4 is known as the wild type due to the absence of any of the mentioned substitutions. *NAT2*genotype

and phenotype differ a lot among different ethnicities. For example, while *G191A* substitution is found in the African Americans and native Africans, it barely exists in Caucasians (Hein D.W. et al., 2000) (Table 3). It is important to note that new polymorphisms and new *NAT2* haplotypes are continuously being identified.

4. Identification of acetylation phenotype of NAT2

Measurement of NAT2 activity can be performed by measurement of several substrates/metabolites of drugs such as isoniazid, sulfamethazine, procainamide in both *in vitro* and *in vivo* studies, whereby, the concentration of the parent drug and metabolites reflect enzyme activity and hence the phenotype. Caffeine introduced by Cascorbi in 1984, is the safest and easiest substrate utilized to measure NAT2 activity. Caffeine undergoes several oxidation/demethylation processes by CYP enzymes whereby an intermediate molecule is formed known as paraxanthine changing to 1-methylxanthine (Walker K. et al., 2009). Also, paraxanthine can be N-acetylated via NAT2 to 5-acetylamino-5-formylamino-3-methyluracil. Paraxanthine can also be metabolized through other pathways (Grant D.M. et al., 1984). Therefore, NAT2 activity is measured by obtaining the ratio of 5-acetylamino-5-formylamino-3-methyluracil to 1-methylxanthine. This ratio revealed a reliable association between *NAT2* genotype and enzyme activity (Walker K. et al., 2009).

The caffeine test tends to be superior over the other tests that were previously applied (Grant D.M. et al., 1984) and is currently the most commonly used *in vivo* tool to measure NAT2 phenotype status (Walker K. et al., 2009).

5. NAT2 genotype – phenotype relationship

Any individual carrying 2 alleles of rapid phenotype is considered to be a fast acetylator. Whereas individuals who carry 2 alleles of slow NAT2 phenotype are considered to be slow acetylators. Heterozygous genotype, having of one rapid and one slow allele, determines the intermediate acetylator (Heck J.E. et al., 2012) (Table 6).

6. NAT2 haplotypes distribution worldwide (figure 4)

A study published in 2008 on 41 populations worldwide revealed the distribution of several *NAT2* haplotypes (Sabbagh A. et al., 2008). The 7 common SNPs were genotyped for a total of 6,727 individuals.

One of the observations was the Linkage Disequilibrium (LD) of the 3 SNPs that constitute the *NAT2*5A* haplotype: *T341C, C481T*, and *A803G* in Europe, Asia, and America. Although *NAT2*5A* is relatively common in Europe, it is rarely found in populations of Asian origin. This LD is somehow lost in Sub-Saharan Africa, since there is low correlation between *A803G* and the two other SNPs. As a result, a higher frequency of *NAT2*12A* that is represented by *A803G* alone is shown there. Also, *NAT2*14* haplotype which is defined by the *G191A* SNP is seen in sub-Saharan Africa

only, while the *G857A* forming the *NAT2*7A* haplotype is usually found in Central America and Asia. On the other hand, the *G590A* SNP is relatively common in all the studied samples except in American Indians. Also, the *C282T* SNP is equally distributed among different populations (Sabbagh A. et al., 2008).

Sub-Saharan populations clearly display a wider diversity of haplotypes than Europeans or Asians; hence more combinations of *NAT2* haplotypes are formed. On the other hand, only *NAT2*4,*6A, and *7B* are the most common haplotypes observed in Asian populations. The Europeans haplotype diversity falls between those of Sub-Saharan and East Asian populations (Sabbagh A. et al., 2008).

*NAT2*4* haplotype coding for the fast acetylator enzyme is widely found in East Asia and forms 50% of global variation except for the Thai population. On the other hand, Africa and Europe display haplotypes coding for the slow acetylator phenotype more than the *NAT2*4* haplotype. Moreover, the fast acetylator phenotypes encoded by *NAT2*12A* and *NAT2*13* haplotypes are mainly found in Africa especially in Baka and Bakola Pygmies, and in similar distribution as in East Asia (Sabbagh A. et al., 2008). Table 10 shows the minimal allele frequencies (MAF) of the 7 common *NAT2* SNPs of the 28 studies in comparison to our study.

7. NAT2 haplotypes distribution in the Middle East

Few studies are available on *NAT2* haplotype distribution in Middle Eastern Arab populations (Table 12). For example, Woolhouse et al evaluated *NAT2* genotypes

and phenotypes on 106 unrelated Emirati subjects (Woolhouse N.M. et al., 1997). The most common *NAT2* allele was the *NAT2*5* allele with a frequency of 0.530 which was more frequent when compared to other ethnic groups. This was followed by the *NAT2*6* allele with a frequency of 0.210. Similarly, Hamdy et al genotyped for *NAT2* polymorphisms, among other genes, in 199 unrelated Egyptians (Hamdy S.I. et al., 2003). The *NAT2* variants were comparable to that of Caucasians (Cascorbi et al., 1995), and the most common haplotypes were *NAT2*4* with a frequency of 0.215, *NAT2**5 with a frequency of 0.497, and *NAT2**7 with a frequency of 0.260. The slow acetylator phenotype was the dominant phenotype with a frequency of 0.605.

In addition, Tanira et al genotyped 172 Omani individuals and showed that the distribution of *NAT2* polymorphisms is comparable to other neighboring countries like the United Arab of Emirates. Other than identifying the frequency of the 7 common polymorphisms and haplotypes of *NAT2*, an interestingly new variant was identified; it involved a new combination of polymorphisms: *282C>T*, *341T>C*, and *590G>A*. This variant was called *NAT2*5J* and was accepted by the *NAT2* nomenclature committee. The most frequent *NAT2* allele was *NAT2**5B with a frequency of 0.350. Again, the slow acetylator phenotype was the most common phenotype, followed by the intermediate acetylator, followed at last by the fast acetylator phenotype (0.570 vs. 0.380 vs. 0.050 respectively) (Tanira M. et al., 2003).

Most importantly was a large study conducted by Bu R. et al on mixed Arab populations coming from different countries, and focused on genotyping the 7 common

NAT2 genetic polymorphisms as well as other DMEs (Bu R. et al., 2004).The study included 487 healthy subjects: 95% of them were from Saudi Arabia, and 5% were from other Arab countries such as Jordan, Lebanon, Syria, and Yemen. Results for the studied population were similar to Caucasians (Cascorbi et al, 1995). For instance, the most frequent *NAT2* allele observed was the *NAT2**5 with a frequency of 0.470. In addition, the slow acetylator was the dominant phenotype (MAF=0.575) followed by the intermediate phenotype (MAF=0.329).

Table 12 shows the MAF of different *NAT2* alleles of the Arab studies in comparison to those of our study. Also, table 13 shows the different haplotypes and their corresponding phenotypes of the Arab studies in comparison to our results.

8. NAT2 phenotype and drug toxicities

NAT2 is involved in the acetylation of various aromatic amine drugs such as procainamide, dapsone, aminoglutethimide, and hydrazine drugs such as isoniazid, hydralazine, and phenelzine. Toxicity may hence appear even if the drug is administered in therapeutic doses. The reason for that would be the slow NAT2 acetylation status of the individual studied that affects the metabolism of the drug; hence leading to drug accumulation and toxicity (Weber W.W., Hein D.W., 1985).

For example, peripheral neuropathy with chronic administration of isoniazid was linked to slow acetylation of NAT2 enzyme. Another example is the association of lupus erythematosus toxicity with drugs containing primary unsubstituted aromatic

amines. For instance, and following many studies and observations including lupus cases of family members, a final conclusion was made on the association of slow NAT2 phenotype with the incidence of lupus erythematosus when hydralazine or procainamide were administered (Weber W.W., Hein D.W., 1985).

E. *NAT2* genetic polymorphisms and bladder cancer

The correlation between *NAT2* polymorphisms and carcinogenesis is established in the literature for different types of cancers. The strongest evidence comes from studies on bladder cancer. Previous studies have uncovered that individuals with a slow acetylator phenotype are at higher risk of developing bladder cancer (Rothman N. et al., 2007; Hein D.W. 2006; Moore L.E. et al., 2011; Marcus P.M. et al., 2000) and that this risk increases with smoking as NAT2 is involved in the deactivation of aromatic amines found in cigarette smoke. For instance, a study done by Yu M.C. et al in 1994 focused on several aims regarding NAT2 phenotype distribution among different races, and whether slow NAT2 acetylators possess higher levels of activated arylamines than fast NAT2 acetylators. The study involved 133 males living in Los Angeles aged 35 years or above and who were White, Black, or Asian. They were either nonsmokers or smokers with different intensity levels. One of the study conclusions was that a higher mean amino biphenyl hemoglobin adduct levels were observed among slow acetylators in comparison to those carrying fast acetylator phenotypes, and this

was independent of race and smoking levels. This proves that NAT2 slow acetylation phenotype could be one of the causes of bladder cancer.

Recently a study done by Klimcakova L. et al studied the relation of *NAT2* polymorphisms in addition to other risk factors such as age, gender, and smoking with bladder cancer in a Slovak population (Klimcakova L. et al., 2011). The study included 90 bladder cancer cases and 274 healthy controls matched by ethnicity. This study showed that NAT2 slow acetylator phenotype was significantly associated with bladder cancer risk. Also, this increasing risk of bladder cancer and slow acetylator phenotype was shown in males and in older ages, and it additionally increased with a *NAT2*5B/*6A* genotype carriers. Moreover, smoking, a significant risk factor of bladder cancer showed a statistically significant result with slow NAT2 phenotype particularly with the carriers of *NAT2*5B/*6A.* Yet, the literature is sometimes conflicting, and results may be negative in different populations. For example, Mittal et al studied whether *NAT2* genotypes and smoking are risk factor candidates for bladder cancer in a sample of people from rural India. The study included 110 controls and 101 bladder cancer cases diagnosed with transitional cell carcinoma of different grades. Results showed that there was no association between bladder cancer risk development and slow/fast acetylator phenotypes of *NAT2* in comparison to controls, even when including smokers only (Mittal R.D., et al., 2004).

In order to try to have more conclusive results, meta-analyses were conducted (Table 11). For instance, a case-control bladder cancer study was done in 18 hospitals

of different regions in Spain. It was done to see whether there is a correlation between *NAT2* acetylation status and other genetic determinants with bladder cancer risk. The cases were newly diagnosed with carcinoma of the urinary bladder (1998 till 2001) and aged between 21 and 80 years. The controls were chosen from the hospitals coming for several reasons. They were chosen to be non-exposed to tobacco. Then cases/controls were matched according to different criteria. The final number of cases enrolled in the study was 1150 and 1149 for that of the controls. The analysis of this study was further joined with a meta-analysis of previous studies (total=31 studies for NAT2 association and total=22 studies for smoking/NAT2 interaction).Analysis of the Spanish data showed that when taking rapid/intermediate acetylator phenotypes as a reference, NAT2 slow acetylators had a higher risk of bladder cancer (OR:1.4 [95%CI=1.2-1.7]), and this was more with smokers compared to nonsmokers (OR: 1.8[95%CI=1.2-2.8]). As for the meta-analysis, a statistically significant result was reported for NAT2 association: OR= 1.400 [95%CI=1.200-1.600]; furthermore, interaction of NAT2 and smoking status analysis showed statistical significance of OR: 1.200 [95%CI=1.100-1.500] (Garcia-Closas M. et al., 2005).

Another interesting meta-analysis was done by Marcus et al and shed the light not only on the association of slow NAT2 phenotype and bladder cancer risk, but also on the variation of this association among different ethnic populations (Marcus P.M. et al., 2000). Twenty two case-control studies published between the period of 1979 and 1998 were analyzed in this study. Studies where subjects were exposed to aromatic amines or arsenics were excluded. The regions participating in this study were mainly

from European countries, but also included Asia, USA, India and the Middle East. The number of cases and controls enrolled was 2496 and 3340 respectively. The odds ratio was conducted by pooling the populations together, sub pooling them excluding those of India and the Middle East since each had only one study, and also sub pooling the population to include Caucasians only. Results showed that the association of NAT2 slow acetylator phenotype and bladder cancer was found in some, but not all populations. The highest odds ratio reported was that of that of the 3 studies in Asia (OR 2.1[95%CI=1.2-3.8]), followed by that of the 13 studies in Europe (OR 1.4[95%CI=1.2-1.6]), then followed by that of the 3 studies in USA with an odds ratio of no statistical significance (OR 0.9[95%CI=0.7-1.3]) although the population size of both USA (North Carolina study) and Europe was large. Therefore, the *NAT2*/bladder cancer association varies among different geographic areas. According to this study, the reason behind this could be the variable exposure to aromatic amines between different regions, to include smoking habits or occupational exposure. This however does not explain the differences in the results between Europe and USA. Also another reason could be the potential role of other genetic factors such as *NAT1*.

To our knowledge, and except for the study mentioned in the meta-analysis, no other data are available from any other Middle Eastern Arab population.

CHAPTER II

SPECIFIC AIMS

The correlation between *NAT2* polymorphisms and carcinogenesis is established in the literature for different types of cancers. The strongest evidence comes from studies on bladder cancer. Previous studies have uncovered that individuals with a slow acetylator phenotype are at higher risk of developing bladder cancer (Rothman N. et al., 2007; Hein D.W. 2006; Moore L.E. et al., 2011; Marcus P.M. et al., 2000) and that this risk increases with smoking.

The increased incidence of bladder cancer in Lebanese patients seen compared to the figures from neighboring regions delineates a need to search for possible environmental, lifestyle or genetic risk factors in the Lebanese population.

To date, only few genetic polymorphisms cancer association studies have been performed in the Middle East. Most of the research has been performed on Turkish people. The few studies done on the Middle Eastern population for *NAT2* polymorphism showed unusually high prevalence of *NAT2*5* and *NAT2*6* alleles in comparison to other ethnicities. Woolhouse *et al*. reported a frequency of 0.53 for *NAT2*5* and 0.21 for *NAT2*6* in the Emiratis (Woolhouse N.M. et al., 1997).

Therefore, an independent study for the Lebanese population is interesting and necessary.

In this study, we aimed at measuring the frequencies of several *NAT2* genetic polymorphisms in a sample of Lebanese patients with bladder cancer (cases) and Lebanese patients or healthy individuals without bladder cancer (controls), and test if these polymorphisms are predictors for the development of bladder cancer in the Lebanese population. The following hypothesis was proposed:

NAT2 slow acetylator phenotype is associated with an increased risk of bladder cancer in patients who ever smoked.

CHAPTER III

MATERIALS AND METHODS

A. Human subjects

This study includes 100 bladder cancer patients treated and followed up by physicians at AUBMC and 100 patients who were being treated for other diseases at AUBMC. Participants were asked to fill a questionnaire regarding family history, medical history, and history of exposure to potential bladder cancer risk factors such as smoking history and frequency, alcohol and fat intake, use of Chinese herbal tea, intake of caffeine and matte, meat intake, long term use of chlorinated water, exposure to arsenic, fluid intake, analgesic abuse, and exercise. Chart review was also performed for the cases for histologic diagnosis, grading and staging.

The study was approved by the Institutional Review Board of Human Rights. All subjects accepted to sign an informed consent during recruitment.

B. Experimental methods

1. Sample processing

About 1ml of whole blood was withdrawn and processed by dividing the available volume into aliquots that were stored in eppendorf tubes at -80°C until DNA isolation.

2. DNA isolation

DNA was extracted from 300ul whole blood and stored at -20°C until genotyping. The DNA extraction method was done according to the guidelines of the Flexigene DNA kit (Qiagen GmBH D-40724 Hilden) with minor adjustments. Quantity and purity of DNA were measured using the Nanodrop spectrophotometer.

3. Genotyping

Genotyping was performed by restriction fragment length polymorphism (RFLP) based on Deitz et al., 2000.

a. Initial polymerase chain reaction

Every reaction required 3ul of DNA (approximately 50-250ng), 25ul of ready Taq mix, and 0.8ul of forward and reverse primer each. Nuclease–free water (approximately 20.4ul) was added till 50ul final volume is reached. A No Template Control sample was prepared in every PCR run to confirm the absence of any contamination. The samples were run on the thermal cycler according to the following protocol:

Stage 1: Step 1 94ºC for 5 minutes

Stage 2 consisting of 35 cycles: Step 1: 94ºC for 1 minute

Step 2: 55ºC for 1 minute

Step 3: 72ºC for 1 minute

Stage 3: Step 1 72ºC for 5 minutes

Stage 4: Step 1 4 ºC infinite hold

Before proceeding with the RFLP experiment, some non-cut PCR products were run on 2%agarose for 50 minutes. An 866 bp band was clearly detected confirming that the resulting PCR product is surely the *NAT2* gene.

b. Restriction Fragment Length Polymorphism (RFLP)

G191, A434C, and *C481T* polymorphisms of *NAT2* were attained by digesting the PCR product with MspI and KpnI restriction enzymes. Sample digestion required a mixture of 15ul PCR product, 1ul of MspI and KpnI each, 4ul buffer solution, and 9ul nuclease-free water in a total volume of 30ul. The digested samples were incubated for 4 hours at 37 ºC.

T111C, G590A, C759T, and *G857A* were detected by digesting *NAT2* PCR product with TaqI and BamHI restriction enzymes. The digestion mixture is the same as the previous digestion method but the incubation temperature is 37 ºC kept for 4 hours followed by 65 ºC kept for another 4 hours.

C282T and *A845C* polymorphisms were obtained by digesting PCR product with FokI and DraIII restriction enzymes. Also, the digestion mixture is similar to above mixtures but the incubation temperature is 37 ºC kept for 4 hours.

c. Nested PCR and RFLP

T341C and *A803G* polymorphisms of *NAT2* were detected by nested PCR. Every reaction required a 20ul total volume containing 1ul *NAT2* amplified PCR product, 9ul ready taq mix (RED TAQ mix), and 0.4ul of forward and reverse primer each, and 9.2ul of DEPC water. Also, a No Template Control sample was prepared in every PCR run to confirm the absence of any contamination. The samples were run on a PCR thermal cycler. Several optimization steps were performed until the following protocol was used:

Stage 1: Step 1 94ºC for 5 minutes

Stage 2 consisting of 35 cycles: Step 1: 94ºC for 1minute

Step 2: 58ºC for 1minute

Step 3: 72ºC for 1minute

Stage 3: Step 1 72ºC for 5 minutes

Stage 4: Step 1 4 ºC infinite hold

Few non cut nested PCR products were run on 1%gel agarose gel for 1 hour. A 141 base pair was obtained confirming that the *T341C* allele was amplified.

The *T341C* amplified nested PCR product was digested with AciI restriction enzyme. The *A803G* amplified nested PCR product was digested with DdeI restriction enzyme. The digestion cocktail for both polymorphisms was a 30ul volume containing 15ul of nested PCR, 1ul AciI/ DdeI, 2ul digestion buffer, and 12ul nuclease-free water. The product was incubated for 4 hours at 37 ºC.

The following table shows the primers' sequences of each PCR experiment.

Experiment	Primer	Sequence
NAT2 PCR	forward	5'-GGCTATAAGAACTCTAGGAAC-3'
	Reverse	5'-AAGGGTTTATTTGTTCCTTATGAAC-3'
Nested *T341C* PCR	forward	CACCTTCTCCTGCAGGTGACCG-3'
	Reverse	5'-TGTCAAGCAGAAAATGCAAGGC-3'
Nested *A803G* PCR	forward	5'-TGAGGAGAGGTTGAAGAAGTGCT-3'
	Reverse	5'-AAGGGTTTATTTTGTTCCTTATTCTAAAT-3'

d. Bands analysis according to the enzyme restriction site (figures 5, 6, 7, 8, 9)

AciI cuts the 141bp into 120 and 21 bp if T is substituted by C at position of 341 of the 866 bp coding region. If there is no mutation, then the restriction site will be lost yielding a 141bp band. If the two alleles are found, the 141,120, and 21 bp bands will be obtained.

DdeI cuts the 120 bp band into 97 and 23 bps if A is substituted by G at position of 803 on the 866 bp coding region. If no mutation exists, then a 120 bp band will be obtained since DdeI will lose its restriction site in this case. Heterozygous genotype AG will yield 120, 97, and 23 bp bands.

As mentioned previously FokI and DraIII are used to detect the polymorphism at *C282T* and *A845G* sites respectively. If C is substituted by T at position 282, then FokI loses it restriction site yielding 667 and 199 bp bands. If no mutation is found, then 429, 238, and 199 bp bands will be obtained. DraIII only cuts when A is substituted by C at position 845 since this mutation adds a DraIII restriction site cutting the 199bp band into 153 and 146 bp bands.

Using these two enzymes together aids in detecting the two polymorphisms at the same time. MspI is used to detect the mutations of *G191A*, *A434C*, and KpnI is used to detect the mutation of *C481T*. Using them together will help us detect the three mutations at the same time. If G is replaced by A at position 191, then 1 MspI site will be lost yielding 416, 384, and 66 bp bands. If the genotype is GG then the MspI restriction site will be regained yielding 416, 291, 93, and 66 bp bands. If A is replaced by C in position 434, then additional MspI site will be obtained inside the 191bp band yielding 416, 244, 47, 93, and 66 bp bands. If C is substituted by T at position 481, then KpnI restriction site will be lost, thus yielding 707, 93, and 66 bp bands.

TaqI is used to detect the mutations at *T111C*, *G590A*, and *C759T* positions. BamHI is used to detect the mutation of *G857A.* When used together, the

polymorphisms at the previous mentioned sites will be detected at the same time. A TT genotype at position 111, will yield a 332, 226, 170, 98, and 40 bp bands. While a CC genotype will bring an additional TaqI restriction site yielding 252, 80, 226, 170, 98, and 40 bp bands. When G is replaced by A at position 590, one TaqI site will be lost yielding 332, 396, 98, and 40 bp bands. If C is substituted by T at position 759, then a TaqI site will be also lost but at a different position yielding 332, 226, 268, and 40 bp bands. When the genotype is AA at position 857, then BamHI will lose its restriction site. Thus, 332,226,170, and 138 bp bands will be obtained.

4. Data analysis

Data were analyzed using SPSS. Baseline demographics of cases and controls including mean age, sex, and smoking status were computed and compared between cases and controls using Student-t test and Fisher exact test as applicable (Table 7). Minimal allele frequencies (MAF) of all *NAT2* SNPs were calculated and checked for Hardy Weinberg Equilibrium (HWE) using an online calculator.

Haplotypes were manually allocated using excel and based on previously reported data (http://www.louisville.edu/medschool/pharmacology/NAT html; Sabbagh A. et al., 2008; Cascorbi I. et al., 1995) (Table 5). *C282T, C481T, and A803G* SNPs were not included in the haplotype allocation since no variant alleles were found; therefore, we were left with 7 SNPs: *C282T, G191A, C481T, G590A, G857A, A803G,* and *T341C* (Table 4). Haplotypes were then lumped into 2 phenotype groups: fast

acetylators that included those who carry at least one *NAT2*4* allele, and the rest were categorized as slow acetylators (http://www.louisville.edu/medschool/pharmacology/NAT html; Sabbagh A. et al., 2008; Cascorbi I. et al., 1995) (Table 6). We did not include an intermediate acetylator phenotype because of the small sample size.

Association analysis was performed by first comparing the frequency of the 2 different phenotypes in cases vs. controls using Fisher exact test (Table 8). Then, a logistic regression was performed to include NAT2 phenotype and the demographic characteristics (Table 9).

Odds ratios (OR) and *P* values were used as indicators of statistical significance. $P \leq 0.05$ and OR whereby the 95% confidence interval does not include 1 indicate a statistical significant result.

CHAPTER IV

RESULTS

Demographic data

The number of control subjects enrolled in this study was 81 while the number of bladder cancer cases was 49. A significantly higher percentage of bladder cancer cases were males (79.6% vs. 53.1%) and smokers (67.3% vs. 50.6%) with a *P* value of 0.002 and 0.045 respectively. There was no difference in the mean age between cases of controls (70.14 ± 11.867 vs. 71.95 ± 17.978 respectively) where the *P* value was 0.533 (Table 7).

1. Genotyping and phenotyping results:

Genotyping using RFLP was successful for the 10 *NAT2* SNPs (*G191A, T341C, A434C, G590A, A803G, G857A, T111C, C282T, C481T,* and *C759T),* and examples are shown in figures 5, 6, 7, 8, and 9.

All SNPs were in HWE, and the corresponding MAFs identified in this sample population were in concordance with those of Caucasians as well as the other studies done in the Middle East. Seven haplotypes were computed: *NAT2**4, *5A, *5B, *5C, *6A, *7B, and *13. *NAT2*5B* was the most common (41.975%), followed by *NAT2*6A* (28.395%), followed by *NAT2*4*(19.136) (Table 12). Then 15 different haplotype

combinations were found: *NAT2 *4/*4, *4/*5B, *4/*5C*, and **4/*6A* that were coded as fast acetylators, and **5A/*5C, *5B/*5B, *5B/*5C, *5B/*6A, *5C/*6A, *5B/*7B, *6A/*7B, *6A/*6A, *6A/*13,* and **13/*13* that were coded as slow acetylators. In the, analysis, we coded NAT2*13/*13 as a slow acetylator which is wrong. However, this mistake will not have a big effect on the total analysis, since only one subject (control) had this genotype (Table 13). *NAT2*5B/*6A* was the most common genotype reported in this study which is in concordance to that of the Caucasians as well as the studies done in the Middle East.

2. Association analysis

The percentage of slow acetylators was 65% in cases and 62% in controls, and this was not statistically significantly different with and an odds ratio 1.199 [95% CI: 0.575-2.570)] (Table 8). Logistic regression results showed that only sex was statistically significantly associated with bladder case whereby females were at a lower risk of bladder cancer: OR= 0.31(females=0, males =1) and 95% confidence interval 0.131-0.736. *NAT2* phenotype, age, and smoking did not show any significant differences among bladder cases and controls by logistic regression (Table 9).

CHAPTER V

DISCUSSION

The reason for shedding light on bladder cancer goes to the high incidence of this disease in the Middle East, especially in Lebanon. Several risk factors are associated with bladder cancer development such as cigarette smoking and slow *NAT2* phenotype. NAT2 enzyme, being one of the phase II drug metabolizing enzymes, plays a key role in detoxifying many xenobiotics, chemicals, and most importantly the carcinogenic arylamines from cigarette smoking.

A. Cigarette smoking and bladder cancer

This study proved that smoking is an important factor for bladder cancer development. The percentage of smokers in bladder cancer cases was higher than that of the controls with a *P*- value of 0.045. This shows that our study results are consistent with other association studies regarding cigarette smoking and bladder cancer risk. For example, a study done in 2001 by Castelao J.E. et al. proved the high association of cigarette smoking to bladder cancer risk. They have shown that smokers have 2.5 times higher risk of bladder cancer than non-smokers with and an odds ratio of 2.5 [95% CI: 2.1-3)] (Castelao J.E. et al., 2001).

B. Gender and bladder cancer

Similarly, male gender showed a statistically significant association with bladder cancer risk with a *P*-value of 0.002 being more significant than cigarette smoking habit. The percentage of males in the cases was 79.6, while the percentage of males in controls was 53.1. This confirms the results of other studies that also showed a significant association with male gender and bladder cancer risk. For example, a study done on 1269 bladder cancer patients highlighted male gender as a risk factor for bladder cancer incidence. The outcome was that males tend to develop bladder cancer more than females with a ratio of 2.2:1 (Hortsmann M. et al., 2008).

C. *NAT2* and bladder cancer

The association of NAT2 phenotype with bladder cancer risk was studied in many countries worldwide. Several studies were affirmative about the significant association of bladder cancer with the slow *NAT2* phenotype especially if other factors were additionally studied, mainly smoking habit. Our study included a total number of 130 subjects (81 controls and 49 cases); however, no statistical significance was reported regarding *NAT2* slow phenotype and bladder cancer development. This is most probably due to our relatively low sample size and insufficient power. This is especially relevant knowing that the expected effect size is not too large. For instance, and as shown in Table 9, all ORs did not exceed 2 and most of them were close to 1.

Our results may also be true as no association was found with other investigators on different populations. For example, a study done in India by Mittal et al in 2010 didn't show any statistical significance as well (Table 11). Another analysis done in 1997 by Okkels H. et al also didn't reach to an association between *NAT2* genotype and bladder cancer. The study analyzed the association of polymorphisms of *NAT1* and *NAT2* and smoking with bladder cancer risk. The effect of *NAT2* slow acetylator genotype solely on bladder cancer risk was not statistically significant. Also, when adjusting for age, gender, and sex, *NAT2* genotype was not statistically significant regarding bladder cancer risk (Okkels H. et al., 1997).

Although no statistically significant difference regarding NAT2 slow acetylator phenotype and its association to bladder cancer was shown in this study, the high frequency of slow NAT2 acetylator phenotype is very relevant in the Lebanese population. Therefore, *NAT2* genotype and its effect on enzyme activity should be further studied.

D. Limitations

Limitations are always found in every study. The sample number of the studied population (81 controls and 49 cases) is so far too low, hence further recruitment is needed to reach statistical power for the hypothesis tested (target sample size= 300 cases and 300 controls). Another limitation includes the fact that we did not measure actual enzyme activity as this may differ among populations, even within the same

genotype. This can be done by performing the caffeine test for every subject and measuring the caffeine metabolite in urine samples. Moreover, other risk factors could have been analyzed to see their effect on bladder cancer (such as coffee consumption, arylamine exposure other than smoking, further stratifying people who smoke into subgroups such as moderate and heavy, etc…). These risk factors are being collected but were not analyzed in this study. Finally, we did not analyze pathology data as many data points are missing, and further data entry is in progress.

CHAPTER VI

CONCLUSION

This is the first study done in Lebanon emphasizing the association of *NAT2* polymorphisms with bladder cancer risk. Although no significant statistical results were found yet, this study shows how prevalent is the NAT2 slow acetylator phenotype in the Lebanese population. Also, this study constitutes a solid base for upcoming studies to highlight the importance of *NAT2* polymorphisms and bladder cancer. Results of this study, after reaching adequate statistical power, may guide practices for prevention of bladder cancer.

Table 1. TNM classification of the urinary bladder cancer, UICC 2002 (Ploeg M., Witjes J.A., 2011)

T-primary tumor	**Disease extend**
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Ta	Non-invasive papillary carcinoma
Tis	Carcinoma in situ: “flat tumor”
T1	Tumor invades subepithelial connective tissue
T2	Tumor invades detrusor muscle
T2a	Superficial muscle (inner half)
T2b	Deep muscle (outer half)
T3	Tumor invades perivesical tissue
T3a	Microscopically
T3b	Macroscopically
T4a	Tumor invades prostate, uterus, vagina, pelvic, or abdominal wall Prostate, uterus, or vagina
T4b	Pelvic or abdominal wall
N-lymph nodes	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single lymph node ≤ 2 cm in greatest dimension
N2	Metastasis in a single lymph node > 2 cm but ≤5 cm in greatest dimension, or multiple lymph nodes ≤ 5 cm in greatest dimension
N3	Metastasis in a lymph node > 5 cm in greatest dimension
M-distant metastasis	
Mx	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Table 2. Preferred substrates for human NAT enzymes (Walker K. et al., 2009)

NAT1	**NAT2**
p-Aminobenzoic acid (PABA)	Isoniazid
p-Aminosalicylic acid (PAS)	Sulfamethazine
Sulfamethoxazole	Dapsone
Sulfanilamide	Hydrazine
2-Aminofluoxene	Aminoglutethimide
Caffeine	Procainamide
	2-Aminofluoexene
	Caffeine

Table 3. Human *NAT2* alleles (Hein D.W. et al., 2000; http://www.louisville.edu/medschool/pharmacology/ consensus-human-arylamine-n-acetyltransferase-gene-nomenclature)

Allele (haplotype)	Nucleotide Change(s)	Amino Acid Change(s)	Phenotype
NAT2*4	None	None	Fast
NAT2*5A	341T>C; 481C>T	Ile114Thr	Slow
NAT2*5B	341T>C; 481C>T; 803A>G	Ile114Thr; Lys268Arg	Slow
NAT2*5C	341T>C; 803A>G	Ile114Thr; Lys268Arg	Slow
NAT2*5D	341T>C	Ile114Thr	Slow
NAT2*5E	341T>C; 590G>A	Ile114Thr; Arg197Gln	Slow
NAT2*5F	341T>C; 481C>T; 759C>T; 803A>G	Ile114Thr; Lys268Arg	Slow
NAT2*5J	282C>T ;341T>C; 590G>A	Ile114Thr; Arg197Gln	Slow
NAT2*6A	282C>T ; 590G>A	Arg197Gln	Slow
NAT2*6B	590G>A	Arg197Gln	Slow
NAT2*6C	282C>T ; 590G>A; 803A>G	Arg197Gln; Lys268Arg	Slow
NAT2*6D	111T>C; 282C>T; 590G>A	Arg197Gln	Slow
NAT2*7A	857G>A	Gly286Glu	Slow
NAT2*7B	282C>T; 857G>A	Gly286Glu	Slow
NAT2*12A	803A>G	Lys268Arg	Fast
NAT2*12B	282C>T; 803A>G	Lys268Arg	Fast
NAT2*12C	481C>T; 803A>G	Lys268Arg	Fast
NAT2*13	282C>T	None	Fast
NAT2*14A	191G>A	Arg64Gln	Slow
NAT2*14B	191G>A; 282C>T	Arg64Gln	Slow
NAT2*14C	191G>A; 341T>C; 481C>T; 803A>G	Arg64gGln; Ile114Thr; Lys268Arg	Slow
NAT2*14D	191G>A; 282C>T; 590G>A	Arg64Gln; Arg197Gln	Slow
NAT2*14E	191G>A; 803A>G	Arg64Gln; Lys268Arg	Slow
NAT2*14F	191G>A; 341T>C; 803A>G	Arg64Gln; Ile114Thr; Lys268Arg	Slow
NAT2*14G	191G>A; 282C>T; 803A>G	Arg64Gln; Lys268Arg	Slow
NAT2*17	434A>C	Gln145Pro	Slow
NAT2*18	845A>C	Lys282Thr	Fast

Table 4. Minimal allele frequency of the *NAT2* SNPs in this current study

SNP	rs number	Restriction Enzyme	MAF%	HWE *P* value
282 C>T	1041983	FokI	32.099	0.860
191 G>A	1801279	MspI	0	N/A
434 A>C	N/A	MspI	0	N/A
481 C>T	1799929	KpnI	42.593	0.890
111 T>C	N/A	TaqI	0	N/A
590 G>A	1799930	TaqI	28.395	0.772
759 C>T	N/A	TaqI	0	N/A
857 G>A	1799931	BamHI	2.469	0.820
803 A>G	1208	DdeI	47.531	0.894
341 T>C	1801280	AciI	48.148	0.921

MAF= Minimal Allele Frequency
HWE=Hardy Weinberg Equilibrium

Table 5. Common *NAT2* haplotypes (Cascorbi I. et al., 1995)

Allele	**Nucleotide at position**						
	G191A	***C282T***	***T341C***	***C481T***	***G590A***	***A803G***	***G857A***
***4**	G	C	T	C	G	A	G
***5A**	G	C	C	T	G	A	G
***5B**	G	C	C	T	G	G	G
***5C**	G	C	C	C	G	G	G
***6A**	G	T	T	C	A	A	G
***7B**	G	T	T	C	G	A	A
***13**	G	T	T	C	G	A	G

Table 6. NAT2 haplotypes and their corresponding phenotypes among cases and controls in this study

Genotype	Phenotype	Cases N=49	Controls N=81
***4/*4**	Fast acetylator	1	3
***4/*5B**	Fast acetylator	9	11
***4/*5C**	Fast acetylator	1	4
***4/*6A**	Fast acetylator	6	10
***4/*7B**	Fast acetylator	2	0
***13/*13**	Fast acetylator	0	1
***5A/*5C**	Slow acetylator	0	1
***5B/*5B**	Slow acetylator	6	15
***5B/*5C**	Slow acetylator	0	3
***5B/*6A**	Slow acetylator	17	21
***5C/*6A**	Slow acetylator	0	1
***5B/*7B**	Slow acetylator	1	3
***6A/*7B**	Slow acetylator	0	1
***6A/*6A**	Slow acetylator	6	6
***6A/*13**	Slow acetylator	0	1

Table 7. Demographic data of subjects enrolled in this study

Demographic data		**Cases N=49**	**Controls N=81**	***P*-value**
Age	Mean ± SD	70.14 ± 11.867	71.95 ± 17.978	0.533
Sex	Male	39(79.592)*	43(53.086)*	**0.002**
	Female	10(20.408)	38(46.914)	
Smoking status	Smoking	33(67.347)	41(50.617)	**0.045**
	Non-smoking	16(32.653)	40(49.383)	

*N (%)

Table 8. NAT2 phenotype distribution among cases and controls

NAT2 acetylation	Cases N=49	Controls N=81	*P*-value	OR(95 % CI)
Fast acetylator	38.776	34.568	0.382	1.199 (0.575-2.570)
Slow acetylator	61.224	65.432		

Table 9. Regression modeling association between bladder cancer and the risk factors in this study

Risk factor	Odds ratio	95% Confidence interval
Sex*	**0.31**	**0.131-0.736**
Age	0.992	0.968-1.015
Smoking	1.45	0.656-3.207
Phenotype	1.2999	0.595-2.834

*: Coding was used as such: males=0 females=1

Table 10. Number and minimal allele frequency of *NAT2* haplotypes of the 28 previously studied populations in comparison to our study sample (Sabbagh A. et al., 2008)

Ethnicity	Country	Sample size	Reference	NAT2*4	NAT2*5A	NAT2*5B	NAT2*5C	NAT2*6A	NAT2*7B	NAT2*13
Sub-Saharan Africa	Tswana South Africa	101	Lukhnova et al 2002	27(0.134)#	1(0.005)	47(0.233)	17(0.084)	34(0.168)	0	13(0.064)
	Ateke Bantu Gabon	50	Patin et al 2006	10(0.100)	1(0.01)	37(0.370)	3(0.030)	16(0.160)	2(0.020)	6(0.060)
	Baka'da Pygmies Gabon	40	Patin et al 2006	8(0.050)	0	4(0.05)	0	11(0.138)	0	13(0.163)
	Baka Pygmies Cameroon	30	Patin et al 2006	4(0.067)	0	13(0.217)	5(0.083)	8(0.133)	0	6(0.100)
	Mandenka Senegal	97	Sabbagh et al 2008	18(0.093)	2(0.010)	65(0.335)	3(0.015)	32(0.165)	13(0.067)	10(0.052)
	Dogons Mali	50	Dolinenko et al 1996	13(0.130)	0	22(0.220)	8(0.080)	32(0.320)	1(0.010)	4(0.040)
	Somali Somalia	24	Patin et al 2006	3(0.063)	0	19(0.396)	0	17(0.354)	1(0.021)	0
North Africans	Moroccans Morocco	44	Patin et al 2006	13(0.148)	0	45(0.511)	0	22(0.250)	3(0.034)	1(0.011)
Europeans	Spanish Spain	258	Agundez et al 1996	133(0.258)	1(0.002)	242(0.469)	0	127(0.246)	3(0.006)	0
	Sardinians Sardinia	49	Patin et al 2006	20(0.204)	1(0.010)	52(0.531)	1(0.010)	24(0.245)	0	0
	French France	60	Dolinenko et al 1996	22(0.183)	6(0.050)	54(0.450)	2(0.017)	30(0.250)	1(0.008)	5(0.042)
	UK Caucasians United Kingdom	112	Lukhnova et al 2002	44(0.196)	8(0.036)	104(0.464)	6(0.027)	54(0.241)	5(0.022)	1(0.004)
	US Caucasians USA	387	Deitz et al 2000	187(0.242)	20(0.026)	318(0.411)	17(0.022)	206(0.266)	15(0.019)	7(0.009)
	Swedes Sweden	50	Patin et al 2006	11(0.110)	6(0.060)	50(0.50)	3(0.030)	28(0.280)	2(0.020)	0
	Saami Finland	48	Patin et al 2006	23(0.240)	3(0.031)	42(0.438)	0	15(0.156)	13(0.135)	0
	Germans Germany	844	Cascorbi et al 1995	383(0.227)	70(0.041)	647(0.383)	68(0.040)	470(0.278)	22(0.013)	26(0.015)
	Polish Poland	248	Mrozikiewicz et al 1996	109(0.220)	26(0.052)	164(0.331)	30(0.060)	149(0.300)	17(0.034)	0
	Ashkenazi Jews	40	Patin et al 2006	9(0.113)	0	41(0.513)	0	29(0.363)	1(0.013)	0
	Russians Russia	290	Gaikovitch et al 2003	134(0.231)	12(0.021)	213(0.367)	17(0.029)	184(0.317)	17(0.029)	0
	Turks Turkey	303	Aynacioglu et al 1997	140(0.231)	8(0.013)	216(0.356)	29(0.048)	185(0.305)	27(0.045)	0
Central/South Asians	Gujarati India	50	Patin et al 2006	13(0.130)	1(0.010)	29(0.290)	4(0.040)	43(0.430)	6(0.060)	0
	Turkmen Uzbekistan	50	Patin et al 2006	31(0.310)	0	23(0.230)	3(0.030)	30(0.300)	12(0.120)	0
East Asians	Han Chinese China	112	Gao et al 2004	132(0.589)	0	40(0.018)	0	46(0.205)	38(0.170)	3(0.013)
	Chinese China	44	Patin et al 2006	46(0.523)	0	6(0.068)	0	22(0.250)	13(0.148)	1(0.011)
	Japanese Japan	144	Tanaka et al 2002	208(0.722)	0	1(0.003)	0	55(0.191)	22(0.076)	1(0.003)
	Koreans Korea	288	Lee et al 2003	353(0.613)	1(0.002)	5(0.009)	0	126(0.219)	70(0.122)	6(0.010)
	Thai Thailand	44	Patin et al 2006	26(0.295)	0	10(0.114)	0	34(0.386)	17(0.193)	1(0.011)
Central Americans	Nicaraguans Nicaragua	137	Martinez et al 1998	114(0.416)	7(0.026)	86(0.314)	5(0.018)	46(0.168)	0	3(0.011)
Arabs/Caucasians	**Lebanese Lebanon**	**81**	**Present study**	**31(0.191)**	**1(0.006)**	**68(0.420)**	**9(0.056)**	**46(0.284)**	**4(0.025)**	**3(0.019)**

#: N (%)

Table 11. Association studies from different references on *NAT2* phenotype and bladder cancer risk

Study name	Description	Country	Population size	Association of NAT2 acetylation phenotype with bladder cancer risk(yes/No)	OR (95% CI)	Adjusting for confounding factors	Statistical Significance	OR(95% CI)
Current study	**Case-control**	**Lebanon**	**81 controls and 41 cases**	**No**	**1.199(0.575-2.570)**	**Sex and smoking**	**No**	**1.299(0.595-2834)**
Mittal et al 2004	Case-control	North India	110 controls and 101 cases	No	1.180(0.690-2.030)	smoking	No	0.835(0.328-2.125)
Klimcakova et al 2010	Case-control	Slovakia	90 cases and 274 controls	Yes	1.900(1.150-3.160)	age, gender, and smoking	Yes but less	1.830(1.020-3.290)
Garcia-Closas et al 2005	Case-control of 18 hospitals[1]	Spain	1150 cases and 1149 controls	Yes	1.400(1.200-1.700)	Interaction of NAT2/smoking status	Yes	1.800(1.200-2.800)
	meta-analysis of 31 studies[2]	Different countries	Cases*	Yes	1.400(1.200-1.600)	Interaction of NAT2/smoking status	Yes	1.200(1.100-1.500)
Mimi et al 1994	Association study	USA(Los Angeles)	133 males of different ethnicities	Yes	significant	N/A	N/A	N/A
Marcus et al 1999	meta-analysis of 22 case-control studies[3]	different countries	2496 cases and 3340 controls	Yes but not for all countries	2.1(1.2-3.8) for Asia studies and 1.400(1.200-1.600)for Europe studies	N/A	N/A	N/A

*: 5096 cases from 31 studies for *NAT2* association, and 4305 cases from 22 studies for *NAT2*/smoking interaction
[1]: 5 different areas in Spain (Austria, Barcelona metropolitan areas,Vallès/Beges, Alicante, Tenerife)
[2]: Europe (18 studies), USA (4 studies), Asians (6 studies), and the Spanish study
[3]: Europe (14 studies), Asia (3 studies), USA (3 studies), India (1study), Middle East (1 study)

Table 12. Minimal allele frequency of different *NAT2* haplotypes of different populations in comparison to our study sample

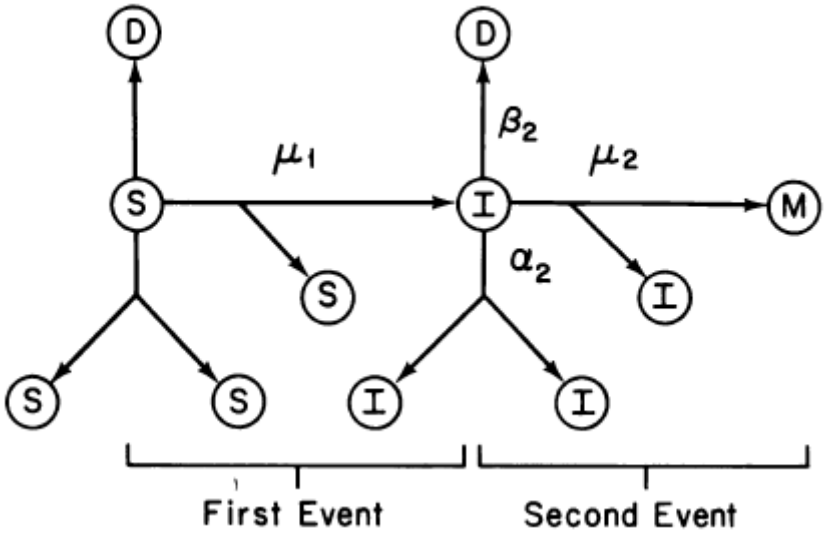
Allele	Present study 2013 Lebanese N=81	Tanira et al 2003 Omanis N=127	R Bu et al 2004 Arabs N=487	Woolhouse et al 1997 Emiratis N=106	Hamdy et al 2003 Egyptians N=199	Cascorbi et al 1995 Causasians N=844
***4**	31 (0.191)*	45(0.177)	sum of *4 and other alleles (<0.030)	39(0.180)	79(0.215)	383(0.227)
***5A**	1 (0.006)	8(0.031)	34(0.035)	0	154(0.497)	70(0.042)
***5B**	68 (0.420)	89(0.350)	287(0.406)	112(0.530)		647(0.383)
***5C**	9 (0.056)	9(0.035)	21(0.024)	4(0.020)		68(0.040)
***6A**	46 (0.284)	63(0.248)	186(0.216)	44(0.210)	sum of 6A and 6B 83(0.326)	470(0.278)
***7B**	4 (0.025)	10(0.039)	13(0.013)	9(0.040)	sum of 7A and 7B 11(0.028)	22(0.013)
***13**	3 (0.019)	6(0.024)	N/A	0	N/A	26(0.015)

Table 13. Number and frequency of *NAT2* phenotypes of different populations in comparison to our study sample

Genotype	Phenotype	Present study 2013 Lebanese N=81	Tanira et al 2003 Omanis N=127	Woolhouse et al 1997 Emiratis N=106	Cascorbi et al 1995 Causasians N=844
***4/*4**	Fast acetylator	**3(0.037)***	3(0.024)	2(0.020)	36(0.043)
***4/*5B**	Fast acetylator	**11(0.136)**	15(0.118)	23(0.220)	162(0.192)
***4/*5C**	Fast acetylator	**4(0.049)**	0	0	17(0.02)
***4/*6A**	Fast acetylator	**10(0.123)**	14(0.110)	10(0.090)	107(0.127)
***4/*7B**	Fast acetylator	**0**	3(0.024)	2(0.020)	7(0.008)
***13/*13**	Fast acetylator	**1(0.012)**	0	0	1(0.001)
***5A/*5C**	Slow acetylator	**1(0.012)**	0	0	6(0.007)
***5B/*5B**	Slow acetylator	**15(0.185)**	21(0.165)	26(0.250)	128(0.152)
***5B/*5C**	Slow acetylator	**3(0.037)**	3(0.024)	3(0.030)	22(0.026)
***5B/*6A**	Slow acetylator	**21(0.259)**	20(0.157)	26(0.250)	169(0.200)
***5C/*6A**	Slow acetylator	**1(0.012)**	2(0.016)	0	19(0.023)
***5B/*7B**	Slow acetylator	**3(0.037)**	4(0.031)	6(0.060)	5(0.006)
***6A/*7B**	Slow acetylator	**1(0.012)**	0	0	9(0.011)
***6A/*6A**	Slow acetylator	**6(0.074)**	10(0.079)	4(0.040)	68(0.081)
***6A/*13**	Slow acetylator	**1(0.012)**	2(0.016)	0	12(0.014)

*N (%)

Figure 1. Two-stage model of carcinogensis (Moolgavkar S.H., 1983)



D: Differentiated cell

S: Stem cell

I: Intermediate cell

M: Mutagenic cell

μ1: rate of transformation of a stem cell into an intermediate cell

μ2: rate of transformation of an intermediate cell into a malignant cell

β2: rate of transformation of an intermediate cell into a dead or differentiated cell, or rate for both actions

Figure 2. Overview of current understanding of arylamine-induced bladder cancer pathogenesis (Yu M.C. et al., 2002)

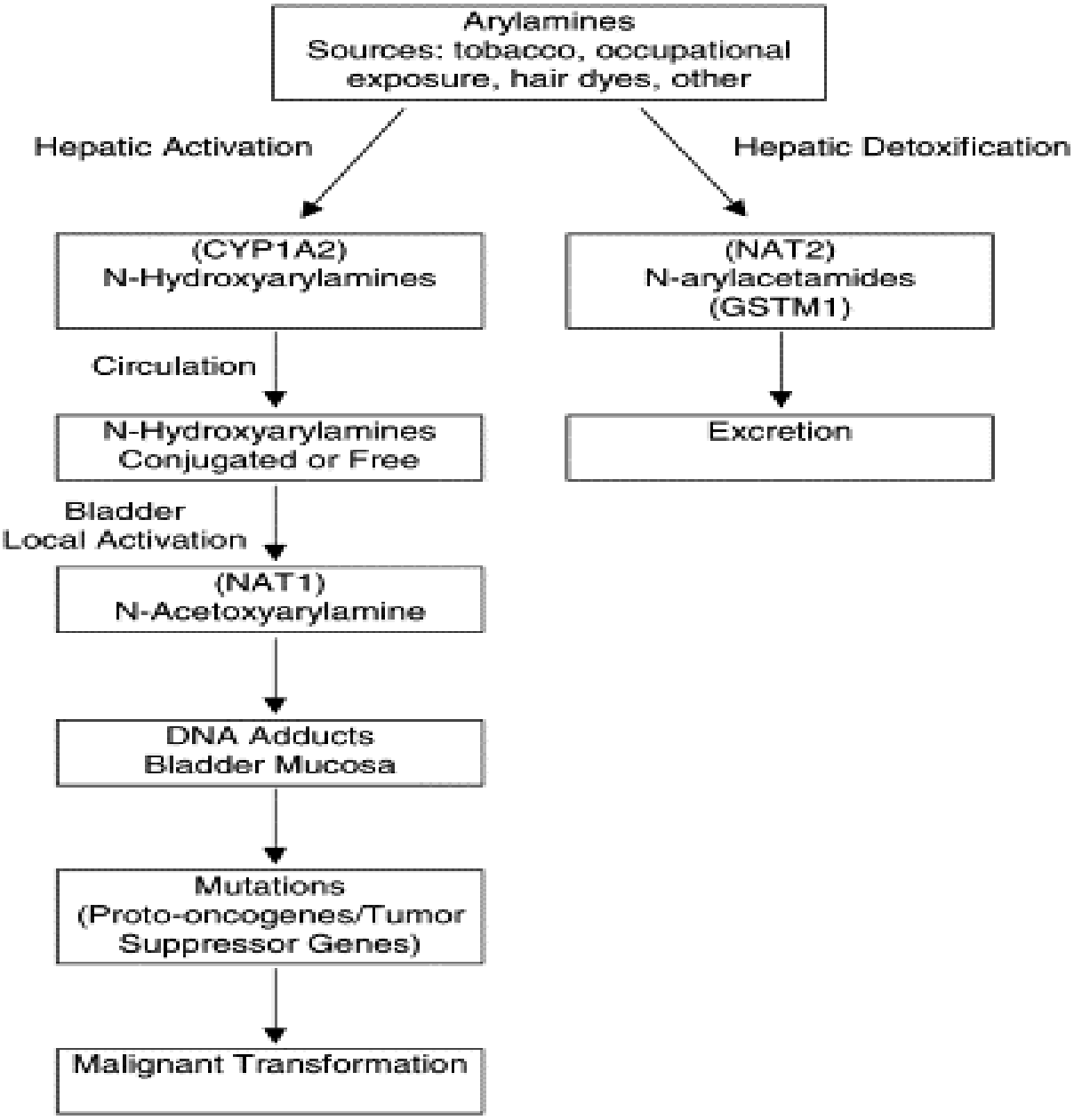


Figure 3. Arylamine metabolism and its relevance to bladder carcinogenesis (Taylor J.A. et al., 1998)

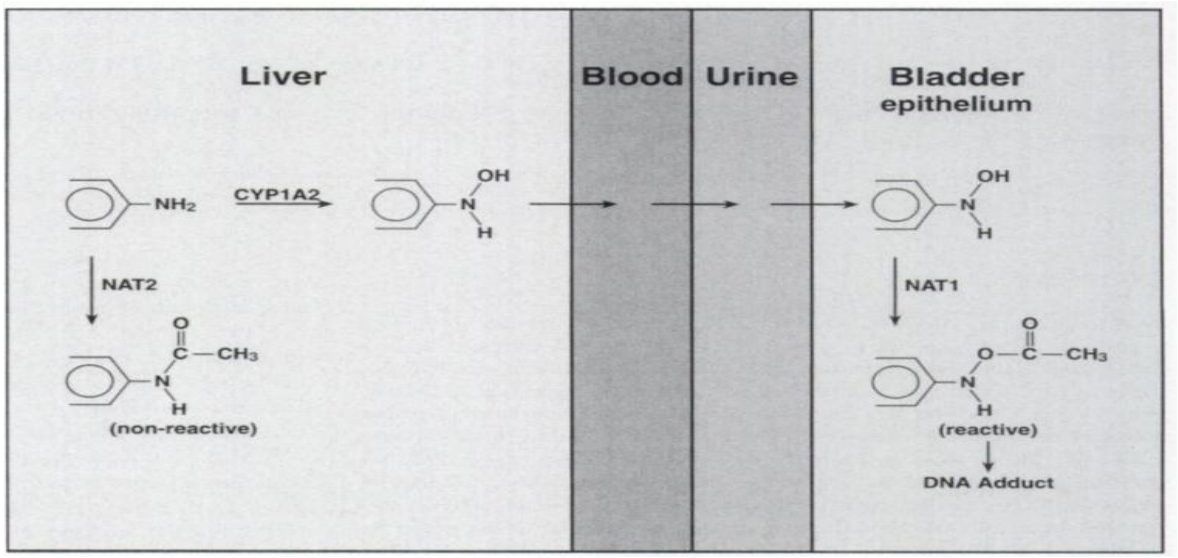


Figure 4. Chemical equations of aromatic and heterocyclic amines activation and deactivation (Aldridge J.E et al, 2003)

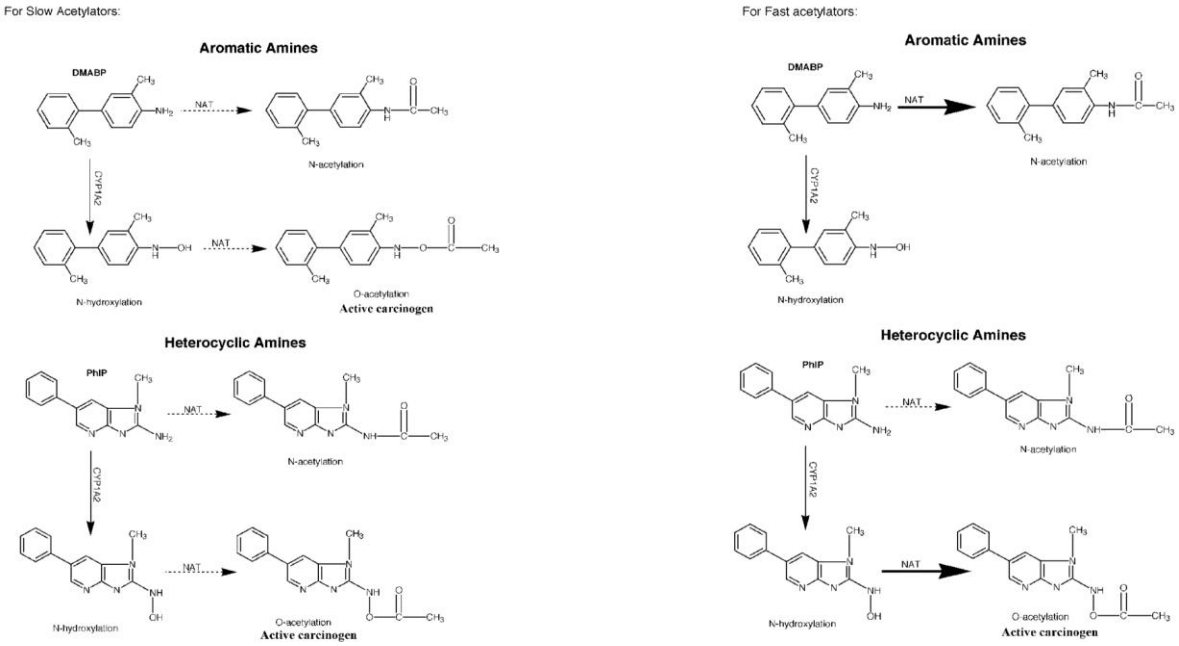


Figure 5. *NAT2* haplotype frequency distribution for the 28 population samples genotyped for the 7 common *NAT2* SNPs (Sabbagh A. et al., 2008)

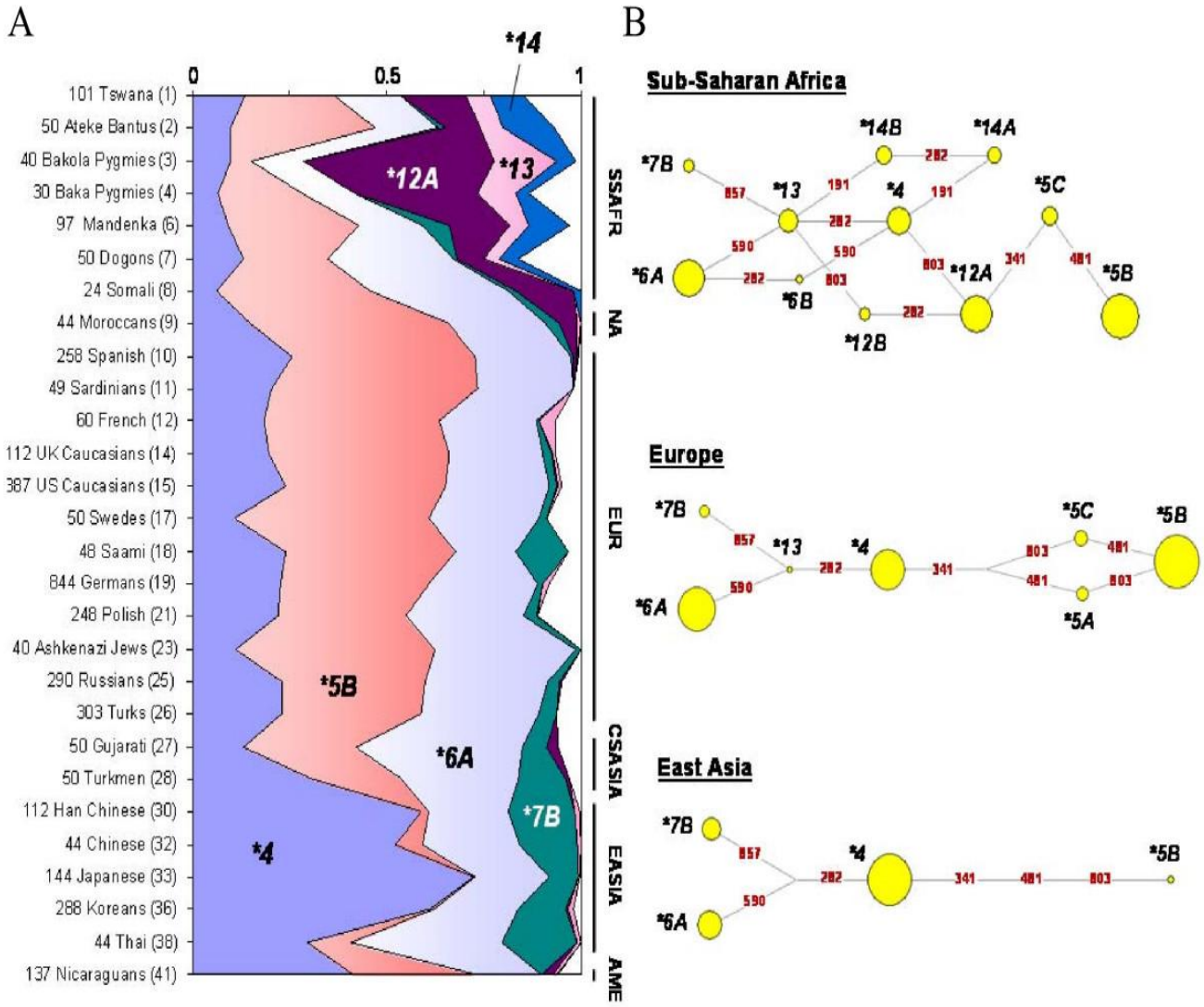
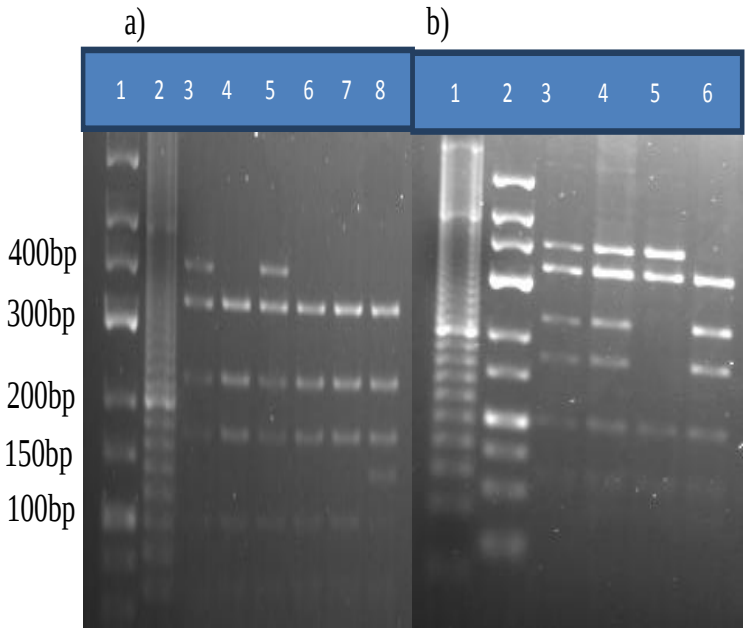
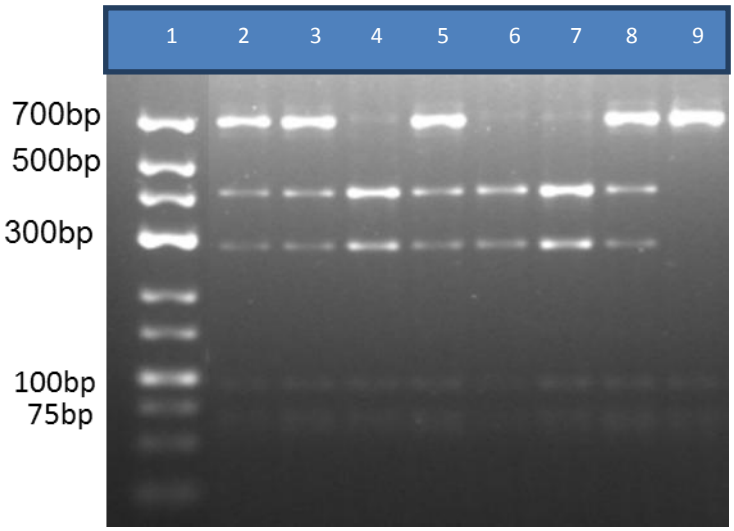


Figure 6. Gels results for *T111C*, *G590A*, *C759T*, and *G857A NAT2* polymorphisms



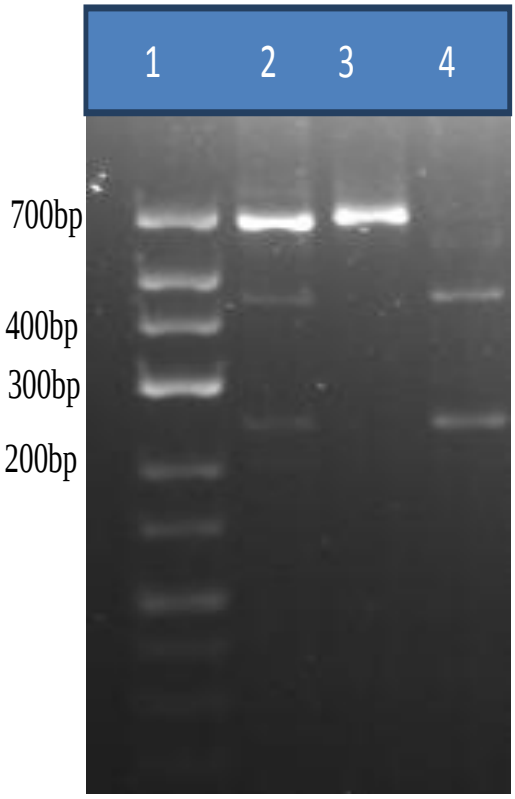
Well	Bands: base pairs (bp)	Description to genotype
1(a)		Ladder
2(b)		Ladder
3, 5(a)&3,4(b)	396-332-226-170-98-40	TT-GA-CC-GG
4, 6(a)&6(b)	332-226-170-98-40	TT-GG-CC-GG
8(a)	332-226-170-138-98-40	TT-GG-CC-GA
5(b)	396-332-98-40	TT-AA-CC-GG

Figure 7. Gel results for *G191A, A434C,* and *C481T NAT2* polymorphisms



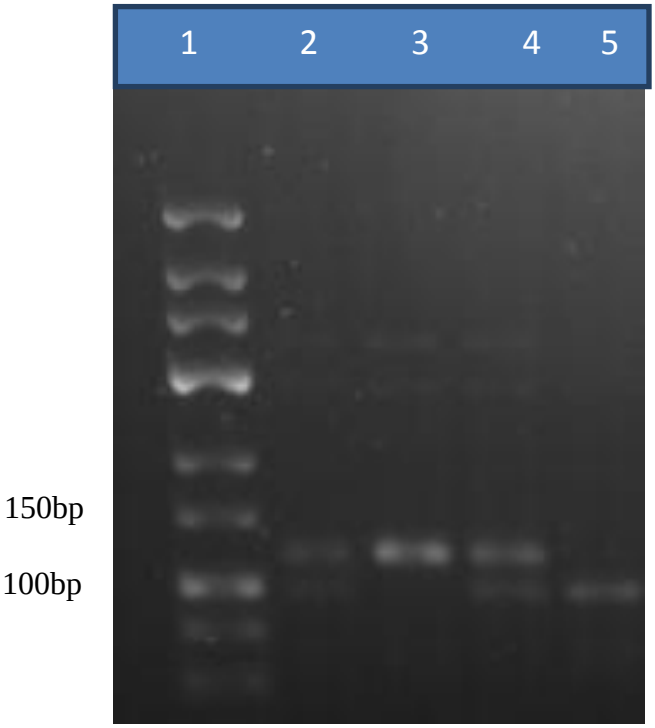
Well	Bands: base pairs (bp)	Description to genotype
1		Ladder
2,3,5,8	707-416-291-93-66	GG-AA-CT
4,6,7	416-291-93-66	GG-AA-CC
9	707-93-66	GG-AA-TT

Figure 8. Gel results for *C282T NAT2* polymorphism



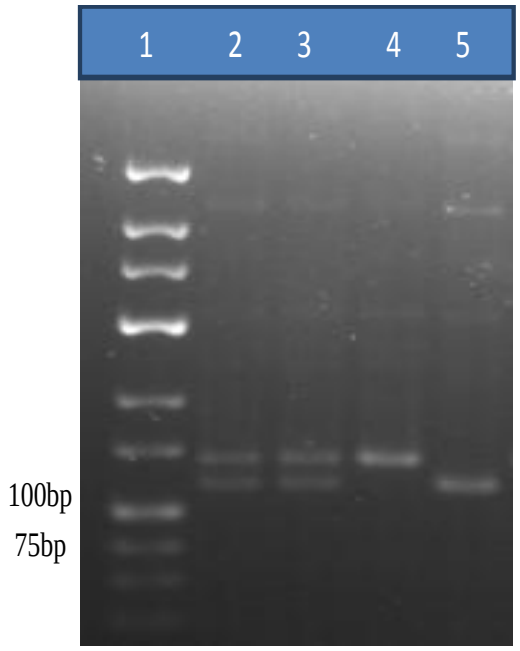
Well	Bands: base pairs (bp)	Description to genotype
1		Ladder
2	667-429-238	CT
3	667	TT
4	429-238	CC

Figure 9. Gel results for *A803G NAT2* polymorphism



Well	Bands :base pairs (bp)	Description to genotype
1		Ladder
2 &4	120-97-23 (not shown)	AG
3	120	AA
5	97-23 (not shown)	GG

Figure 10. Gel results for *T341C NAT2* polymorphism



Well	Bands base pairs(bp)	Description to genotype
1		Ladder
2 & 3	141-121-20 (not shown)	TC
4	141	TT
5	121-20 (not shown)	CC

Figure 11. TaqI cutting the *NAT2* gene at nucleotide number 111 being TT, at nucleotide number 590 being GG , and at nucleotide number 759 being CC. BamHI cutting the *NAT2* gene at nucleotide number 857 being GG

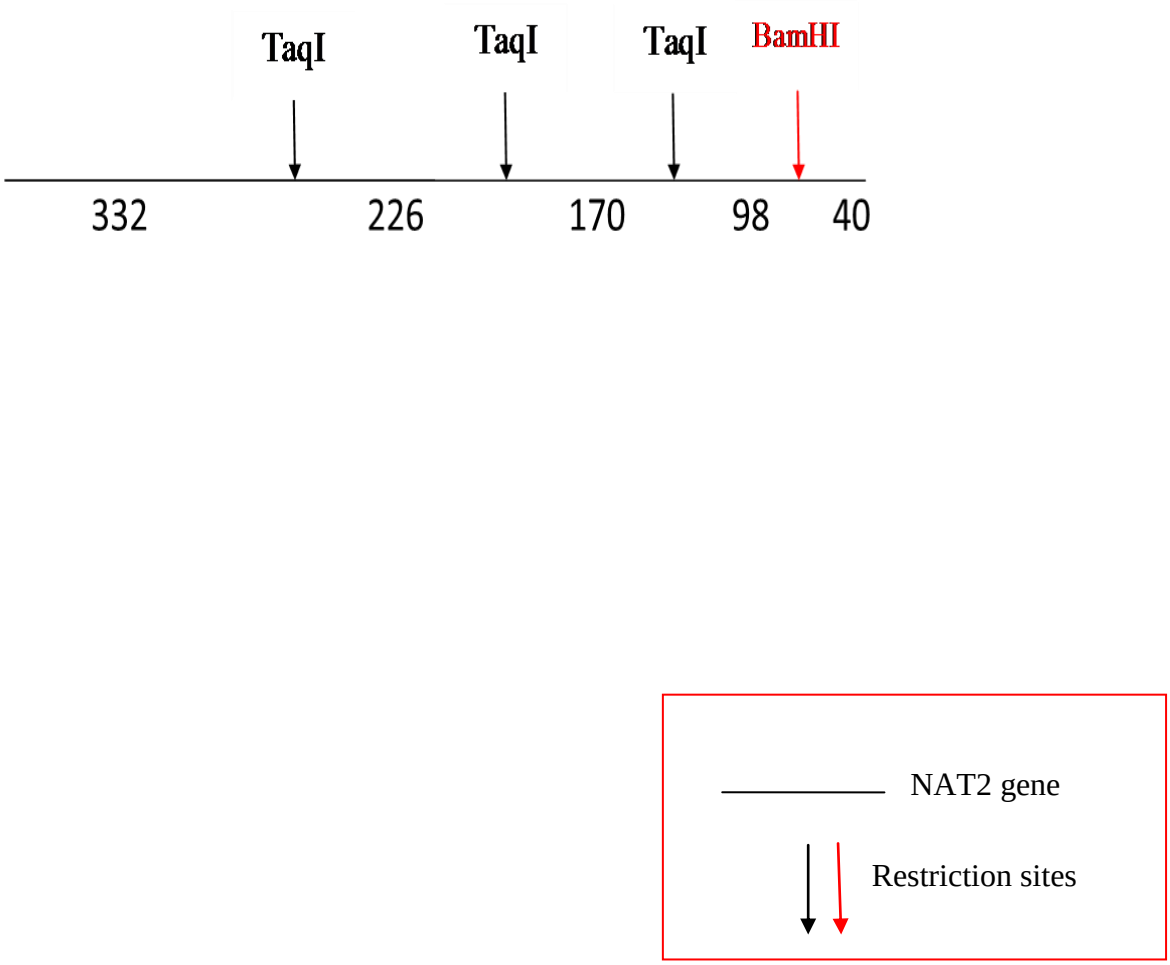


Figure 12. FokI cutting the *NAT2* gene at nucleotide number 282 being CC genotype

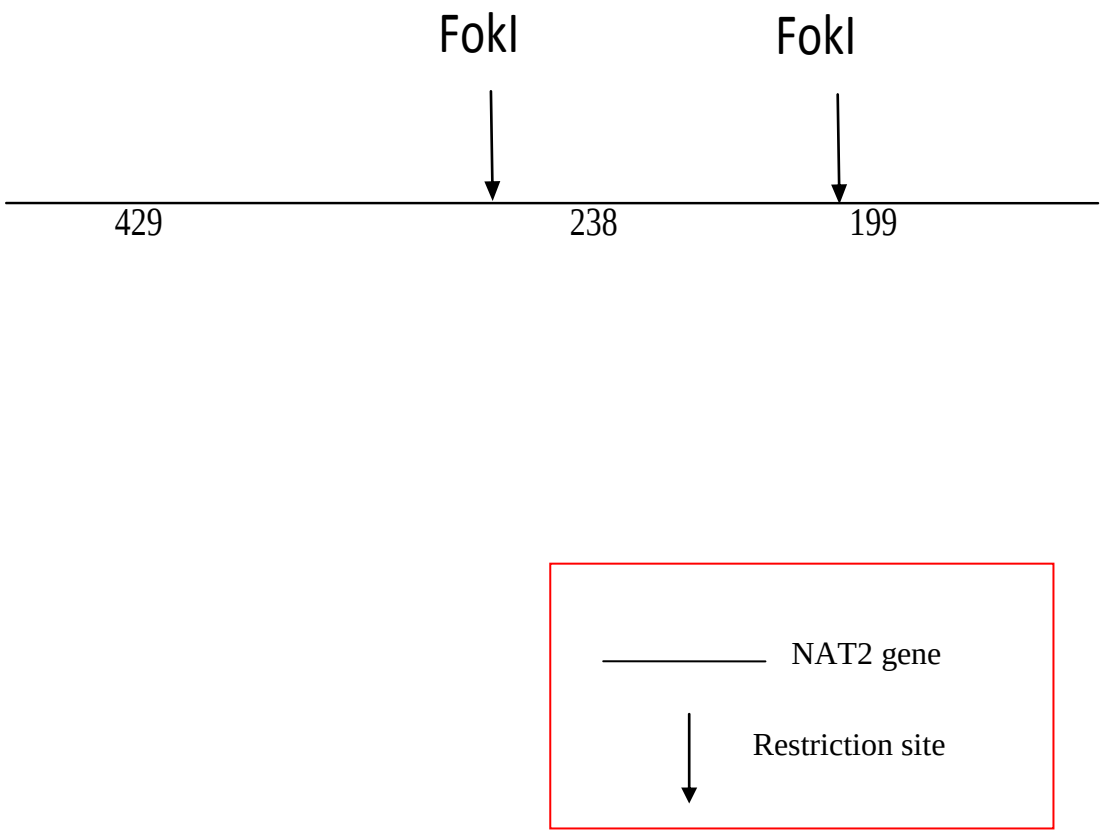


Figure 13. MspI cutting the *NAT2* gene at nucleotide number 191 and 434 being GG and AA respectively. KpnI cutting the NAT2 gene at nucleotide number 481 being CC

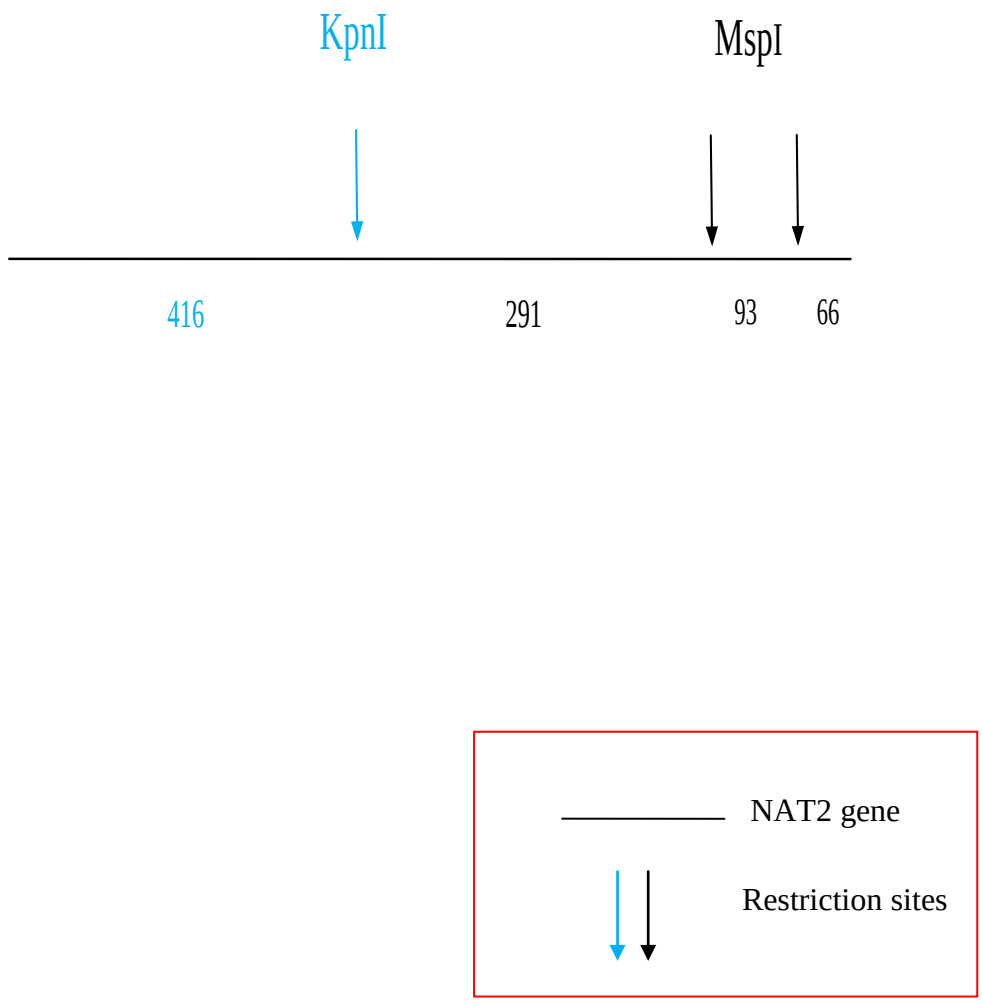


Figure 14. AciI cutting the *NAT2* 141bp size band at nucleotide 341 being CC

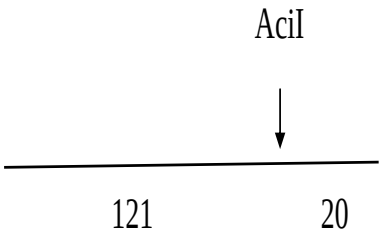
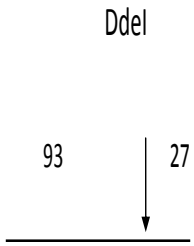
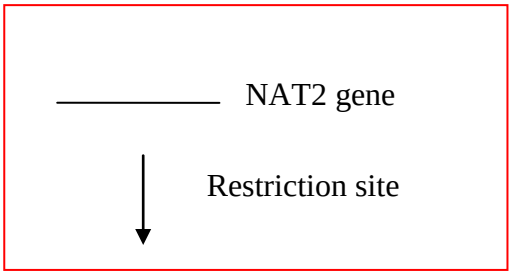


Figure 15. DdeI cutting the *NAT2* 120bp size band at nucleotide number 803 being GG genotype.





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