The Islamic University – Gaza
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الجامعة الأسلامية – غزة
عمادة الدراسات العليـــا
كلية العلـــوم
قسم العلوم الحياتية
تحاليــل طبيــة

CYP2C19 Polymorphism in Childhood Hematological Malignancy

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Submitted in partial fulfillment of Requirements for the degree of Master of Biological Sciences/Medical Technology
Faculty of science

1427/2006هـ

بسم الله الرحمن الرحيم

"وما اوتيتم من العلم الا قليلا"

صدق الله العظيم

Declaration

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Abstract

Cytochrome P450 2C19 (CYP2C19) participates in the metabolism of many clinically important drugs and many xenobiotic compounds. Genetic polymorphisms of the CYP2C19 gene are described to have possible effect on drug treatment and increasing susceptibility to carcinogenic substances. The aim of this study was to determine the frequencies of the major polymorphic CYP2C19 alleles (CYP2C19*2 and CYP2C19*3) and to investigate their association with occurrence of childhood hematological malignancies and/or age of onset of the disease in the investigated patients with comparison to normal subjects. The polymorphism of CYP2C19 was analyzed by a genotyping technique, based on polymerase chain reaction (PCR) followed by restriction enzyme analysis. EDTA blood samples were obtained from 52 previously diagnosed hematological malignancy children (45 from El-Nasser hospital and 7 from the European Gaza hospital) and from 52 normal subjects. The DNA was extracted from the EDTA blood sample for each child. The frequencies of each allele in the cancer group and the control group were compared. In the patient group the frequencies of CYP2C19*2 and CYP2C19*3 were 9.62% and 0.96%, respectively, while in the control group the respective frequencies were 5.77% and 2.88%. These percentages show that there is no significant difference between the control group and the patient group in terms of the frequencies of CYP2C19*2 and CYP2C19*3. The genotyping analysis showed the following results: 15.39% (1*/2*), 1.92% (1*/3*), 1.92% (2*/2*), and 80.77% (1*/1*) in the patients while in the normal subjects the results were 7.69% (1*/2*) , 5.77% (1*/3*), 1.92% (2*/2*), and 84.61% (1*/1*). Data also showed that there is no significant Correlation between the onset age and the polymorphism of the CYP2C19. Moreover, the frequency of affected males was more than that of females. In conclusion, no significant association was found between the CYP2C19 polymorphism and the occurrence or the onset age of the childhood hematological malignancies. Further studies are needed to investigate more CYP2C19 alleles and other important CYP genes polymorphisms in cancer patients.

Key words: Cytochrome P450, CYP2C19, Polymorphism, Genotyping, Alleles, Onset age, Hematological malignancy, Poor metabolizer, Extensive metabolizer, PCR-RFLP.

الملخص

يشارك الأنزيم CYP2C19 في عمليات البناء والهدم لكثير من الأدوية و المواد الكيميائية الغريبة التي يتعرض لها الإنسان. ولقد أشير إلى احتمالية تأثير تعدد الأشكال الجيني للجين CYP2C19 على العلاج بالأدوية وكذلك على زيادة الحساسية للمواد المسرطنة. ولذلك كان الهدف من هذه الدراسة تحديد نسبة وجود الأشكال الجينية الرئيسية الغير طبيعية للجين CYP2C19 وهي رقم 2 و رقم 3 (CYP2C19*2, CYP2C19*3) والبحث في علاقتهما بحدوث أمراض الدم السرطانية لدى الأطفال من جهة وعلاقتهما بعمر الأطفال عند الإصابة من جهة أخرى وذلك بالمقارنة مع أشخاص طبيعيين غير مصابين بأي من سرطانات الدم. و لقد تم استخدام طريقة PCR-RFLP (إكثار الجزء من الحامض النووي الديؤكسي ريبوزي (الدنا) ومن ثم قطعه بواسطة إنزيمات قاطعة متخصصة)، حيث تم جمع 52 عينة دم في أنابيب محتوية على مانع التجلط EDTA من مرضى سرطانات دم مشخصين مسبقا من مستشفى النصر للأطفال (45) ومن مستشفى غزة الأوروبي (7) بالإضافة إلى جمع 52 عينة دم من أشخاص طبيعيين. ثم تم فصل المادة الو راثية (الدنا) من عينات الدم. تم تحديد نسبة وجود كل من الشكل رقم 2 ورقم 3 للجين CYP2C19 عند المرضى وعند الأشخاص الطبيعيين. ولقد وجد أن نسبة وجود الشكل CYP2C19*2 عند المرضى كانت 9.62% بينما كانت النسبة في المجموعة الضابطة 5.77%، أما بالنسبة للشكل CYP2C19*3 كانت النسبة لدى المرضى 0.96% بينما لدى المجموعة الضابطة كانت النسبة 2.88%، وتثبت هذه النسب انه لا يوجد فرق ذو دلالة إحصائية بين المجموعتين فيما يتعلق بنسبة وجود كل من الشكلين. ويشير التوزيع الجيني لدى مجموعة المرضى إلى النتائج التالية: 15.39% (1*/2*)، 1.92% (1*/3*)، 1.92% (2*/2*)، 80.77% (1*/1*). بينما لدى المجموعة الضابطة كانت النتائج كالتالي: 7.69% (1*/2*)، 5.77% (1*/3*)، 1.92% (2*/2*)، 84.61% (1*/1*). تشير النتائج كذلك إلى عدم وجود علاقة بين العمر عند بداية الإصابة بسرطانات الدم قيد الدراسة وتعدد الأشكال للجين CYP2C19، ولقد كانت نسبة المصابين من الذكور أكثر منها من الإناث.

والخلاصة أنه لا توجد علاقة أو ارتباط ذو دلالة إحصائية بين تعدد الأشكال للجين CYP2C19 و حدوث سرطانات الدم عند الأطفال أو العمر عند الإصابة لديهم. وتجدر الإشارة هنا إلى أننا بحاجة ماسة للمزيد من الأبحاث و الدراسات لمعرفة المزيد عن الأشكال الغير طبيعية الأخرى للجين CYP2C19 ولتعدد الأشكال الجينية لجينات مهمة أخرى من الـ CYP عند مرضى سرطانات الدم والسرطانات الأخرى.

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Dedication

To my great parents for their

Nurturing and Inspiration.

To my beloved wife for her

Patience and Support.

To my brothers and sisters.

To my teachers.

Acknowledgements

I would like to acknowledge many people who helped me through my long journey to the master degree.

First, I would like to express my sincerest gratitude to my supervisor Professor Fadel A. Sharif for his close guidance, patience, continuous support, generous help, fruitful and constructive suggestions during all times of my work. I would like also to express my sincere gratitude to my co-supervisor Dr. Abdalla abed for his support and help throughout this work.

My deep and sincere appreciations are also to Dr. Zakaria K. Alsik, head of the pediatric hematological oncology department in the European Gaza hospital, Late Dr. Elham Al-Borno head of the pediatric oncology department (Al-Nasser hospital), and Dr. Abdel khaliq M. Harb for their help and advice.

I'd also like to express my sincere gratitude to all the members of the research and PCR lab in the medical technology department in particular Mr. Nasser Aldein S. Abu- Shaaban and Mr. Mohamed Aouda.

My deep and sincere appreciations are to all the laboratory stuff in the European Gaza hospital for their help and support.

My special deep and sincere gratitude to my parents, wife, brothers, sisters, and friends for their encouragement, continuous support and help.

We are grateful to Sky Advertising co. for their financial support.

Abbreviations

6-MP	6-mercaptopurine
A	Alanine.
aa	amino acid.
ACTH	Adrenocorticotropic hormone.
ALL	Acute lymphoplastic leukemia.
AML	Acute myeloid leukemia.
BamH I	Restriction enzyme.
Bcr-abl	Breakpoint cluster region- Abelson.
bp	Base pair.
cAMP	Cyclic Adenosine Monophosphate.
cDNA	Complementary Deoxyribonucleic acid.
CI	Confidence Interval.
CML	Chronic myelogenous leukemia.
CNS	Central Nervous System.
CYP	Cytochrome P450.
CYP2C19	Cytochrome Protein, Family 2, Subfamily C, Polypeptide 19.
D	Aspartic acid.
dATP	Deoxyadenosine Triphosphate.
dCTP	Deoxycytidine Triphosphate.
dGTP	Deoxyguanosine Triphosphate.
DNA	Deoxyribonucleic acid.
DS	Down syndrome.
dTTP	Deoxythymidine Triphosphate.
E	Glutamic acid.
EDTA	Ethylenediaminetetraacetic acid.
EM	Extensive metabolizer.
EPI INFO	Epidemiology Information, (Database and statistics software for public health professionals).
Et Br	Ethidium promide.
F	Forward.
FAB	French-American-British.
GSTM	Glutathione S-transferase M1.
GSTP	Glutathione S-transferase P.
GSTs	Glutathione-S-Transferases.
GSTT	Glutathione S Transferase Theta 1.
HCC	Hepatocellular carcinoma.
HCV	Hepatitis C virus.
I	Isoleucine .
Ig	Immunoglobuline.
IVS	Intervening sequence.
JMML	Juvenile Myelomonocytic leukemia.
Kb	kilo base.
KDa	Kilo Dalton.
K-ras	Kirsten -retrovirus-associated DNA sequences.
MDS	Myelodysplastic syndromes.
MEHs	Mammalian Epoxide Hydrolases.

MM	Multiple myeloma.
MOH	Ministry of Health.
MTHFR	Methylenetetrahydrofolate reductase.
MTX	Methotrxate.
N	Asparagine.
NAD(P)H	Nicotinamide Adenine Dinucleotide Phosphate.
NADH	Nicotinamide Adenine Dinucleotide.
NATs	N-acetyltransferases.
NQO1	Quinone Oxidoreductase.
OR	Odd ratio.
P	Proline.
PAP	*Papanikolaou* test.
P53	Protein 53.
PAS	Periodic acid Schiff.
PM	Poor metabolizer.
PPI	Proton Pump Inhibtor.
R	Reverse.
RFC	Reduced folate carrier.
RFLP-PCR	Restriction Fragment Length Polymorphism- Polymerase Chain Reaction.
rpm	Round per minute.
ROS	Reactive Oxygen Species.
RR	Relative risk.
S	Serine.
SCE	Sister Chromatid Exchanges.
SD	Standard deviation.
Sma I	Restriction enzyme.
SNPs	Single nucleotide polymorphisms.
SPSS	Statistical Product and service solutions
TAE	Tris – Acetate – EDTA.
Taq polymerase	Thermus aquaticus DNA polymerase.
TBMT	Thiopurine methyltransferase.
UK	United kingdome.
UDP	Uridine diphosphate.
USA	United States of America.
V	Valine.
W	Tryptophan.
WHO	World health organization.
X	Unknown or 'other' amino acid.
XMEs	Xenobiotic metabolizing enzymes.

CHAPTER – 1

INTRODUCTION

Chapter one

Introduction

1.1. Overview

Cytochrome P450 (CYP) proteins are heme enzymes that function in diverse pathways, from carbon source assimilation to hormone biosynthesis. In all P450s, heme is bound in a structurally conserved protein core, allowing them to catalyze regioselective and stereoselective oxidation of hydrocarbons. CYP enzymes, named for the absorption band at 450 nm of their carbon-monoxide-bound form, are one of the largest superfamilies of enzyme proteins. CYP enzymes superfamilies are actively involved in drug and xenobiotic metabolism **[1]**.

Humans have been estimated to have at least 57 different CYP genes **(Table 1.1)** and 47 pseudogenes **[2]**, but the major drug metabolizing human P450s are CYP1A, CYP2B, CYP2C, CYP2D, CYP2E, and CYP3A **[3, 4, 5]**.

The etiology of hematological malignancies is largely unknown. Biological and epidemiological data implicate an important role of exogenous toxicants including cytotoxic drugs, benzene, radiation and cigarette smoking. Most of these substances are detoxified by CYP enzymes which are present mainly in the liver cells **[6]**.

Pediatric cancer is a treatable disease with cure being a realistically obtainable goal. However, children still die of cancer and it is one of the leading causes of death in childhood. It is a chronic illness with long term morbidity both from the disease, and treatment or both **[7]**. Pediatric cancers differ from adult cancers and different types of cancer occur at different ages. General aspects of childhood and adult cancers are provided in **Table 1.2**.

Table 1.1. Major CYP genes and their main functions.*

CYP Gene	Main functions
CYP1	Xenobiotic metabolism
CYP2	Xenobiotic metabolism, Arachidonic acid metabolism
CYP3	Xenobiotic and steroid metabolism
CYP4	Fatty acid hydroxylation
CYP5	Thromboxane synthesis
CYP7	Cholesterol 7-hydroxylation
CYP8	Prostacyclin synthesis
CYP11	Cholesterol side-chain cleavage, Steroid 11-hydroxylation, Aldosterone synthesis.
CYP17	Steroid 17-hydroxylation
CYP19	Androgen aromatization
CYP21	Steroid 21-hydroxylation
CYP24	Steroid 24-hydroxylation
CYP26	Retinoic acid hydroxylation
CYP27	Steroid 27-hydroxylation
CYP46	Cholesterol 24-hydroxylation
CYP51	Sterol biosynthesis

* Compiled from Nelson, (1999) **[8].**

The CYP enzymes are the most important enzyme system that metabolize and inactivate drugs, and in some cases activate them to active, toxic or carcinogenic products **[3, 8, 9]**. Polymorphisms (the presence of more than one allele at a particular locus at a frequency of 1% or higher in a population) have been shown to affect the half-life, efficacy, and toxicity of clinically used drugs and affect susceptibility to environmentally caused disease states such as cancer **[10, 11]**.

Polymorphisms in genes have developed during the evolution through various events, such as point mutations (missense, nonsense, frameshift, and silent mutation), gene conversions, deletions, and insertions. Numerous single nucleotide polymorphisms (**SNPs)** are also found throughout the genome **[12]**.

Most xenobiotic metabolizing enzymes (XMEs) have been found to display considerable genetic heterogeneity. Environment and diet are thought to be important modifiers during the evolution, the genome being adapted to the living circumstances through natural selection **[12]**.

Table 1.2. General aspects of childhood and adult cancers **[7]**.

Parameter	Child	Adult
Primary site	Haemopoietic, CNS, Lymphatic, (mesodermal).	Breast, Lung, Colon (ectodermal).
Stage at diagnosis	80% disseminated.	Local or regional.
Screening test	Usually not useful.	Useful *e.g*; mammography, PAP smear, self examination.
Early detection	Mostly accidental Usually late.	Improves with education
Response	Very responsive to chemotherapy.	Less responsive to chemotherapy.
Outcome	~65% 5 yr survival (all types).	<50% 5 yr survival (all types).
Prevention	Unlikely	Many preventable.

In the Gaza Strip in the period (1995-2000) the incidence rate of the childhood hematological malignancies were 31.4% (61.9% in males, 38.1% in females). Most common types of pediatric cancer were lymphomas (27.3%), bone marrow (23.8%), brain and nervous system (17.6%) **[13]**.

In the United States of America (USA) the most common childhood malignancies are acute lymphoblastic leukemia, central nervous system (CNS) tumors and lymphomas **(Table 1.3)**. Together, these cancers account for 63 % of cases. Childhood cancers occur at a slightly higher rate in males and at a significantly higher rate in whites **[14]**. The incidence of leukemia and lymphoma is the highest, representing 41.1% of all types of childhood cancers.

Table 1.3. The incidence of childhood cancers in the USA.*

Cancer	Incidence (%)
Leukemia	30.2
Central nervous system tumor	21.7
Lymphoma	10.9
Neuroblastoma	8.2
Soft tissue sarcoma	7.0
Renal tumor	6.3
Bone tumor	4.7
Others	11.0

*Adopted from Linet *et.al*. (1999) **[15]**.

1.2. Scope

CYP2C19 gene is highly polymorphic and its polymorphism contributes to assigning humans to different genotypes. The three normal alleles are functionally active in metabolizing certain drugs and xenobiotic compounds which may be carcinogenic. The other CYP2C19 alleles are functionally inactive. Accordingly, the aim of this study is to investigate the occurrence of mutant (abnormal) alleles in children with hematological malignancies in comparison to the normal population. There are two main enzyme deficient alleles called CYP2C19*2 (CYP2C19m1) and CYP2C19*3 (CYP2C19m2). They represent more than 99% of all the abnormal CYP2C19 alleles in Asian population and 87% in Caucasian population **[16, 17, 18]**. In the present study the Identification of the various alleles

will be done by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique.

1.3. Objective

The overall aim of the study is to investigate the association between CYP2C19 gene polymorphism and occurrence of childhood hematological malignancies.

The specific objectives of the study are to:

- Investigate the association between the major CYP2C19 alleles and occurrence of childhood hematological malignancies.

- Investigate the correlation between the major CYP2C19 alleles and the age of onset of the disease in the investigated patients.

1.4. Importance of the study

The study will reveal the correlation between the childhood hematological malignancies and the CYP2C19 polymorphism. This will help in identifying the alleles that may predispose to childhood hematological malignancies.

The identification of susceptible populations through knowledge of their alleles could lead to the elucidation of mechanisms of disease and help to design the preventive strategies that are of greatest benefit.

The medical practice today focuses mostly on the “treatment after the disease onset” while the molecular medicine promises “the prevention before the disease onset”. Now the genes responsible for diseases are being mapped on chromosomes and mutations precipitating the clinical conditions are being identified **[19]**. The homozygous child for the defective CYP2C19 alleles may be protected from exposure to the carcinogenic materials and drugs which are metabolized by the CYP2C19 enzyme.

CHAPTER – 2

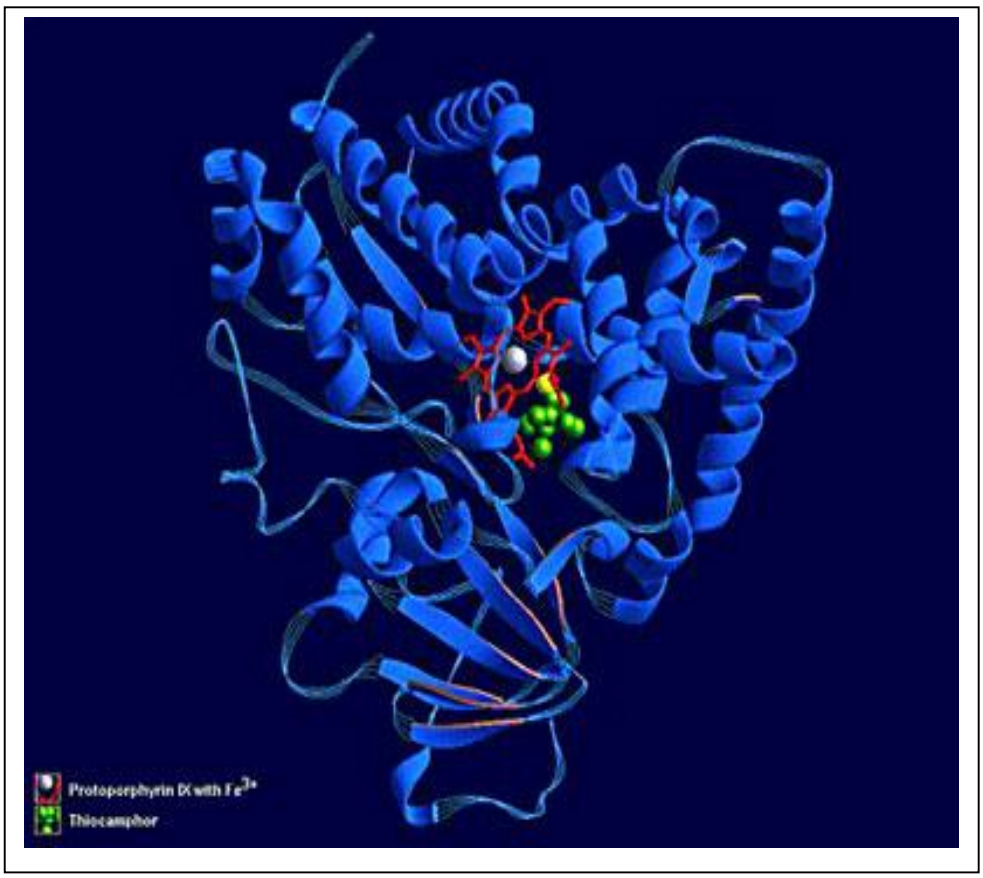
Literature Review

Chapter 2

Literature Review

2.1. CYP enzyme system

CYPs are heme-thiolate proteins **(Figure 2.1)**; their most conserved structural features are related to heme binding and common catalytic properties, the major feature being a completely conserved cysteine serving as fifth (axial) ligand to the heme iron **[1]**.



Protein chain= blue
Protoporphyrin = red
Iron (III) = silver
Thiocamphor = green

Figure 2.1. CYP Protein structure **[20].**

The CYP genes are found in the genomes of virtually all organisms, but their number has exploded in plants **[1]**. The human genome encodes more than 57 members of the family, whereas the genome of the plant *Arabidopsis* encodes more than 250 members **[21, 22]**. In humans, the CYP genes are expressed in numerous tissues that metabolize a large number of drugs and are under the control of genetic and non-genetic factors **[17]**.

In plants, chemical defense seems to be a major reason for CYP diversification. In prokaryotes, CYPs are soluble proteins while in eukaryotes, they are usually bound to the endoplasmic reticulum or inner mitochondrial membranes. The electron carrier proteins used for conveying reducing equivalents from NAD(P)H differ with the subcellular localization **[1]**.

2.1.1. Nomenclature

The nomenclature of CYP enzymes are based on the similarity in the genetic sequence: I) Isoenzymes with greater than 40% genetic sequence similarity are grouped into families denoted by CYP, and a number *e.g.*, CYP2. II) Isoenzymes within a family that have greater than 55% sequence similarity are grouped in a subfamily designated by a capital letter *e.g.*, CYP2C. III) Individual isoenzymes that have been specifically identified are given a further number *e.g.*, CYP2C19 **[23, 24]**.

2.1.2. Drug Interactions

In humans, the CYP gene family contains at least 57 different genes, of which only a small group is involved in drug and chemical transformations **[25]**. The most important CYP isoenzyme is CYP3A4 (50% of the CYP metabolism) followed by CYP2D6 (20%), CYP2C9 and CYP2C19 (together 15%). The remaining is carried out by CYP2E1, CYP2A6 and CYP1A2 **[5]**.

CYP enzymes are present in every cell, but are primarily located in the endoplasmic reticulum of the hepatocytes and in the small intestines, with smaller quantities in the kidneys, lungs and brain**.** The liver is the main site of drug

metabolism. CYPs in the gut are also involved in drug interactions, for example constituents of grapefruit juice are known to inhibit the metabolism of some drugs in the gut wall **[23]**.

CYP enzymes play a dual role in the organism. On the one hand, they inactivate the drug/xenobiotic and prepare it for excretion. On the other hand, they are also capable of activating foreign chemicals to highly reactive toxic intermediates that might act as carcinogens or mutagens **[26]**.

2.1.3. Phase I and Phase II enzymes

The reactions catalyzed by xenobiotic-biotransforming enzymes are generally divided into two groups, called phase I and phase II **(Table 2.1)**. Phase I reactions involve oxidation, reduction and hydrolysis. These reactions expose or introduce a functional group (-OH, -NH_2, -SH or -COOH) **(Figure 2.2)**, and usually result in only a small increase in hydrophilicity **[26]**. Phase II biotransformation reactions include glucuronidation, sulfation, acetylation, methylation, conjugation with glutathione (mercaptopuric acid synthesis), and conjugation with amino acids (such as glycine, taurine, and glutamic acid) **[26]**.

Among the phase I biotransformation enzymes, the CYP system ranks first in terms of catalytic versatility and the sheer number of xenobiotics it detoxifies or activates to reactive intermediates **[26]**.

Phase I and Phase II enzymes do not act according to the highly specific "lock-and-key" mode. Fortunately for us, they are capable of entering into a wide variety of chemical reactions because they encounter an enormous range of natural and man-made chemicals **[27]**.

Table 2.1. List of reactions catalyzed by some drug metabolizing enzymes **[26]**.

Phase I reaction	**Reaction type**	**Enzyme**
Oxidation	Hydroxylation, *N*-oxidation, *S*-oxidation, *N*-dealkylation, *O*-dealkylation, desamination, desulfation, oxidative dehalogenation	Cytochrome P450-monooxygenases
	Dehydration	Alcohol dehydrogenase
	dehydration of amines	Monoaminoxidases
	N-oxidation, *S*-oxidation	Flavin monooxygenases
Reduction	Dehalogenisation of nitrogroups	Cytochrome P450-monooxigenases
Hydrolysis	Hydrolysis of epoxides	Epoxide hydrolases
Others	Oxidation of radicals	Superoxide dismutases
	peroxidation	glutathione peroxidases
Phase II reactions		
Conjugation	Glucosylation	UDP-glucuronosyltransferase
	sulfation	sulfotransferases
	acetylation	*O*-, *N*-acetyltransferases
	methylation	*O*-, *N*-, *S*-methyltransferases
	glutathione *S*-conjugation	glutathione *S*-transferases

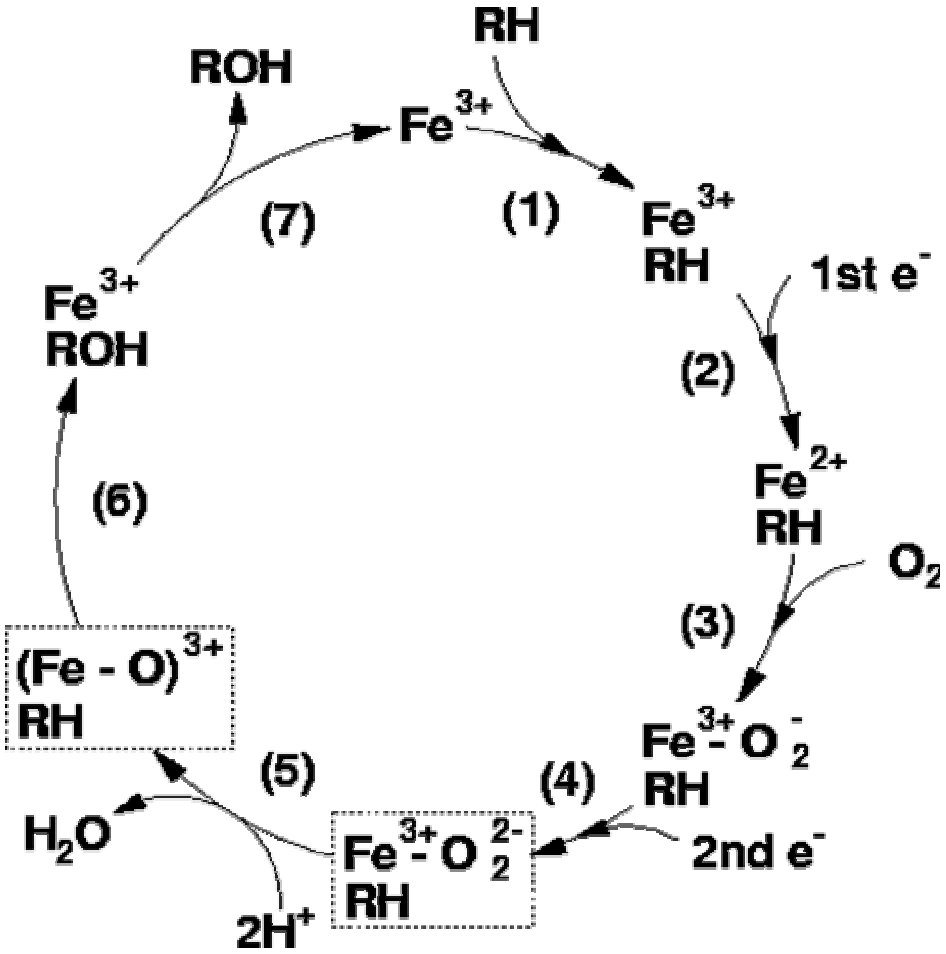


Figure 2.2. The catalytic cycle of cytochrome P450. The intermediate states enclosed in dashed boxes have not been directly observed and are hypothetical **[28]**. **(I)** As the substrate (RH) binds it converts the iron (III) from low spin 6 coord to high spin ferric five coord. **(II)** The addition of an electron (NADH) to give the high spin ferrous substrate bound enzyme. **(III)** Oxygen binding occurs to give a ferric superoxide intermediate. **(IV)** Intermediate VI is less well characterized and normally written as a ferricperoxide. **(V)** O-O bond cleavage occurs to give an oxygen atom in formal oxidation state. **(VI)** The substrate (RH) is oxidized and converted to (ROH). **(VII)** The final step is the releasing of the alcohol and the resting state of the protein (CYP).

2.2. CYP2C19

The human CYP2C subfamily consists of four members (CYP2C8, -9, -18, and -19), which share >82% amino acid identity **[29]**.

The CYP2C19 gene is located within a cluster of CYP genes on the 4th band of region 2 of the long arm of chromosome 10 (10q24) **(Table 2.2)**. This gene encodes a member of the CYP superfamily of enzymes **[30]**.

Table 2.2. Description of the CYP2C19 gene **[30]**.

Symbol	CYP2C19
Gene name	Cytochrome p450, family 2, subfamily C, polypeptide 19.
Alternate Names	cytochrome P450, subfamily IIC (mephenytoin 4-hydroxylase), polypeptide 19; flavoprotein-linked monooxygenase; mephenytoin 4'-hydroxylase; microsomal monooxygenase; xenobiotic monooxygenase.
Location	10q24.1-q24.3.
Geneatlas name	Xenobiotic monooxygenase.
Synonym symbol(s)	CPC1, CYP2C, P450C2C, CPCJ.
DNA structure	90.21 kb, 9 Exons.
mRNA size	1473 bp.
Protein size	55.9 kDa, 490 aa.

2.2.1. History

By somatic cell hybridization and *in situ* hybridization, **Riddell *et al.* (1987)** and **Spurr *et al.* (1987)** assigned a gene for the CYP with mephenytoin 4-prime-hydroxylase activity (CYP2C) to chromosome 10q24.1-q24.3. **Shephard *et al.* (1989)** isolated and sequenced a cDNA clone that codes for a novel member of the CYP2C subfamily in man. Studies of **Wrighton *et al.* (1993)** and of **Goldstein**

et al. **(1994)** had demonstrated a correlation between the levels of CYP2C19 protein and microsomal S-mephenytoin 4-prime-hydroxylase activity in human liver. The molecular defect in CYP2C19 responsible for the poor metabolizer **(PM)** phenotype was determined by **de Morais *et al.* (1994) [31, 32, 33, 34, 35, 36]**.

2.2.2. CYP2C19 Functions

CYP2C19 is responsible for the metabolism of a number of therapeutic agents such as the anticonvulsant drug S-mephenytoin, omeprazole, proguanil, certain barbiturates, diazepam, propranolol, citalopram and imipramine **[37]**. Drugs may be themselves substrates for CYP2C19 enzyme and/or may inhibit or induce the enzyme **[38]**.

CYP2C19 is described according to the catalytic activity as: **I)** CYP 2C19 (EC 1.14.13.80) ((R)-limonene 6-monooxygenase) (EC 1.14.13.48). **II)** ((S)-limonene 6-monooxygenase) (EC 1.14.13.49). **III)** ((S) - limonene 7-monooxygenase) (CYP2C19) (P450-11A) (Mephenytoin 4 - hydroxylase) (CYP2C17) (P450-254C) **[30]**.

I. (+)-(R)-limonene + NADPH + O_2 = (+)-Trans- carveol + NADP (+) + H_2O.
II. (-)-(S)-limonene + NADPH + O_2 = (-)-Trans- carveol + NADP (+) + H_2O.
III. (-)-(S)-limonene + NADPH + O_2 = (-)-perillyl alcohol + NADP (+) + H_2O.

2.2.3. CYP2C19 alleles

The wild type allele of CYP2C19 which was reported by **Romkes *et al.* (1991) [39],** and has been designated CYP2C19*1A. A second wild type allele was described by **Richardson *et al.* (1995) [40]**, and has been designated CYP2C19*1B. A third wild type allele was described by **Blaisdell *et al*. (2002) [41]**, and has been designated CYP2C19*1C. About 24 variants of CYP2C19 are known **(Table 2.3)**. The most important of these alleles are: CYP2C19*2 (681G **à** A), CYP2C19*3 (636G **à** A), and CYP2C19*4 (1A **à** G). The nucleotide

changes in the CYP2C19*2, *3, and *4, lead to a splicing defect, stop codon, and GTG initiation codon, respectively, and therefore to nonfunctional proteins **[42, 43]**.

Table 2.3. The most important CYP2C19 allele nomenclature.

Allele	**Major Nucleotide changes**	**Effect**	**Enzyme activity**		**Reference**
			In vivo	***In vitro***	
CYP2C19*1A	None		Normal	Normal	**[39]**
CYP2C19*1B	99C>T; 991A>G	I331V	Normal		**[40]**
CYP2C19*1C	991A>G	I331V	Normal		**[41]**
CYP2C19*2A	**681G>A**	splicing defect	None		**[44]**
CYP2C19*2B	276G>C;**681G>A**	splicing defect; E92D	None		**[45]**
CYP2C19*2C (also called CYP2C19*21)	IVS1-231G>A; IVS3-23A>G; 481G>C; **681G>A**; IVS5+228A>G; IVS5-51C>G; IVS6-196T>A;	A161P, **splicing defect**, I331V			**[46]**
CYP2C19*3A	**636G>A**; 991A>G; 1251A>C	**W212X**; I331V	None		**[36]**
CYP2C19*3B (also called CYP2C19*20)	**-**889T>G; IVS1-340T>G; IVS1-231G>A; IVS1-47G>A; IVS3+332T>C; **636G>A**; IVS4-205A>G; 1078G>A	**W212X; D360N; I331V**			**[46]**
CYP2C19*4	**1A>G; 99C>T, 991A>G**	**GTG initiation codon; I331V**	None		**[47]**

The principal defect in CYP2C19 responsible for the S-mephenytoin PM phenotype was discovered by **de Morais *et al.* (1994) [36]** to be a single nucleotide (G-to-A) mutation in exon 5 which creates an aberrant splice site. The change alters the reading frame of the mRNA starting with amino acid 215 and produces a premature stop codon 20 amino acids downstream, resulting in a truncated, nonfunctional protein **[16]**.

2.2.4. Genetic polymorphism

Polymorphism within this gene is associated with variable ability to metabolize mephenytoin, known as the PM and extensive metabolizer (EM) phenotypes **[30]**. PMs may suffer adverse effects when treated with a routine clinical dose of a drug inactivated by CYP2C19 or may not gain therapeutic benefit from pro-drugs activated by CYP2C19. For example, the antimalarial drug proguanil is administered as the pro-drug and requires activation by CYP2C19. PMs were found to totally lack the active metabolite cycloguanil in their plasma and are at risk for failed protection from plasmodium infection by proguanil **[48]**. PM subjects may be unable to detoxify some xenobiotic compounds which may be carcinogenic.

Genetic polymorphism in the metabolism of the anticonvulsant drug mephenytoin exhibits marked racial heterogeneity, with a PM phenotype representing 13 to 23% of Oriental populations, but approximately 2 to 5% of Caucasian populations. Two defective CYP2C19 alleles (CYP2C19*2 and CYP2C19*3) account for more than 99% of Oriental PM alleles but only approximately 87% of Caucasian PM alleles **[16]**.

The predominant genetic polymorphisms in CYP2C19 are two null alleles, which result in impaired metabolism of CYP2C19 substrates. The substitution of G681A in exon 5 of CYP2C19*2 variant allele creates an aberrant splice site resulting in an alteration of the reading frame of mRNA and consequently a truncated non-functional protein. The substitution of G636A in exon 4 of CYP2C19*3 allele results in a premature stop codon, which is common in oriental populations but very rare in Caucasians. PMs of CYP2C19 represent approximately 3-5% of

Caucasians. Higher frequencies of PMs (13-23%) are found in most Asian populations **[49]**.

2.2.5. Genetic regulation of CYP2C19

CYP enzymes have a variety of gene regulatory mechanisms. Many of these genes can be turned on or induced by a chemical signal. The steroid hormones are under strict endocrine control. Their levels are tightly regulated. One example is the induction of steroid biosynthetic CYPs by adrenocorticotropic hormone ACTH. ACTH stimulates production of cyclic adenosine monophosphate (cAMP) that presumably activates a protein kinase that phosphorylates some unidentified protein, leading to an increase in gene transcription **[21]**.

2.3. Chemical-induced diseases and mechanisms of chemical carcinogenesis

Cancer is a genetic disease that proceeds in three main steps: initiation, promotion and progression **[50, 51]**. Procarcinogen activators (including many XMEs) are targets of research elucidating risk factors in carcinogenesis initiation **[12]**. Toxic chemicals are active as such or are activated into reactive metabolites by XMEs **[12]**. Toxicity affects target tissues by causing damage to cells, especially the nucleus. The damage caused can be reversible or irreversible **[12]**.

The basic association between metabolism of toxic substances and various toxic outcomes is presented in **Figure 2.3.**

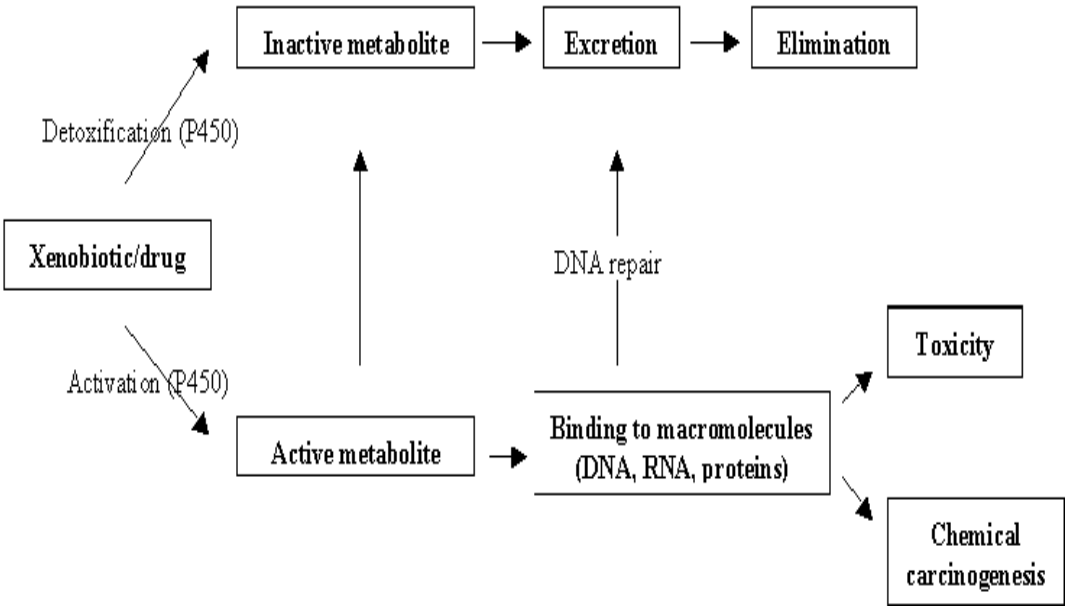


Figure 2.3. Association between metabolism and toxicity of chemical compounds **[12]**.

In chemical carcinogenesis, metabolic activation of procarcinogens by XMEs is often needed to initiate the process **[12].** Procarcinogens converted to reactive intermediates are usually electrophilic and bind covalently to DNA, thus damaging the genetic material and activating oncogenes. Metabolic activation may produce reactive oxygen species (ROS), which may directly damage the DNA or activate carcinogens to reactive intermediates. A third way is through activation of the protein kinase C cascade, leading to the phosphorylation of key nuclear proteins (transcription factors) involved in the regulation of DNA replication, changes in the epidermal growth factor, immunosuppression, dedifferentiation and hyperplasia **[52]**. Briefly, the process of carcinogenesis is divided into the following steps: (I) metabolic activation of carcinogens by XMEs, (II) DNA adduct formation, (III) unsuccessful DNA damage recognition, (IV) unsuccessful DNA repair and (V) failure of the Immune system to recognize tumor formation **[12]**.

2.4. Role of genetic factors in chemical toxicity and carcinogenesis

Individuals are known to differ in their susceptibility to cancer and birth defects. Genetic polymorphisms are often the reason for this phenomenon, including

mutations in XME genes, oncogenes and tumor suppressor genes (e.g., p53, k-ras) **[12]**. Variability in the genome occurs at the levels of genes (mutation/deletions/insertions/DNA adducts) or chromosomes (SCE, sister chromatid exchanges). Some of these changes may be associated with interindividual differences in susceptibility to toxicity and tumor initiation **[12]**.

Several epidemiological studies in cancer patients have shown that individual susceptibility to cancer might partly depend on the genetically determined high or low activity of certain enzymes. This association may be based on interindividual variation in the metabolism of carcinogens that are either detoxified, or metabolically activated to ultimate carcinogens **[26]**. However, the association studies can produce spurious results if cases and controls have differing allele frequencies for genes that are not related to the disease being studied **[53, 54]**.

CYPs, epoxide hydrolases (mEHs), glutathione-S-transferases (GSTs), N-acetyltransferases (NATs) and NAD(P)H Quinone Oxidoreductase (NQO1) are the main polymorphic XME groups found to be associated with certain diseases **[12]**.

2.5. Cancer susceptibility related to ethnicity or race

Racial and ethnic groups can exhibit substantial average differences in disease incidence, disease severity, disease progression, and response to treatment **[55]**. Epidemiological data show that ethnic and racial groups differ significantly in terms of cancer incidence and mortality rates **[26]**. Cancer incidence rates of esophageal cancer in black Americans are approximately three times higher than in white Americans; incidence rates of multiple myeloma (mm), liver, cervical, and stomach cancer are twice as high; and those of cancers of the oral cavity and pharynx, larynx, lung, prostate, and pancreas are 50% higher. The incidence of chronic lymphocytic leukemia and premenopausal breast cancer is also higher in black Americans. In contrast, white Americans have higher incidence rates of melanoma, leukemia, lymphoma, and cancers of the endometrium, thyroid, bladder (in males), ovary, testis, and brain, as well as postmenopausal breast cancer **[26]**. Also African Americans have the highest death rate from all cancer

sites combined and from malignancies of the lung and bronchus, colon and rectum, female breast, prostate, and cervix of all racial or ethnic groups in the United States. The death rate from cancer among African American males is 1.4 times higher than that among white males; for African American females it is 1.2 times higher **[56]**.

Among the biological factors that might contribute to the higher cancer risks in certain ethnic or racial groups are variations in the prevalence of genetic traits affecting carcinogen metabolism and DNA repair **[26]**.

Genetic differences in the regulation, expression and activity of phase I and phase II genes, encoding drug metabolizing enzymes might be crucial factors in defining cancer susceptibility, as well as in determining the toxic or carcinogenic potential of drugs and other environmental pollutants **[26]**.

Metabolism of many drugs influences their pharmacological and toxicological effects. One of the major causes of interindividual variation of drug effects is genetic variation of drug metabolism. Polymorphisms, which cause decreased, increased, or lacking enzyme expression or activity by multiple molecular mechanisms, are generated by mutations in the genes for drug-metabolizing enzymes **[26]**.

2.6. Hematological malignancies

A malignant tumor is the result of a series of DNA alterations in a single cell, or clones of that cell, which lead to loss of normal function, aberrant or uncontrolled cell growth and often metastasis **[57]**. Hematological malignancies are the types of cancer that affect blood, bone marrow and lymph nodes **[58]**.

2.6.1. Leukemia

Leukemia refers to a group of cancers which affect the white blood cells (leukocytes), in other words it is a cancer of the blood or bone marrow characterized by an abnormal proliferation of leukocytes **[59]**. It is clinically and pathologically split into its acute and chronic forms.

Acute leukemias are characterized by the rapid growth of immature white blood cells. This crowding makes the bone marrow unable to produce healthy cells. It is a more common cause of death for children in the US than any other type of malignant disease **[59]**. Chronic leukemias are distinguished by the excessive buildup of relatively mature, but still abnormal white blood cells **[59]**.

Leukemias are classified according to the type of abnormal cells found most in the blood into lymphocytic leukemia and myelogenous leukemia.

There are two approaches appeared in classification of leukemia. One was published by a cooperative group of hematologists and hematopathologists from France, America, and Britain and was designated the French-American-British **(FAB)** classification. The other was the WHO approach. Although the WHO classification was never widely used, the FAB proposal was adopted internationally. It provided long needed standard terminology for the acute leukemias and was quickly accepted by most of the multiinstitutional study groups **[60]**.

The classification of acute leukaemias is now widely based on combined morphological, cytochemical, immunophenotyping, and cytogenetics assessment studies **[60, 61]**. The first two facets of the diagnosis of acute leukemia can be achieved by careful morphological assessment of blood and bone marrow smears and marrow trephine biopsy sections. The usual cytologic features of AML and ALL are listed in **Table 2.4 [60]**.

Table 2.4. Usual cytologic features of AML and ALL.*

	AML	ALL
Blast size	Large and uniform	Small to medium; variable
Chromatin	Finely dispersed	Rather coarse
Nucleoli	1 to 4, often prominent	Absent or 1 or 2; indistinct
Cytoplasm	Moderately abundant Granules often present	Scant to moderate Granules lacking in nearly all cases
Auer rods	60–70% of cases	Absent
Myelodysplasia	Often present	Absent

***Adopted from Mckenna, (2000) **[60]**.

Cytochemical stains are often useful in distinguishing poorly differentiated AML from ALL and in identifying subsets of AML, **Table 2.5 [60]**.

Table 2.5. Cytochemical profiles in acute leukemia.*

	Myeloperoxidase and Sudan Black B	Nonspecific esterase	Periodic acid Schiff (PAS)
AML	**+**	**+** (Monocytic, diffuse)	**±**
ALL	**-**	**±** (Focal)	**+** (75%)

***Adopted from Mckenna, (2000) **[60]**.
+ positive, - negative, ± not definitive.

The lineage of most cases of morphologically and cytochemically poorly differentiated acute leukemia can be accurately characterized by immunophenotyping (involves the labeling of white blood cells with antibodies directed against surface proteins on their membrane). Additionally,

immunophenotypic subsets of AML and ALL can be determined by this technique, **Table 2.6 [60, 62]**.

Table 2.6. Immunophenotypic classification of ALL.*

	% of ALL	FAB category	T-cell-associated antigens	B-cell-associated antigens	Surface Ig[1]
B-cell precursor	**>80**	**L1, L2**	**-**	**+**	**-**
B cell	**<5**	**L3**	**-**	**+**	**+**
T-cell precursor	**15**	**L1, L2**	**+**	**-**	**-**

***Adopted from McKenna, (2000) **[60]**.
1. Ig, immunoglobulin. -, negative; +, positive

2.6.1.1. Acute lymphoplastic leukemia (ALL)

ALL is a cancer of the lymphocyte, characterized by the overproduction and continuous proliferation of malignant and immature lymphocytes (lymphoblasts) in the bone marrow. It is fatal if left untreated as ALL spreads into the bloodstream and other vital organs quickly (hence "acute"). ALL can affect children at any age, but is more common in children aged 1–4. ALL is more common in boys than girls **[63]**. ALL is more common in children with certain chromosomal and genetic abnormalities such as Down syndrome, Bloom syndrome, Ataxia-telangiectasia, and Fanconi syndrome **[64]**. This leukemia is more common in males and in whites **[14]**.

2.6.1.2. Myeloid leukemias in children

The myeloid leukemias in childhood represent a spectrum of hematopoietic malignancies. Over 90% of myeloid leukemias are acute and the remainders include chronic and/or subacute myeloproliferative disorders such as chronic myelogenous leukemia (CML) and juvenile myelomonocytic leukemia (JMML). Myelodysplastic syndromes (MDS) are rare in children **[65]**.

2.6.1.3. Acute myeloid leukemia (AML)

AML is defined as a clonal disorder caused by malignant transformation of a bone marrow-derived, self-renewing stem cell or progenitor, which demonstrates a decreased rate of self-destruction and also aberrant differentiation **[65]**.

2.6.1.4. Chronic Myelogenous Leukemia (CML)

Chronic myelogenous leukemia (CML) accounts for only 5% of all childhood leukemia, and 80% of the cases occur after 4 years of age. The cytogenetic abnormality most characteristic of CML is the Philadelphia chromosome, which represents a translocation of chromosomes 9 and 22 [t(9;22)] resulting in a bcr-abl fusion protein. CML is characterized by a marked leukocytosis and is often associated with thrombocytosis, sometimes with abnormal platelet function. Bone marrow aspiration or biopsy reveals hypercellularity with relatively normal granulocytic maturation and no significant increase in leukemic blasts. Although reduced leukocyte alkaline phosphatase activity is seen in CML, this is not a specific finding **[66]**.

2.6.2. Lymphoma

Lymphoma is any of a variety of cancers that begins in the lymphatic system. In technical terms, lymphoma denotes malignancies of lymphocytes or, more rarely, of histiocytes. Traditionally, lymphoma is classified as Hodgkin's lymphoma, discovered by Thomas Hodgkin in 1832, and non-Hodgkin's lymphoma (all other types of lymphoma). Modern classifications of lymphoma have moved away from this artificial division **[67]**.

The two main groups of lymphoma in humans are Hodgkin's disease (characterized by the growth of Reed-Sternberg cells in the cancer) and the Non-Hodgkin's Lymphomas which lack the Reed-Sternberg cells, both types are very rare in children aged less than 3 years. Boys are affected slightly more often than girls **[68]**.

2.6.3. Genetic polymorphism and hematological malignancies

Several authors have studied the correlation between gene polymorphism and hematological malignancies. For example **Canalle *et. al.,* (2004)** and his colleagues have shown that carriers of the rare GSTP1 V allele were at higher risk for ALL while no difference has been found in the prevalence of the GSTM1 and GSTT1 null genotypes between ALL patients and the controls. Moreover, no association has been found between CYP1A1*2 and CYP2E1*3 variants and ALL **[69]**.

The frequency of an inactivating polymorphism in NQO1 appears to be increased in a cohort of myeloid leukemia patients with abnormalities of chromosomes 5 and/or 7, but not in those with balanced translocations, other clonal abnormalities, or normal karyotypes **[70]**.

There is no observed differences in the prevalence of either the methylenetetrahydrofolate reductase (MTHFR) 677 or 1298 genotypes between the cases (ALL and AML) and controls in the total population **[71]**.

2.7. CYP2C19 and cancer

The association between CYP2C19 polymorphism and hematological malignancy has not been studied before, as no articles discussing this matter have been published yet. There are, however, many articles that tackled the association between the CYP enzymes and cancer.

Genetic polymorphisms, including **SNPs,** are extensively used in case-control studies of practically all cancer types. They are used for the identification of inherited cancer susceptibility genes and those that may interact with environmental factors. However, being genetic markers, they are applicable only on heritable conditions, which is often a neglected fact **[72]**.

Genetic polymorphism of CYPs can lead to severe toxicity or therapeutic failure of medications as well as to a possible increase in an individual's susceptibility to certain types of chemically induced cancers and other diseases **[73]**.

The CYP2C19 gene is involved in the primary route of metabolism of the anticonvulsant phenytoin, the anxiolytic agent diazepam, tricyclic antidepressants, proton pump inhibitors (PPI) such as omeprazole, lansoprazole and pantoprazole, and the antimalarial drug proguanil **[42]**.

Several epidemiological studies in cancer patients have shown that an individual susceptibility to cancer might partly depend on the genetically determined high or low activity of certain enzymes. This association may be based on inter-individual variation in the metabolism of carcinogens that are either detoxified, or metabolically activated to ultimate carcinogens **[26]**. CYP2C19 may participate in the activation of procarcinogens of esophagus, stomach and lung cancers, but may be involved in the detoxification of carcinogens of bladder cancer **[74]**. CYP2C19 polymorphism is associated with pharmacological effects of thalidomide in prostatic neoplasm **[74]**. On the other hand, some CYPs remove cancer-causing agents by turning them into benign chemicals within the body **[75]**.

The variant m1 allele (CYP2C19*2A) of the CYP2C19 gene was evaluated for its association with prostate cancer risk among a Swedish population, but no significant differences were found between cancer patients and controls **[76]**. A very small study on Japanese patients revealed a significant association of the (PM) genotype with squamous cell carcinoma of the lung, but the association with bladder cancer seen in Caucasians was not found **[77]**. CYP2C19 PM with HCV-seropositive subjects was found to be associated with a higher risk for developing hepatocellular carcinoma (HCC) **[12]**.

CHAPTER – 3

Materials and Methods

Chapter 3
Materials and Methods

3.1. Study population

The present study was carried out on 52 children (49 ALL, 2 Lymphoma, and 1 CML) and 52 unrelated children as a control. The patient's genotyping data will be compared with that of the normal subjects.

3.1.1. Patients

All patients were collected from outpatient and inpatient hematological oncology departments from El-Nasser hospital (45 patients) and the European Gaza hospital (7 patients) from the period of June 2005 to November 2005. A copy of the approval letter from MOH is given in **Appendix A**. All subjects were informed about the contents and aims of the study and gave their written consent **(Appendix B)**. When the participants were under 18 years old, the written consent was given by their parents. The study protocol was approved by the local ethics committee (Palestinian National Authority - MOH - Helsinki Committee). A copy of the approval letter is given in **Appendix C**.

The patient's data including: name, age, sex, disease, onset age, place of living, and the family history were gathered and coded for each subject **(Appendix D) Table 3.1**.

3.1.2. Controls (Normal subjects).

Controls were selected to be healthy individuals without a history of hematological malignancies. Fifty two unrelated subjects from the European Gaza hospital, El-Nasser hospital and Gaza diagnostic center were selected to be the normal control for this study regardless of sex and age. The genotyping of the control was carried out by **Gharbiyoh A**. **(2006) [78]**.

Table 3.1. Characteristics of the study subjects.

Patients	n	Mean age (years)	Mean Onset age	North area	Middle area	South area
Total	52	8.5	5.7	30	13	9
Male	31	8.87	5.77	16	8	6
Female	21	8	5.63	14	5	3
Leukemia	49	8.72	5.81	28	13	8
Lymphoma	2	5.25	4	1	0	1
Meylocytic leukemia	1	5	4	1	0	0

3.2. Materials

3.2.1. Chemicals

Chemicals and reagents used in this study are listed below **(Tables 3.2 and 3.3)**. All chemicals were of analytical and molecular biology grade.

3.2.2. PCR primers

All PCR primers are indicated from 5' to 3' end. Sense primers are marked with (F) while antisense primers are marked with (R). The primer sequences were obtained from published literature **[44, 79]** and are provided in **Table 3.4**.

Table 3.2. Chemicals and reagents.

Chemicals and reagents	Supplier
Wizard Genomic DNA Purification Kit	Promega (Madison, USA)
PCR Master mix	Promega (USA)
Agarose gel	Promega (USA)
Nuclease free water	Promega (USA)
Sma I Restriction enzyme	New England Bio Labs (USA)
50 bp DNA ladder	New England Bio Labs (USA)
Low molecular weight marker	New England Bio Labs (USA)
BamH I restriction enzyme	Sigma diagnostics (USA)
PCR primers	Operon (Germany)

Table 3.3. Solutions and their contents.

Solution	Contents
DNA gel loading buffer (Bromophenol blue) (6X)	0.25% Bromophenol blue 40% (w/v) sucrose in water
DNA gel loading buffer (Orange G) (10X)	0.2% Orange G 40% (w/v) sucrose in water
Tris – Acetate – EDTA (TAE) (50X)	2M Tris base (pH 8), 1M Glacial acetic acid, 0.05M EDTA (pH 8)
Ethidium Bromide (**EtBr**)	(10mg/ml)

Table 3.4. PCR Primers used in the study.

Sequence Name	Nucleotide Sequence	Reference
CYP2C19*2 (F)	AATTACAACCAGAGCTTGGC	**[44, 79]**
CYP2C19*2 (R)	TATCACTTTCCATAAAAGCAAG	
CYP2C19*3 (F)	AAATTGTTTCCAATCATTTAGCT	**[79]**
CYP2C19*3 (R)	ACTTCAGGGCTTGGTCAATA	

3.2.3. Equipments

The experimental part of the work was done in the Islamic University genetics lab. The important equipments that were used are listed in **Table 3.5**.

Table 3.5. List of the major equipments used in the study.

Instruments	Manufacturer
Thermocycler, mastercycler personal	Eppendorf, Germany
Thermocycler, mastercycler gradient	Eppendorf, Germany
Electrophoresis chambers and Electrophoresis power supply	Biorad, USA
Microcentrifuge	Sanyo, U.K
Hoefer MacroVue UVis-20	Hoefer, USA
Microwave oven	L.G, Korea
Digital camera	Canon (Japan)

3.3. Methods

3.3.1. DNA extraction

3.3.1.1. Sample collection and preparation

About 2.5ml of venous blood were drawn into sterile EDTA tubes and mixed gently. The blood samples were then stored at -70˚C until time of DNA extraction and purification.

3.3.1.2. DNA extraction and purification

Genomic DNA was isolated from blood using Wizard Genomic DNA Purification Kit (Promega, USA), the contents of which are given in **Table 3.6,** according to the manufacturer's protocol. The materials that should be supplied by the user are indicated in **Table 3.7**.

Table 3.6. Wizard genomic DNA purification kit solutions.

Solutions	Volume
cell lysis solution	100 ml
nuclei lysis solution	50 ml
protein precipitation solution	25 ml
DNA rehydration solution	50 ml
RNase solution (4mg/ml)	250 µl

Table 3.7. Materials that should be supplied by the user.

Sterile 1.5 ml microcentrifuge tubes
Water bath, (37^{o}C, 65 ^{o}C)
Isopropanol, room temperature.
70% ethanol, room temperature.
Microcentrifuge.

3.3.1.3. Isolation of genomic DNA

A volume of 300µl well mixed blood were added to 1.5 ml microcentrifuge tube containing 900 µl of cell lysis solution (lyses the red blood cells and white blood cells), mixed by inverting the tube gently and the mixture was incubated at room temperature for 10 minutes. During the incubation period the tube was periodically mixed (2-3 times) by inversion.

The mixture was centrifuged at 13,000 rpm for 20 seconds, then the supernatant was removed and discarded without disrupting the visible white pellet, after that the white pellet was resuspended by vigorous vortexing (10-15 seconds).

A volume of 300 µl nuclei lysis solution (lyses the nuclear membrane of white blood cells nuclei) were added to the resuspend pellet with pipeting the solution 5-6 times. The solution should become very viscous; the samples with visible clumps should be incubated at 37^{o}C until the clumps are disrupted.

A volume of 1.5 µl RNase solution (digests the RNA) was added to the nuclear lysate and they were mixed by inverting 2-5 times, then incubated at 37^{o}C for 15 minutes, and the mixture was cooled to room temperature.

One hundred µl protein precipitation solution (precipitates all the cellular and nuclear proteins) were added to the cooled nuclear lysate and they were mixed vigorously for 10-20 seconds by the vortex -Small protein clumps may be visible after vortexing- then centrifuged at 13,000 rpm for 3 minutes. A dark brown protein pellet should be visible.

The supernatant was then transferred to a clean 1.5 ml microcentrifuge tube containing 300 µl isoprobanol and was mixed gently by inversion until white thread-like strands of DNA form a visible mass, then centrifuged at 13000 rpm for 1 minute. The DNA will be visible as a small white pellet.

The supernatant was removed and 300µl of 70% ethanol were added to the small white pellet and gently inverted several times to wash the DNA pellet, then centrifuged at 13,000 rpm for 1 minute.

The ethanol was aspirated carefully using a micropipette, and then the tube was inverted on a clean absorbent paper. The pellet was air-dried for 10-15 minutes.

The DNA pellet was rehydrated by addition of 100µl DNA rehydration solution and incubation at 65^{o}C for 1 hour. The DNA pellet should be mixed periodically by gently tapping the tube.

Finally The DNA was stored at 2-8^{o}C until PCR analysis.

3.3.2. PCR procedure

The two most common allelic variants (CYP2C19*2 and CYP2C19*3) of CYP2C19 were investigated using PCR followed by restriction analysis i.e., PCR-RFLP **[79]**.

For CYP2C19*2 detection (**Figure 3.1)**, a segment of exon 5 which contains the base pair substitution **681G>A** was amplified using the primer set presented in **Table 3.4** and the PCR product (168bp) was restricted using Sma I restriction enzyme which cuts the normal allele into two segments (118 bp and 50 bp) but doesn't cut the abnormal allele. The CYP2C19*2 homozygotes should yield one band (168 bp) while the CYP2C19*2 heterozygotes should produce three bands (168 bp, 118 bp, and 50 bp).

For CYP2C19*3 detection, a segment of exon 4 which contains the base pair substitution **636G>A** was amplified using the primer set presented in **Table 3.4** and the PCR product was restricted using BamH I restriction enzyme which cuts the normal allele into two segments (175 bp and 96 bp) but doesn't cut the abnormal allele. The CYP2C19*3 homozygote should yield one band (271 bp) while the CYP2C19*3 heterozygotes should produce three bands (271 bp, 175 bp, and 96 bp).

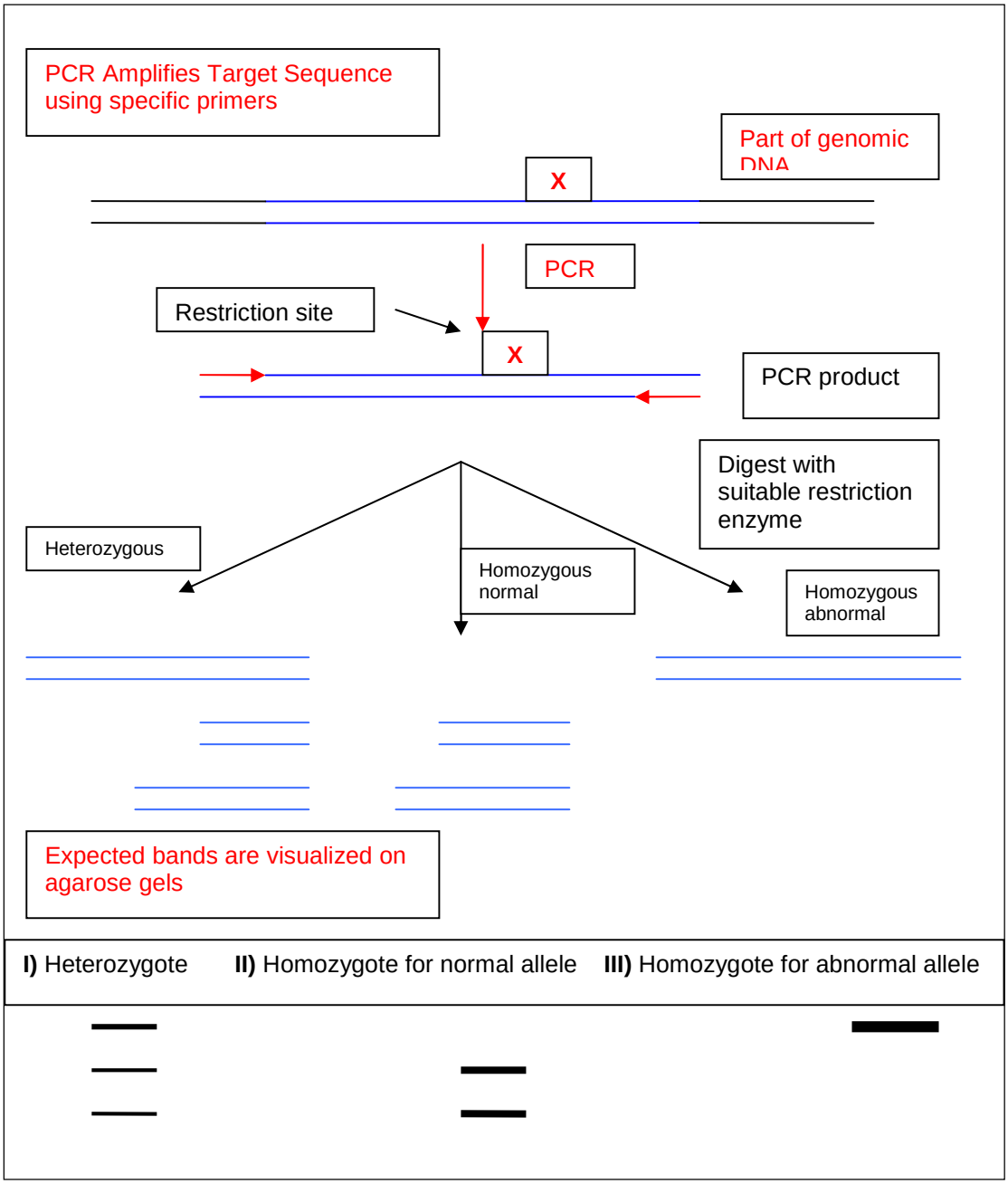


Figure 3.1. The PCR-RFLP principle of detecting mutant and wild type alleles. **I) Heterozygote**: three bands indicating that half of the PCR products were cut (normal allele) and the other half weren't (abnormal allele).**II) Homozygote for normal allele**: two bands indicating that the product was completely cleaved while **III) Homozygote for abnormal allele**: one band indicating that there was no cleavage happened.

The amplicons of CYP2C19*2 and CYP2C19*3 were prepared separately using the PCR master mix (Promega), **Table 3.8**, and appropriate primer sets at a concentration of 0.4 µM each. The quantity of the DNA used was determined using the spectrophotometer at 260 nm wavelength (1.0 absorbance = 50 µg/ml). The concentrations and volumes of the PCR mix are presented in **Table 3.9**. The negative controls (doesn't contain a DNA template) were applied with each PCR run. Every homozygote and heterozygote sample was repeated in triplet for PCR and restriction.

Table 3.8. PCR master mix buffers and solutions.

Concentration	Solution
50 units/ml	Taq DNA Polymerase [supplied in a proprietary reaction buffer (pH 8.5)]
400 µM	Each: dATP, dGTP, dCTP, dTTP
3 mM	$MgCl_2$

Table 3.9. The concentrations and volumes of the PCR mix for 25 µl reaction volume.

Component	Volume	Final Concentration
PCR master mix, 2x	12.5 µl	1x
Forward primer (10µM)	1 µl	0.4 µM
Reverse primer (10µM)	1 µl	0.4 µM
DNA template	4 µl	≈ 200 ng
Nuclease free water	6.5 µl	

3.3.2.1. CYP2C19*2 PCR-RFLP procedure

The PCR mix was amplified as follows: pre-heating (40 ^{o}C, 2 min), and initial denaturation (94 ^{o}C, 5 min) were followed by 35 cycles of (denaturation at 94 ^{o}C for 1 min, annealing at 54 ^{o}C for 1 min, and extension at 72 ^{o}C for 1 min) followed by a final extension step (72 ^{o}C, 5 min) **[79]**.

Electrophoresis was carried out on a Biorad electrophoresis setup and electrophoresis power supply system. The chambers of the system were filled with 1x TAE buffer (pH 8.0). The PCR products were analyzed on 2% agarose gel (1gm agarose, 1ml TAE (50X), and 49 ml distilled water) containing (2 µl of 10 mg/ml) ethidium bromide. 5.6 µl of the PCR product was mixed with 1.4 µl (10x Orange G) and the mixture was loaded on the gel along with 100 bp DNA ladder which is used for determining the size of the PCR product, then the gel was run on 80 volts for one hour. By using Hoefer MacroVue UVis-20 and Canon digital camera the results of the migration were detected and documented. The PCR product (168 bp) migrated just near the 200 bp ladder band. The PCR products (168 bp) were cut with Sma I which cleaves the ccc/ggg sequence, **Figure 3.2** shows the sequence, recognition site of Sma I and the location of the primers used to amplify the CYP2C19*2 fragment. **Figure 3.3** shows the recognition site for Sma I restriction enzyme.

The restriction enzyme digestion of the PCR product for (CYP2C19*2) was performed as follows: in a total of 25 µl volume, 10 µl PCR-amplified DNA were mixed with 0.5 µl Sma I (20 µ/µl), 2.5 µl 10x enzyme buffer, and 12µl nuclease free water, the reaction was incubated at 25 ^{o}C overnight, then the products were analyzed by a 3% agarose gel electrophoreses **(Figure 3.1)**, two fragments (118 bp and 50 bp) are produced from the digestion of the normal allele.

AATTACAACCAGAGCTTGGCATATTGTATCTATACCTTTATTAAATGCTTTAATT
TAATAAATTATTGTTTTCTCTTAGATATGCAATAATTTTCCCACTATCATTGATT
ATTT**CCCGGG**AACCCATAACAAATTACTTAAAAACCTTGCTTTTATGGAAAGT
GATA

Figure 3.2. CYP2C19*2 product (168 bp) and Sma I recognition site underlined with red color, PCR primers used are shown in blue color.

5'….CCC▼GGG….3'

3'….GGG▲CCC….5'

Figure 3.3. The Recognition site of Sma I restriction enzyme.

3.3.2.2. CYP2C19*3 PCR-RFLP procedure

The PCR protocol used for CYP2C19*3 was the same as that for CYP2C19*2 except that using the appropriate CYP2C19*3 primers set, and using the Bromophenol blue (6X) as loading dye (2 µl 6x Bromophenol blue, 5µl PCR product) which migrates with the 200bp DNA fragments while Orange G migrates along with 50bp DNA fragments. Detection and documentation of the PCR products were the same as described for CYP2C19*2. The products (271 bp) were cut with BamH I which cleaves the G/GTACC sequence **(Figures 3.4** and **3.5)**.

The restriction enzyme digestion of the PCR product for (CYP2C19*3) has been performed as follows: in a total of 25 µl volume, 10 µl PCR-amplified DNA were mixed with 1.5 µl BamH I (20 µ/µl), 2.5 µl 10x enzyme buffer, and 11 µl nuclease free water. The reaction was incubated at 37°C overnight, and then the products were analyzed by a 3% agarose gel electrophoresis. **Figure 3.4** shows the sequence, recognition site of BamH I and the location of the primers used to amplify the CYP2C19*3 fragment. **Figure 3.5** shows the recognition site for BamH I restriction enzyme.

AAATTGTTTCCAATCATTTAGCTTCACCCTGTGATCCCACTTTCATCCTGGGCT
GTGCTCCCTGCAATGTGATCTGCTCCATTATTTTCCAGAAACGTTTCGATTATA
AAGATCAGCAATTTCTTAACTTGATGGAAAAATTGAATGAAAACATCAGGATT
GTAAGCACCCCCTGGATCCAGGTAAGGCCAAGTTTTTTGCTTCCTGAGAAAC
CACTTACAGTCTTTTTTTCTGGGAAATCCAAAATTCTATATTGACCAAGCCCTG
AAGT

Figure 3.4. CYP2C19*3 product (271 bp) and BamH I recognition site underlined with blue color, primers used are in blue color.

5'….G▼GATCC….3'

3'….CCTAG▲G….5'

Figure 3.5. The Recognition site of BamH I restriction enzyme.

Homozygotes for the mutant allele are predicted to have the PM phenotype. In all other cases the subjects were assumed to have the EM phenotype.

3.4. Statistical analysis

The statistical packages SPSS, EPI INFO, and Excel were used to perform all the analyses and charts. Descriptive statistics were reported as absolute frequencies and percentages for qualitative data. Comparisons of genotypes between patients and controls were performed by means of the odds ratio and the Chi-square (χ^2) test or Yates corrected Chi-square in the case of expected frequencies less than 10 or the Fisher's exact test in the case of expected frequencies less than 5. All the statistical tests were two sided; a *p* value of <0.05 was considered as statistically significant. Multivariate analysis, correlation coefficient, odds ratios, p value (two-sided tests) and 95% CI were used to describe the strength of association.

CHAPTER – 4

Results

Chapter 4

Results

4.1. Patient's Personal data analysis

The total number of patients enrolled in this study was fifty two, 21 (40.38%) of them were females and the other 31 (59.62%) were males **(Table 4.1)**. Statistical analysis showed that there is no significant difference between male and female distribution ($p > 0.05$). The age of the subjects ranged from 3 to 20 years with a mean of 8.52, the male's age mean was 8.87 while that of the females was 8.00 years **(Table 4.2)**. There is no significant difference between males and female's age means (p value > 0.05).

Table 4.1. Distribution of patients according to sex.

Sex	n	Percentage (%)	P value
Male	31	59.62	0.17
Female	21	40.38	
Total	52	100	

Table 4.2. Statistical analysis for male and female age means.

Sex	n	Mean ± SD	95% C I	P value
Male	31	8.87 ± 2.99	(0.31 - 1.44)	0.42
Female	21	8.00 ± 4.76		
Total	52			

4.1.1. The onset age distribution

The onset age of hematological malignancies ranged from 1 to 16 years with a mean of 5.70 years **(Table 3.1)**. Male's onset age mean was 5.86 year, and that

of the females was 5.63 years. Statistical analysis using ANOVA-I test indicated that there is no significant difference between males and females in terms of the age of onset of malignancy ($p > 0.05$), but there is a significant correlation between the number of cases and the onset age ($\chi^2 = 43.39$, $p < 0.05$) where 9 patients have 5 years onset age, 7 patients have 3 years onset age, and 5 patients have 6 years onset age while the other onset age frequency have less than 3. There is also no significant difference between the onset age and the type of disease ($p > 0.05$). According to the subjects sex **(Table 4.3)** there is no significant difference between males and females and the onset age means ($p > 0.05$).

Table 4.3. Statistical analysis for male and female onset age means.

Sex	n	Mean ± SD	CI	P value
Male	31	5.86 ± 3.10	(0.43 – 0.7)	0.89
Female	21	5.63 ± 4.46		
Total	52			

4.1.2. The living area

Distribution of the study subjects according to the living area showed that the Northern area (Gaza city and north Gaza Strip) has the highest frequency (30) of the cases representing 57.69%, followed by the Middle area with 13 cases (25%), and finally by the Southern area (Rafah and Khanyouns) with 9 patients representing 17.3% **(Table 4.4**). Statistically there is a significant difference between the distribution of the hematological malignancies patients and the living area in Gaza Strip ($p < 0.05$), but there is no significant difference between the distribution of patients in Gaza Strip and the sex of the patient ($p > 0.05$). Also there is no significant difference between the living area and the type of the disease ($P > 0.05$).

Table 4.4. Distribution of cases according to the living area.

Living area	n	Percentage (%)	P value
Northern area	30	57.7	0.001
Middle area	13	25.0	
Southern area	9	17.3	
Total	52	100%	

4.1.3. The family history and the type of hematological malignancy

The family history of the patients shows that only two ALL patients were sibs (brother and sister). According to the type of malignancy, 49 of the cases were ALL representing 94.2%, 2 cases (3.85%) were Lymphoma, and one case was CML which represented 1.92% **(Figure 4.1)**. Statistically, there is a significant difference between the types of the hematological malignancies ($p < 0.05$). Also there is a significant difference according to the family history ($p < 0.05$).

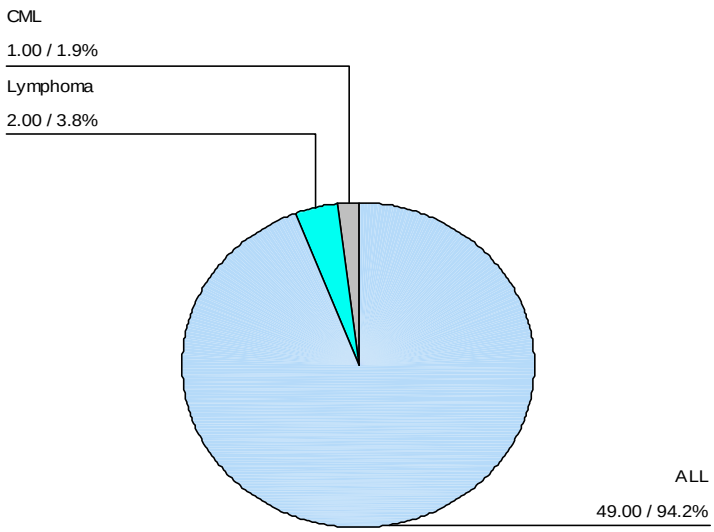


Figure 4.1. Distribution of patients according to the type of hematological malignancy.

4.2. CYP2C19 Genotyping results

DNA extraction, PCR amplification and restricted PCR products were done and recorded for all samples. **Figure 4.2** shows the DNA extracted from representative patients samples. CYP2C19 exon 5 PCR products and the restricted PCR products are presented in **Figures 4.3 and 4.4**, respectively. The PCR amplification product and the restricted product of CYP2C19 exon 4 are presented in **Figures 4.5 and 4.6**, respectively.

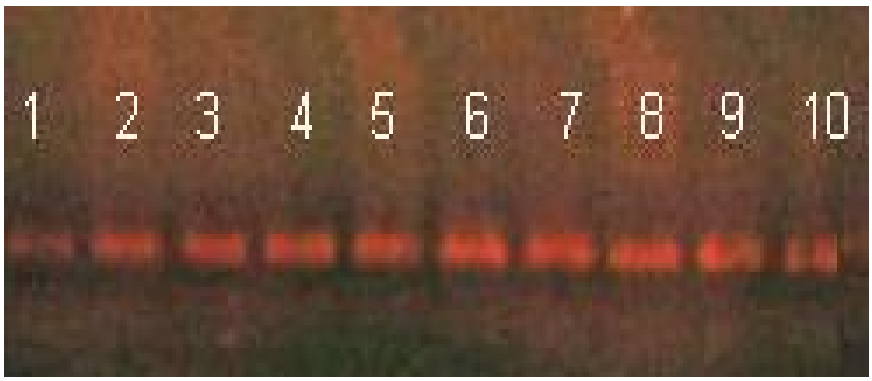


Figure 4.2. A photograph of DNA extracted from 10 EDTA - blood samples run on 1% ethidium bromide stained agarose gel and visualized by UV.

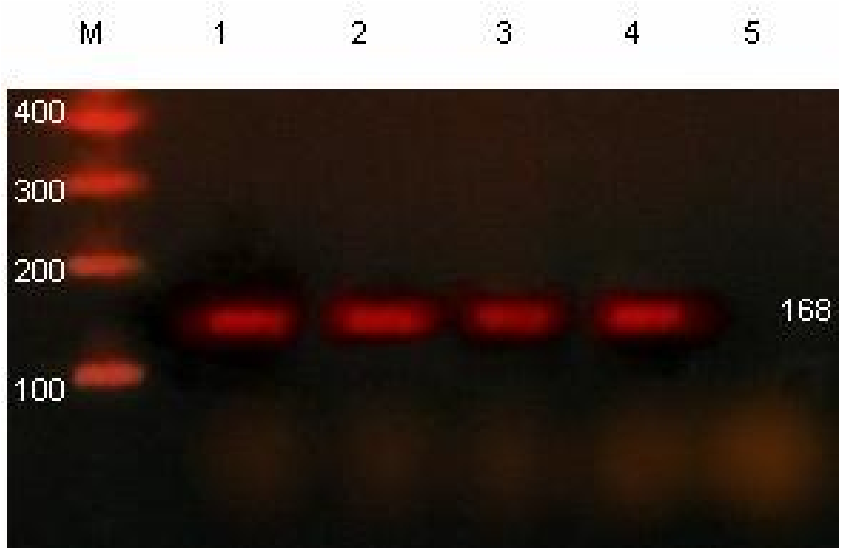


Figure 4.3. Amplification products of CYP2C19*2 by PCR. Amplified products of each reaction were analyzed by electrophoresis on 2 % ethidium bromide stained agarose gel. Lane M: 100 bp marker, lanes 1-4 amplified CYP2C19*2 fragment (168 bp). Lane 5: Negative control.

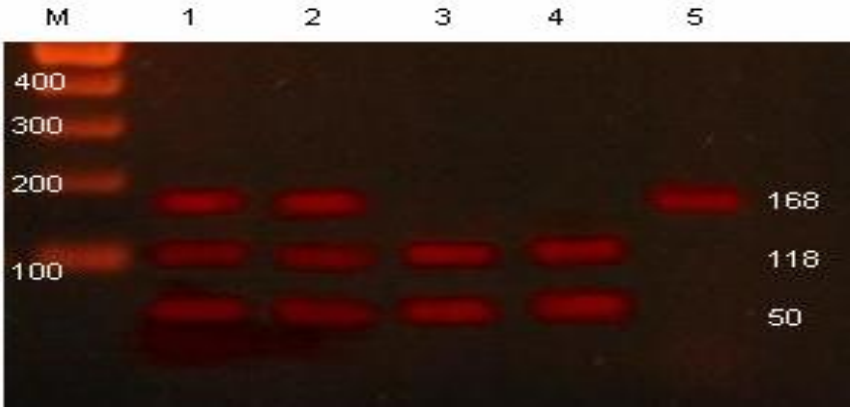


Figure 4.4. Restriction products for CYP2C19*2 by Sma I. Restricted products were analyzed by electrophoresis on 3% ethidium bromide stained agarose gel. Lane M: 100 bp marker, lanes 1and 2: Heterozygote, lanes 3 and 4: Normal Homozygote, lane 5: CYP2C19*2 homozygote.

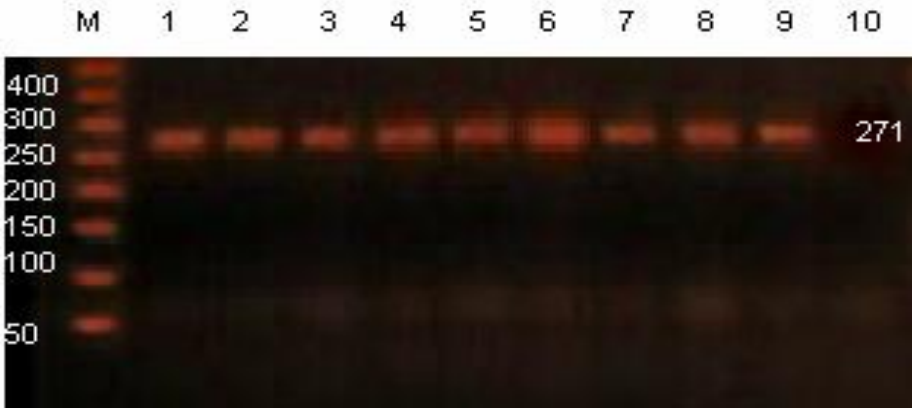


Figure 4.5. Amplification products of CYP2C19*3 by PCR. Amplified products of each reaction were analyzed by electrophoresis on 2 % ethidium bromide stained agarose gel. Lane M: 50 bp DNA marker, lanes 1-9 are amplified CYP2C19*3 fragment (271 bp), lane 10: Negative control.

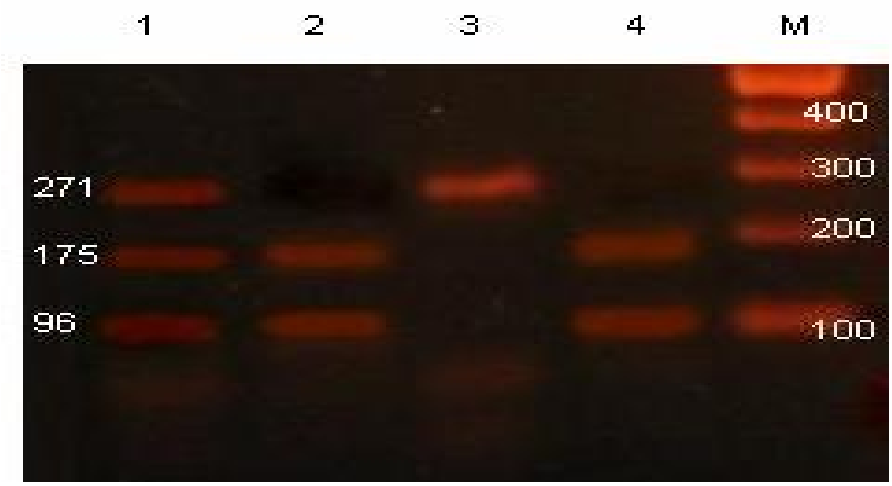


Figure 4.6. Restriction products for CYP2C19*3 by BamH I. Restricted products were analyzed on 3% ethidium bromide stained agarose gel. Lane 1: Heterozygote sample, lane 2: Normal Homozygote, lane 3: CYP2c19*3 PCR product as a negative control without adding a BamH I restriction enzyme. Lane 4: Normal homozygote, lane M: 100 bp marker.

4.3. Genotyping analysis

The genotyping (PCR-RFLP) analysis of patients DNA samples showed the following results: heterozygotes (1*/2*): 15.39% (8/52), heterozygotes (1*/3*): 1.92% (1/52), homozygotes (2*/2*): 1.92% (1/52), and the remaining cases were homozygotes (1*/1*): 80.77% (42/52) **(Figure 4.7 and Table 4.5)** while in normal subjects **(Figure 4.5 and Table 4.6)** the following results were obtained by **Gharbyeh A. (2006) [78]**: heterozygotes (1*/2*): 7.69% (4/52), heterozygotes (1*/3*): 5.77% (3/52), homozygotes (2*/2*): 1.92% (1/52), and the rest of the cases were normal homozygotes (1*/1*) 84.61% (44/52), no homozygotes for CYP2C19*3 or compound heterozygotes (2*/3*) has been encountered in any of the investigated individuals.

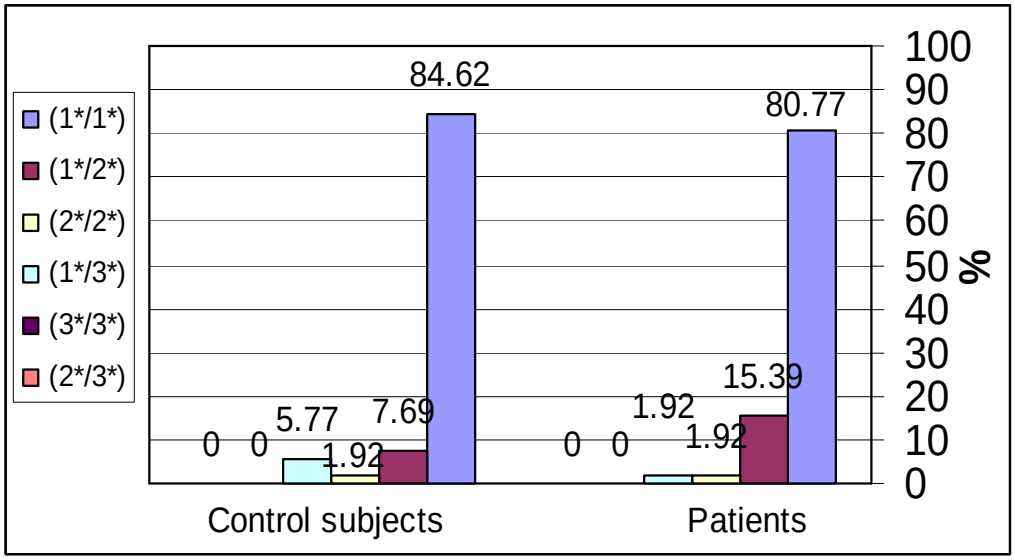


Figure 4.7. Distribution of patients and normal subjects according to their CYP2C19 genotypes.

The derived allele frequency for CYP2C19*2 (9.62%) in patients population was calculated as follows:

$$\frac{\text{The total number of the CYP2C19*2 alleles}}{\text{The total number of the CYP2C19 alleles}} \times 100\% = \frac{10}{52 \times 2} = \frac{10}{104} = 9.62\%$$

The total number of the CYP2C19*2 alleles (10) was computed by adding the number of alleles present in the heterozygotes (8) to the number of alleles present in the homozygotes (2x1=2).

Table 4.5. CYP2C19 genotypes and derived allele frequencies in the patients.

Genotype	n	Observed frequency	Allele	Derived allele frequency
CYP2C19*1/ CYP2C19*1	42	80.77%		
CYP2C19*1/ CYP2C19*2	8	15.39%	CYP2C19*1	89.42%
CYP2C19*2/ CYP2C19*2	1	1.92%	CYP2C19*2	9.62%
CYP2C19*1/ CYP2C19*3	1	1.92%	CYP2C19*3	0.96%
CYP2C19*3/ CYP2C19*3	0	0%		
CYP2C19*2/ CYP2C19*3	0	0%		
Total	52	100%		100%

The allele frequencies in both groups showed relatively similar distribution, for CYP2C19*2, only 6 alleles (5.77%) were recorded in normal subjects however 10 alleles were encountered (9.62%) in patients. For CYP2C19*3 the normal subjects showed higher frequency than the patients (3 alleles (2.89%) in normal subjects and only one allele (0.96%) in patients). The other alleles, assumed to be normal (CYP2C19*1), showed a distribution of 93 (89.42%) in the patients and 95 (91.35%) in the normal subjects **(Tables 4.5 and 4.6 and Figure 4.8)**.

Table 4.6. CYP2C19 genotypes and derived allele frequencies in the control group.

Genotype	n	Observed frequency	Allele	Derived allele frequency
CYP2C19*1/ CYP2C19*1	44	84.62%		
CYP2C19*1/ CYP2C19*2	4	7.69%	CYP2C19*1	91.35%
CYP2C19*2/ CYP2C19*2	1	1.92%	CYP2C19*2	5.77%
CYP2C19*1/ CYP2C19*3	3	5.77%	CYP2C19*3	2.88%
CYP2C19*3/ CYP2C19*3	0	0%		
CYP2C19*2/ CYP2C19*3	0	0%		
Total	52	100%		100%

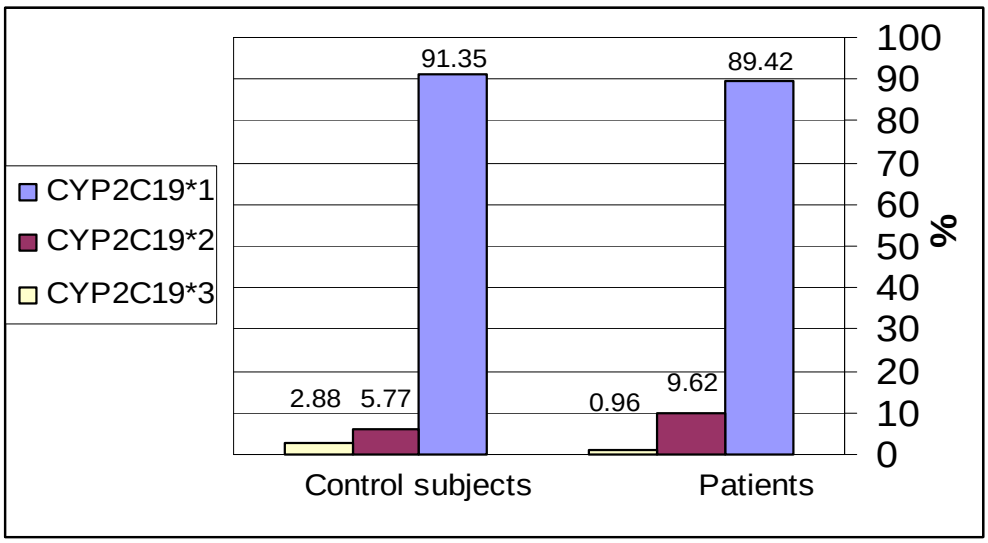


Figure 4.8. Distribution of the CYP2C19 alleles in the patients and the control subjects.

The frequency of CYP2C19*2 in the control group versus the patients group was not significantly different ($p > 0.05$) **(Table 4.7)**. Also the frequency of CYP2C19*3 allele in the control (which is higher than that in patients), showed no significant difference ($p > 0.05$) **(Table 4.8)**.

Table 4.7. Comparison between patients and control subjects in terms of the CYP2C19*2 allele frequency.

Group	CYP2C19*2 allele	Other alleles	P value
Patients	**10**	**94**	**0.435***
Control	**6**	**98**	

***Chi-square (Yates corrected).

Table 4.8. Comparison between patients and control subjects in terms of the CYP2C19*3 allele frequency.

Group	CYP2C19*3 allele	Other alleles	P value
Patients	**1**	**103**	**0.621***
Control	**3**	**101**	

*Fisher exact test.

The comparison between the patients and the control subjects to evaluate the association between the hematological malignancies patients and the CYP2C19*2 polymorphism was done statistically, the patients appear to have the CYP2C19*2 allele by 1.97 higher than the controls with relative risk of 1.8 **(Table 4.9)**. In contrast for CYP2C19*3, the patients appear to have the CYP2C19*3 allele by 0.32 higher than the control subjects with a risk factor of 0.33 **(Table 4.10)**, but for both alleles there is no significant difference between the patients and the control subjects in terms of having the CYP2C19*2 and CYP2C19*3 alleles (p- value = 0.389 and 0.309, respectively).

Table 4.9. Comparison between the patients and the control subjects in terms of CYP2C19*2 allele distribution.

Group	With CYP2C19*2	Without CYP2C19*2	P value
Patients	**9**	**43**	**0.389**
Control	**5**	**47**	

Odds ratio = 1.97 0.54<OR<7.41
Relative risk = 1.8 0.65<RR<5.01

Table 4.10. Comparison between the patients and the control subjects according to the CYP2C19*3 distribution.

Group	With CYP2C19*3	Without CYP2C19*3	P value
Patients	**1**	**51**	**0.309**
Controls	**3**	**49**	

Odds ratio = 0.32 0.01<OR<3.65
Relative risk = 0.33 0.04<RR<3.1

Regarding the metabolism phenotypes associated with CYP2C19 polymorphism, comparison between the patients and the control subjects showed no difference between the two groups, each having one CYP2C19*2 homozygous (2*/2*) genotype (PM and all the other cases were considered EM **Table 4.11**).

Table 4.11. Comparison between the patients and the control subjects in terms of the phenotypic distribution.

Group	PM	EM	P value
Patients	**1**	**51**	**0.3**
Controls	**1**	**51**	

Odds ratio = 1.0 0.0<OR<37.79
Relative risk = 1.0 0.06<RR<15.57

In brief there is no significant difference between the patients and the controls regarding all the obtained CYP2C19 genotypes **(Table 4.12)**.

Table 4.12. Comparison between the similar genotypes in the patients and the controls. The p-value was calculated for each genotype.

Allele	Genotype	Patients	Controls	P value
CYP2C19*2 681 G→A	GG (Normal)	42	44	0.8†
	GA (Heterozygote)	8	4	0.36†
	AA (Mutant)	1	1	1.0†
CYP2C19*3 636 G→A	GG (Normal)	42	44	0.8†
	GA (Heterozygote)	1	3	0.62*
	AA (Mutant)	0	0	

† Chi-square test.
* Fisher exact test 2-tailed.

Statistical analysis showed that there is no significant difference between the living area and the CYP2C19*2 genotype ($p > 0.05$), there is also no significant difference between the living area and the CYP2C19*3 genotype ($p > 0.05$).

Statistically there is no significant difference between males and females in terms of having the CYP2C19*2 allele ($p > 0.05$) as shown in **Table 4.13**, the same is also true for CYP2C19*3 ($p > 0.05$).

Table 4.13. CYP2C19*2 distribution in males and females.

Sex	CYP2C19*2			Total
	1/1	1/2*	2*/2*	
Male	25	6	0	31
Female	18	2	1	21
Total	43	8	1	52

Moreover, there is no significant correlation between the type of the disease and the presence of the CYP2C19*2 allele ($p > 0.05$) **(Table 4.14)**.

Table 4.14. Type of hematological malignancy and CYP2C19*2 crosstabulation.

Disease	CYP2C19*2			Total
	1/1	1/2*	2*/2*	
ALL	41	7	1	49
Lymphoma	1	1	0	2
CML	1	0	0	1
Total	43	8	1	52

4.4. Correlation between CYP2C19*2 and the malignancy onset age

The mean onset age of the patients who were heterozygotes and homozygotes for CYP2C19*2 was 5.72 years, while the other patients who don't carry the CYP2C19*2 allele had a mean onset age of 5.71 years. The statistical analysis indicated that there is no significant correlation between the onset age and the CYP2C19*2 allele frequency in the patients (Pearson correlation = 0.06, $p = 0.66$). This shows that there is no significant difference between the means of the onset age of the two groups of patients. In other words there is no significant association between the onset age and the CYP2C19 polymorphism.

CHAPTER – 5

Discussion

Chapter 5
Discussion

In our opinion, after completion of the human genome project and the improvement of genetic diagnosis, which overtime will be routine tests for investigating and screening many cancers disposing genetic factors, studies linking cancers with specific genotypes will be easier and more reproductive.

Investigating the association between CYP2C19 polymorphism and cancers have important implications since cytochromes are actively involved in drugs, carcinogens, and procarcinogens metabolism. Some are involved in the activation of procarcinogens, while others may take part in the inactivation of carcinogens. This depends on the kind of carcinogens, cancers, and on the kind of mechanism of carcinogenesis **[74].**

The CYPs are the main drug metabolizing enzymes in human body and the CYP2C19 is one of the most important enzymes in this large family of proteins. The CYP2C19 is responsible for the metabolism of many drugs that some of which are used in treatment of the hematological malignanceis such as cyclophosphamide and glucocorticoids. For the CYP2C19 which is a highly polymorphic locus there is currently 27 alleles, only 3 of which are with normal activity and one with high activity, the others are with no enzymatic activity **[16, 42].** The major CYP2C19 abnormal alleles are CYP2C19*2 and CYP2C19*3 which account for about 99% of all the PM in the Asian population and 87% in the Caucasian population **[16].**

The CYP enzymes have a multi-step role in the metabolism of drugs and carcinogens, several enzymes in various stages are shared to metabolize a given substance, therefore no specific enzyme metabolizes a specific target alone, but it is a cumulative effect of many enzymes some of which are highly polymorphic and many of them may have abnormal alleles e.g., CYP2C19, CYP2C9, CYP3A4, and to a lesser extent other enzymes. Therefore, the metabolism of many chemicals

doesn't depend on one specific enzyme and this highlights the need for investigating all the possible enzymes simultaneously when examining drug metabolism defects.

Our hypothesis postulated that heterozygosity and homozygosity for the base pair substitution in exon 5 (681G **à** A) and exon 4 (636 G **à** A) of CYP2C19 could be associated with a functional decrease in the amount of xenobiotic monooxygenase activity therefore those individuals would have a markedly increased susceptibility to the genotoxic and leukemogenic effects of cytotoxic and xenobiotic substances, that they normally encounter in their daily life.

In this study the CYP2C19*2 frequency in the patients was (9.62%) **(Table 4.5)** and in the controls was (5.77%) **(Table 4.6)**. These frequencies are lower than those reported in Caucasians (14.7%), Egyptian (11.0%), and Asians (30%). Moreover, this study revealed that the CYP2C19*3 frequency in the patients was (0.96%) **(Table 4.5),** and in the controls was (2.88%) **(Table 4.6)** and these frequencies are higher than those recorded in Caucasians (0.04%), Egyptian (0.2%), but lower than those reported in Asians (8%) **[17, 80]**. According to this study the PM phenotype frequencies in the patients and in the controls were the same (1.9 %) **(Table 4.11)** which are lower than those reported in Caucasians (2 to 5%), Saudi Arabians (2.06%), and in Orientals (13 to 23%) **[16, 81]**. The difference in genotype frequencies between the different populations is not unexpected and reflects the different genetic makeup of the different ethnic groups.

Several studies on CYP2C19 polymorphism and its association with carcinogenesis have shown contradictory results. Although this study indicated that the CYP2C19 polymorphism was not significantly associated with childhood hematological malignancies other studies however, associated the CYP2C19 polymorphism with certain types of cancers e.g., esophagus, stomach, lung and bladder cancers **[82]**. Other authors couldn’t associate the CYP2C19 polymorphism with certain types of cancers e.g., Wadelius *et.al.* (1999) evaluated

the association of the variant m1 allele (CYP2C19*2) of the CYP2C19 gene with prostate cancer among a Swedish population, but no significant differences were found between cancer patients and controls **[76]**. In addition, **Bartsch** and his colleagues couldn't find association between the PM genotype in Japanese patients with bladder cancer, but they found a significant association between PM genotype and squamous cell carcinoma of the lung **[77]**. Differences in exposure/consumption of various chemicals (e.g., eating behaviors) could be one explanation for those contradictory results.

Statistical analysis of the frequencies of PMs in the cancer and the control groups showed no significant difference **(Table 4.11)**. However, this doesn't exclude the presence of other predisposing genetic factors to the hematological malignancies that may be involved in the occurrence and progression of the cancer. Therefore, association studies can produce spurious results if cases and controls show different allele frequencies for genes that are not related to the disease being studied **[53, 54].**

Although the reasons for the inconsistencies between the studies are not clear, possible explanations: (I) low frequency of the at risk genotype, which reduces the statistical power, (II) small size of the study population and (III) the type and even the subtype of the hematological malignancies, for example studying the correlation of CYP2C19 polymorphism with the morphological and immunological subtypes of ALL, which may give more valuable results.

CYP2C19 polymorphism, even though not significantly associated with hematological malignancies, should be considered an important public health issue since they may predispose to other types of malignancies or complicate the treatment of existing ones. Research on the role of CYP2C19 polymorphisms in hematological malignancies is not in the last stages, because many genes are involved in the initiation and progression of the hematological malignancy the reason why it is called a multifactorial disease (i.e., determined by a combination

of multiple factors, genetic and environmental) **[50]**, and the CYP2C19 polymorphism could be one step in this process.

The results of the current study showed that there is no significant difference between male and female in having the defective allele, the only patient (female) who is a CYP2C19*2 homozygous (PM) died after a short peroid of time after intiation of the induction therapy which included: dexamethazone, low dose intrathecal methotrexate (MTX), asparaginase , cyclophosphamide, adrimicine, and vincristine. The explanation for this observation could be: I) the drugs which were used in treatment may not be metabolized properly by the CYP2C19 enzyme, so it could have been toxic for her or, II) the drugs may be induced (rapidly converted from the inactive state to the active state) by the CYP2C19, so it would have been useless in the treatment of the disease. One of these drugs is the cyclophosphamide which is a substrate of the CYP2C19 **[83]**. Additionaly, this homozygous patient suffered from Down's syndrome (trisomy 21). Hyperdiploid ALL cells with extra copies of chromosome 21 generate higher levels of the active MTX metabolite, MTX polyglutamates. This is on account of increased intracellular transport of MTX via the reduced folate carrier (RFC) whose gene is localized to chromosome 21 and may also account for the increased MTX-associated toxicity of DS ALL patients **[84]**. But the dose of MTX she had been given is not toxic (low dose intrathecal MTX (12 mg)), so the toxicity of the MTX was excluded. And the toxicity of the other drugs such as cyclophosphamide could be attributed to the absence of the CYP2C19 enzymatic activity **[85]**. Toxicity of drugs for individuals with certain genotypes is well documented for 6-MP and genotyping for the thiopurine methyltransferase (TPMT) gene is mandatory before administration of this drug to ALL patients in many USA hospitals **[86]**.

The polymorphism of CYP2C19 may relate to the type of the hematological malignancy, to the suptypes of it, to the severity and progression of the malignancy, and to the response to treatment. Consequently further studies are needed to link certain genotypes with those variables.

From the results of this study it is clear that the heterozygotes among the patients and the normal subjects, particularly for CYP2C19 *1/*2, have considerable percentages, and this will lead to an increase in homozygotes defective alleles (PM) as a result of mating between carriers.

It appears from our results that the number of the male patients is more than that of the females; this may indicate that males are more susceptible to the hematological malignancies than females. This result is congruent with other findings *e.g*., among newly diagnosed childhood cancers, the standardized (with European reference) incidence rates for all participating registries in Europe yields a boys to girls ratio of 1.22. The incidence of ALL among children younger than 15 years of age is consistently higher among males (approximately 20%) relative to females **[60]**.

Childhood cancers occur at a slightly higher rate in males and at a significantly higher rate in whites **[14]**. The male predominance is a feature of cancer incidence in all ages **[60]**. As observed from this study the hematological malignancies appeared significantly higher in males than in females. Additionally, the Palestinian MOH 1995 – 2000 cancer report showed that the sex distribution of hematological pediatric malignancies is 61.9% in males and 38.1% in females **[13]**. This higher rate could be due to the more frequent exposure of males to carcinogenic materials, or due to the genetic and physiologic differences between sexes. Genotype analysis of the CYP2C19 however, indicated an equal frequency of the CYP2C19*2 and CYP2C19*3 in males and females.

The results also showed that the family history has no significant impact on the occurrence of hematological malignancies because all the patients have no family history of the disease except for one sib. Childhood cancers, with rare exceptions, are not therefore inherited **[60]**. Rates of cancer among offspring of childhood cancer survivors are reported to be similar to those for the general population confirming the lack of a primary genetic cause for most childhood cancers **[60]**. This further confirms the multifactorial nature of haematological malignancies.

The results of the present study showed that the onset age wasn't affected by the sex; the males' onset age mean was 5.77 while that of the females' was 5.63 years. This may indicate that the sex has no role in the beginning of the hematological malignancy. There is a significant correlation however, between onset age of the malignancy and the number of patients where we have encountered 9 patients with 5 years onset age, 7 patients with 3 years, and 5 patients with 6 years. This also indicates that the majority of the examined patients (21 patients representing 40% of the study population) ranged from 3-7 years of onset age which is relatively higher than the (2-5 years) reported by many other investigators **[87].** The environmental and genotypic differences among the different populations should be responsible for such differences.

Regarding the living area of the patients, our results showed that there was a significantly higher number of patients (30 patients representing 57.69%) from the Northern area. This result could be due to environmental, chemical which may include the chemicals used in agriculture and the close proximity to the green area, and/or genetic factors, so these factors must be studied to determine the risk factors in the Northern area.

Certain enzyme genotypes in people without cancer appear to be associated with DNA damage that could lead to cancer. In other words normal human cells with certain enzyme forms show higher levels of DNA mutations, and consequently become predisposed to cancer, than human cells with different enzyme forms **[88].**

The PCR-RFLP technique used in this study is quick and specific. The limitation of this technique however is its inability to detect mutations which occur outside the restriction enzyme recognition site.

Studies on CYP genes polymorphisms are important as they ultimately aim to develop drugs that would accelerate the activity of these enzymes in patients whose enzymes naturally work very slow. Other CYPs do the reverse, taking

procarcinogens (potentially cancer-causing substances) and turning them into cancer-causing chemicals that linger in the body where they can cause damage **[88].**

This field needs more studies on the CYP2C19 (and other CYP genes) polymorphism and their distribution in our population because of their importance in drug and chemical metabolism. Many companies have developed tests for clinical use to detect gene variations - including deletions and duplications - for the CYP2D6 and CYP2C19 genes which is intended to be an aid for physicians in individualizing treatment and dosing of drugs metabolized through these genes **[89].**

The importance of this study emerges from its being the first in investigating the correlation between the CYP2C19 polymorphism and childhood hematological malignancies. Other reports and researches have focused on the correlation between the CYP2C19 genetic polymorphism and solid tumors such as esophagus, stomach, and lung cancers **[74]**.

From our results, though not statistically significant, it is clear that the genotypic characteristics of the CYP2C19 variants in the hematological malignancy patients are different from those of the normal subjects. This makes the results of this study promising but further studies are required for examining more patients and perhaps more CYP genes polymorphisms.

CHAPTER – 6

Conclusions & Recommendations

Chapter 6
Conclusions & Recommendations

The present study focuses on the polymorphism of *CYP2C19* gene in 52 hematological malignancy children in Gaza Strip-Palestine. The results of the study can be summarized as follows:

Comparison between the control group and the hematological malignancy patients showed that there is no association present between the CYP2C19 polymorphism and the occurrence of the hematological malignancies.

Analysis of data showed that hematological malignancies occur at higher frequency in males but the difference is statistically insignificant.

The family history has no role in the occurrence of hematological malignancies, and this proves the importance of the environmental, chemicals and physical factors in the occurrence of ALL and other hematological malignancies.

Analysis of data according to the onset age showed a significant correlation between the number of cases and the onset age where 21 patients have 3-6 years onset age.

The correlation between the CYP2C19*2 allele and the onset age showed no significant correlation. In other words the presence of the CYP2C19*2 allele doesn't affect the onset age of the affected patients.

None of the patients or the normal subjects showed any compound heterozygous genotype (2*/3*). Moreover, none of the 52 patients or the 52 control subjects was homozygous for CYP2C19*3, which reflects the scarcity of this allele in the Gaza Strip population.

The abnormal homozygous phenotype (2*/2*) may affect the treatment of the patients treated with Cyclophosphamide and other chemotherapeutic drugs.

In conclusion we recommend:

- Investigating the correlations between CYP2C19 polymorphism and response to chemotherapeutic drugs, the severity of the disease, and the survival rate after diagnosis.

- Investigating and determining the prevelance of other CYPs genes such as CYP3A4, CYP2D6 in cancer patients as compared to normal individuals.

- Studying of CYP2C19 genotyping of more patients undergoing chemotherapy (mainly cyclophosphamide) to elucidate and prove the effect of the PM phenotype on the survival rate of those patients.

REFERENCES

REFERENCES

1. Reichhart DW and Feyereisen R. (2000). Cytochromes P450: a success story. Genome Biol. 1(6): 3003.1-3003.9.

2. Hukkanen J. (2000). Xenobiotic-metabolizing cytochrome p450 enzymes in human lung. University of Oulu publications. Ph.D. thesis. Oulu University, Finland.

3. Guengerich FP. (2006). Cytochrome P450s and other enzymes in drug metabolism and toxicity. AAPS Journal 8(1): E101-E111.

4. Mcginnity DF, Parker AJ, Soars M and Riley RJ. (2000). Automated definition of the enzymology of drug oxidation by the major human drug metabolizing cytochrome P450s. Drug Metab Dispos. 28(11): 1327-1334.

5. Bertz RJ and Granneman GR. (1997). Use of *in vitro* and *in vivo* data to estimate the likelihood of metabolic pharmacokinetic interactions. Clin Pharmacokinet. 32: 210-258.

6. Bowen DT, Frew ME, Rollinson S, Roddam PL, Dring A, Smith MT, Langabeer SE and Morgan GJ. (2003). CYP1A1*2B (Val) allele is overrepresented in a subgroup of acute myeloid leukemia patients with poor-risk karyotype associated with NRAS mutation, but not associated with FLT3 internal tandem duplication. Blood. 101(7):2770-2774.

7. Hutchinson CL, Menck HR, Burch M and Gottschalk R. (2004). Cancer Registry Management: Principles & Practices. Kendall Hunt. Publisher com. 2nd edition, P 344-360.

8. Nelson DR. (1999). Cytochrome P450 and the individuality of species. Arch Biochem Biophys. 369:1-10.

9. Lin JH and Lu AY. (2001). Interindividual variability in inhibition and induction of cytochrome P450 enzymes. Annu Rev Pharmacol Toxicol 41: 535–567.

10. Goldstein JA. (2005). Human Metabolism Group. University of Texas SouthwesternMedicalSchool,Pharmacology. http://dir.niehs.nih .gov/dirlpc /human.htm. Accessed on May, 2005.

11. Glossary in Genetics, http://helios.bto.ed.ac.uk/bto/glossary/p.htm#p. Accessed on February, 2006.

12. Gullstén H. (2000). Significance of polymorphisms in CYP2A6 gene. University of Oulu publications. Ph.D. thesis, Oulu University. Finland.

13. Al-Najar K, Awad R and Thabet K. (2002). Cancer 1995-2000. MOH publications. Palestine. P vii.

14. Young G, Toretsky JA, Campbell AB and Eskenazi AE. (2000). Recognition of common childhood malignancies. Am Fam Physician. 61(7):2144-2154.

15. Linet MS, Ries LA, Smith MA, Tarone RE and Devesa SS. (1999). Cancer surveillance series: recent trends in childhood cancer incidence and mortality in the United States. J Natl Cancer Inst. 91(12):1051-1058.

16. Online Mendelian Inheritance in Man. www.ncbi.nlm.nih.gov/entrez/dispo mim.cgi?id=124020. Accessed on August, 2006.

17. Rodrigues AD and Rushmore TH. (2002). Cytochrome P450 pharmacogenetics in drug development: *In Vitro* studies and clinical consequences. Curr Drug Metab. 3(3):289-309.

18. He N, Yan FX, Huang SL, Wang W, Xiao ZS, Liu ZQ, and Zhou HH. (2002). CYP2C19 genotype and s-mephenytoin 4'-hydroxylation phenotype in a Chinese Dai population. Eur J. Pharmacol 58:15-18.

19. Sengupta L.K, Sengupta S, and Sarkar M. (2002). Pharmacogenetic applications of the post genomic era department of genetics. Curr Pharmace Biotech. 3(2): 141-150.

20. Smith college. (2004). Biochemistry. http://www.science.smith.edu/ departments/Biochem/. Accessed on April, 2006.

21. Nelson D. (2003). Cytochrome P450s in humans. http://drnelson.utmem. edu/P450lect.html. Accessed on February, 2006.

22. Berg JM, Tymoczko JI, and Stryer L. (2002). Biochemistry. W.H. Freeman and company. 5th edition. p 735.

23. David Woods. (2003). Introduction to drug Interactions. http:// pharminfote ch.co.nz/manual/Interactions/Drug_Int.htm#rf. Accessed on June, 2006.

24. Frye RF. (2004). Probing the world of cytochrome P450 enzymes. Mol Interv. 4(3):157-162.

25. Ingelman-Sundberg M, Oscarson M and McLellan RA. (1999). Polymorphic human cytochrome P450 enzymes: an opportunity for individualized drug treatment. Trends Pharmacol Sci. 20(8):342-349.

26. Gaikovitch EA. (2003). Genotyping of the polymorphic drug metabolizing enzymes cytochrome P450 2D6 and 1A1, and N-acetyltransferase 2 in a Russian sample. http://edoc.huberlin.de/dissertationen/gaikovitch-elena-a-2003-07 14/HTML/chapter4.html#chapter4. Accessed on January, 2006.

27. The Phase I - Phase II Enzyme System: A delicate balance. Brassica protection products LLC. http://www.brassica.com/sci/ph12backgnd.htm. Accessed on July, 2006

28. Segall M. (1997). An ab Initio study of biological systems. Ph.D. thesis, Cambridge University, UK.

29. Yip SP, Lee SY, To SS and Wong ML. (2003). Improved real-time PCR assay for homogeneous multiplex genotyping of four CYP2C9 alleles with hybridization probes. Clin Chem. 49(12):2109-2111.

30. Human Gene CYP2C19 Description and Page Index. http://genome.ucsc.edu/cgibin/hgGene?hgg_gene=L07093&hgg_prot=Q767A3_HUMAN&hg_chrom=chr10&hgg_start=96437900&hgg_end=96602820&hgg_type=knownGene&db=hg18&hgsid=76270719%20TITLE. Accessed on March, 2006.

31. Riddell D. C., Wang H., Umbenhauer D. R., Beaume P. H., Guengerich F. P. and Hamerton J. L. (1987). Regional assignment for the genes encoding human P450IIIA3 (CYP3) and P450IIC9 (CYP2C). (Abstract) Cytogenet. Cell Genet. 46: 682.

32. Spurr N. K., Gough A., Stevenson K., Miles J., Hastie N., Meehan R. and Wolf C. R. (1987). Isolation of human cytochrome P450 cDNA for the study of linkage in human disease. (Abstract) Cytogenet. Cell Genet. 46: 698, 1987.

33. Shephard E. A., Phillips I. R., Santisteban I., Palmer C. N. A. and Povey S. (1989). Cloning, expression and chromosomal localization of a member of the human cytochrome P450IIC gene sub-family. Ann. Hum. Genet. 53: 23-31.

34. Wrighton S. A., Stevens J. C., Becker G. W. and VandenBranden M. (1993). Isolation and characterization of human liver cytochrome P450 2C19: correlation between 2C19 and S-mephenytoin 4-prime-hydroxylation. Arch. Biochem. Biophys. 306: 240-245.

35. Goldstein J. A., Faletto M. B., Romkes-Sparks M., Sullivan T., Kitareewan S., Raucy J. L., Lasker J. M. and Ghanayem B. I. (1994). Evidence that CYP2C19 is the major (S)-mephenytoin 4-prime hydroxylase in humans. Biochemistry *33:* 1743-1752.

36. De Morais S. M. F., Wilkinson G. R., Blaisdell J., Mever U. A., Nakamura K. and Goldstein J. A. (1994). Identification of a new genetic defect responsible for the polymorphism of (S)-mephenytoin metabolism in Japanese. Molec. Pharmacol. 46: 594-598.

37. Ibeanu GC, Blaisdell J, Ferguson RJ, Ghanayem BI, Brosen K, Benhamou S, Bouchardy C, Wilkinson GR, Dayer P and Goldstein JA. (1999). A novel transversion in the intron 5 donor splice junction of CYP2C19 and a sequence polymorphism in exon 3 contribute to the poor metabolizer phenotype for the anticonvulsant drug S-mephenytoin. J Pharmacol Exp Ther. 290(2):635-640.

38. Walker R. (2002). Clinical Pharmacy and Therapeutics. Churchill Livingstone, 3rd edition, P 24.

39. Romkes M, Faletto MB, Blaisdell JA, Raucy JL and Goldstein JA. (1991). Cloning and expression of complementary DNAs for multiple members of the human cytochrome P450IIC subfamily. Biochemistry, 30(13):3247-3255.

40. Richardson TH, Jung F, Griffin KJ, Wester M, Raucy JL, Kemper B, Bornheim LM, Hassett C, Omiecinski CJ and Johnson EF. (1995). A universal approach to the expression of human and rabbit cytochrome P450s of the 2C subfamily in *Escherichia coli*. Arch Biochem Biophysics. 323(1):87-96.

41. Blaisdell J, Mohrenweiser H, Jackson J, Ferguson S, Coulter S, Chanas B, Xi T, Ghanayem B and Goldstein JA. (2002). Identification and functional characterization of new potentially defective alleles of human CYP2C19. Pharmacogenetics. 12(9):703-711.

42. Pirmohamed M and Park BK. (2001). Genetic susceptibility to adverse drug reactions. Trends Pharmacol Sci, 22:298-305.

43. Human Cytochrome P450 *(CYP)* Allele Nomenclature Committee. http://www.imm.ki.se/cypalleles/ (2006). Accessed on June, 2006.

44. De Morais SM, Wilkinson GR, Blaisdell J, Nakamura K, Meyer UA and Goldstein JA. The major genetic defect responsible for the polymorphism of S- mephenytoin metabolism in humans. J Biol Chem 1994; 269:15419–15422.

45. Ibeanu GC, Goldstein JA, Meyer U, Benhamou S, Bouchardy C, Dayer P, Ghanayem BI and Blaisdell J. (1998). Identification of new human CYP2C19 alleles (CYP2C19*6 and CYP2C19*2B) in a Caucasian poor metabolizer of mephenytoin. J Pharmacol Exp Ther. 286(3):1490-1495.

46. Fukushima-Uesaka H, Saito Y, Maekawa K, Ozawa S, Hasegawa R, Kajio H, Kuzuya N, Yasuda K, Kawamoto M, Kamatani N, Suzuki K, Yanagawa T, Tohkin M and Sawada J. (2005). Genetic variations and haplotypes of CYP2C19 in a Japanese population. Drug Metab Pharmacokinet. 20(4):300-307.

47. Ferguson RJ, De Morais SM, Benhamou S, Bouchardy C, Blaisdell J, Ibeanu G, Wilkinson GR, Sarich TC, Wright JM, Dayer P and Goldstein JA. (1998). A new genetic defect in human CYP2C19: mutation of the initiation codon is responsible for poor metabolism of S-mephenytoin. J Pharmacol Exp Ther. 284(1):356-361.

48. Hersberger M, Marti-Jaun J, Rentsch K and Hänseler E. (2001). Two single-tube tetra-primer assays to detect the CYP2C19*2 and *3 alleles of S-mephenytoin hydroxylase. Clinical Chemistry. 47:772-774.

49. Herman D, Dolžan V and Breskvar K. (2003). Genetic polymorphism of cytochromes p450 2C9 and 2C19 in Slovenian population. Zdravvestn. 72: 347-351

50. Nussbaum RI, Mcinnes RR and Willard HF. (2002). Thompson and Thompson Genetics in Medicine. WB Saunders Company, 6th edition. P 311-407

51. Trosko JE. (1992). Does radiation cause cancer? RERF. 4(1):3-5.

52. Parke DV. (1994). The Cytochromes P450 and mechanisms of chemical carcinogenesis. Environ Health Perspect. 102(10):852-853.

53. Marchini J, Cardon LR, Phillips MS and Donnelly P. (2004). The effects of human population structure on large genetic association studies. Nature Genet 36: 512–517

54. Cardon LR and Palmer LJ. (2003). Population stratification and spurious allelic association. Lancet.15;361(9357):598-604.

55. Thomas Alexis LaVeist. (2002). Race, Ethnicity, and Health: A Public Health Reader (San Francisco: Jossey-Bass).

56. Ward E, Jemal A, Cokkinides V, Gopal K. Singh GK, Cardinez C, Ghafoor A, and Thun M. (2004). Cancer disparities by race/ethnicity and socioeconomic status. CA Cancer J Clin. 54:78-93.

57. Brennan P. (2002). Gene–environment interaction and aetiology of cancer: what does it mean and how can we measure it? Carcinogenesis. 23(3):381-387.

58. Wikipedia, the free encyclopedia. http://en.wikipedia.org/wiki/Hematological_malignancy.Accessed on June, 2006.

59. Wikipedia, the free encyclopedia. http://en.wikipedia.org/wiki/Leukemia. Accessed on June, 2006.

60. McKenna RW. (2000). Multifaceted Approach to the diagnosis and classification of acute leukemias. Clinical Chemistry. 46:1252-1259.

61. Scott CS, Den Ottolander GJ, Swirsky D, Pangalis GA, Vives Corrons JL, de Pasquale A, van Hove L, Bennett JM, Namba K and Flandrin G, *et al*. (1993). Recommended procedures for the classification of acute leukaemias. Leukemia & lymphoma, U.K. 11(1-2):37-50.

62. http://en.wikipedia.org/wiki/Immunophenotyping. Accessed on June, 2006.

63. Philip A. Pizzo and David G. Poplack. (2005). Principles and Practice of Paediatric Oncology (5th edition). Lippincott Williams and Wilkins.

64. Nathan DG, Orkin SH, Look AT and Ginsburg D. (2003). Nathan and Oski's Hematology of Infancy and Childhood. WB Saunders Company. 6th edition, p 1694.

65. National Cancer Institute. General information about childhood acute myeloid leukemia and other myeloid malignancies. http://www.nci.nih.gov/cancertopics/pdq/treatment/childAML/healthprofessional. Accessed on June, 2006

66. National Cancer Institute. Chronic myelogenous leukemia. http://www.nci.nih.gov/cancertopics/pdq/treatment/childAML/HealthProfessional/page11. Accessed on August, 2006.

67. Wikipedia, the free encyclopedia. http://en.wikipedia.org/wiki/Lymphoma. Accessed on July, 2006.

68. Lymphoma information network. www.lymphomainfo.net/lymphoma/whatis.html. Accessed on July, 2006.

69. Canalle R, Burim RV, Tone LG and Takahashi CS. (2004). Genetic polymorphisms and susceptibility to childhood acute lymphoblastic leukemia. Environ Mol Mutagen. 43(2):100-109.

70. Larson RA, Wang Y, Banerjee M, Wiemels J, Hartford C, Le Beau MM and Smith MT. (1999). Prevalence of the Inactivating $^{609}C \rightarrow T$ polymorphism in the NAD(P)H Quinone Oxidoreductase (NQO1) gene in patients With primary and therapy-related myeloid leukemia. Blood. 94(2):803-807.

71. Skibola CF, Smith MT, Kane E, Roman E, Rollinson S, Cartwright RA and Morgan G. (1999). Polymorphisms in the methylenetetrahydrofolate reductase gene are associated with susceptibility to acute leukemia in adults. Proc Natl Acad Sci U S A. 96(22): 12810–12815.

72. Hemminki K, Forsti A and Lorenzo Bermejo J. (2005). Single nucleotide polymorphisms (SNPs) are inherited from parents and they measure heritable events. J Carcinog. 4(1):2.

73. Bozina N, Granic P, Lalic Z, Tramisak I, Lovric M and Stavljenic-Rukavina A. (2003). Genetic polymorphisms of cytochromes P450: CYP2C9, CYP2C19, and CYP2D6 in Croatian population. Croat Med J. 44(4):425-428.

74. Shi WX and Chen SQ. (2004). Frequencies of poor metabolizers of cytochrome P450 2C19 in esophagus cancer, stomach cancer, lung cancer and bladder cancer in Chinese population. World J Gastroenterology: 10(13):1961-1963.

75. Research Notes. (1997). Publication of University of Pittsburgh, USA. 29 (20). http://www.pitt.edu/utimes/issues/29/061297/19.html. Accessed on July, 2006.

76. Wadelius M, Autrup JL, Stubbins MJ, Andersson S-O, Johnansson J-E, Wadelius C, Wolf CR, Autrup H and Rane A. (1999). Polymorphisms in NAT2, CYP2D6, CYP2C19, and GSTP1 and their association with prostate cancer. Pharmocogenetics. 9(3):333-340.

77. Bartsch H, Nair U, Risch A, Rojas M, Wikman H and Alexandrov K. (2000). Genetic polymorphism of CYP genes, alone or in combination, as a risk modifier of tobacco-related cancers. Cancer Epidemiol Biomarkers Prev. 9(1):3-28.

78. Gharbiyoh A. (2006). Common CYP2C19 alleles in Gaza strip population. (Master thesis, the Islamic university – Gaza, Palestine).

79. Tamminga WJ, Wemer J, Oosterhuis B, de Zeeuw RA, de Leij LFMH, and Jonkman JHG. (2001). The prevalence of CYP2D6 and CYP2C19 genotypes in a population of healthy Dutch volunteers and psychiatric patients. Eur J Clin Pharmacol. 57: 712–722.

80. Hamdy SI, Hiratsuka M, Narahara K, El-Enany M, Moursi N, Ahmed MS and Mizugaki M. (2002). Allele and genotype frequencies of polymorphic cytochromes P450 (CYP2C9, CYP2C19, CYP2E1) and dihydropyrimidine dehydrogenase (DPYD) in the Egyptian population. Br J Clin Pharmacol. 53(6):596-603.

81. Goldstein JA, Ishizaki T, Chiba K, de Morais SM, Bell D, Krahn PM and Evans DA. (1997). Frequencies of the defective CYP2C19 alleles responsible for the mephenytoin poor metabolizer phenotype in various Oriental, Caucasian, Saudi Arabian and American black populations. Pharmacogenetics. 7(1):59-64.

82. Shi WX and Chen SQ. (2004). Frequencies of poor metabolizers of cytochrome P450 2C19 in esophagus cancer, stomach cancer, lung cancer and bladder cancer in Chinese population. World J Gastroenterol. 10(13):1961-1963.

83. Chang TK, Yu L, Goldstein JA, Waxman DJ. (1997) Identification of the polymorphically expressed CYP2C19 and the wild-type CYP2C9-ILE359 allele as low-Km catalysts of cyclophosphamide and ifosfamide activation. Pharmacogenetics. 7(3):211-222.

84. Taub JW and Ge Y. (2005). Down syndrome, drug metabolism and chromosome 21. Pediatr Blood cancer. 44(1):33-39.

85. Peeters M and Poon A. (1987). Down syndrome and leukemia: unusual clinical aspects and unexpected methotrexate sensitivity. Eur J Pediatr. 146(4):416-422.

86. Coulthard SA, Howell C, Robson J, and Hall AG. (1998). The relationship between thiopurine methyltransferase activity and genotype in blasts from patients with acute leukemia. Blood. 92(8):2856-2862.

87. Lichtman MA, Beutler E, Kipps TJ and Williams WJ. (2003). Williams manual of hematology. The McGraw-Hill Companies, Inc. 6th ed. p 303.

88. Branch R, Adedoyin A, White C, Frye R, Johnson J, Eibling D, Henry B, Fregoso G, Bigbee WL, Gealy R, Melacrino A, Chern H and Persad R. (1997). Genetic differences in metabolizing enzymes may increase cancer risk. http://www.pslgroup.com/dg/23B6E.htm. Accessed on June, 2006.

89. http://www.rochediagnostics.com/products_services/amplichip_cyp450.html. Accessed on July, 2006.

APPENDECES

Appendix A

الجـــــــــامعة الاســـــــلامية – غزة
The Islamic University of Gaza
رئيس قسم الأحياء
برنامج ماجستير العلوم الحياتية

كلية العلوم

التاريخ/ 11/04/2005

الأخ د. فيصل أبو شهلا
مدير عام المستشفيات، حفظه الله،

السلام عليكم ورحمة الله وبركاته،

الموضوع/ تسهيل مهمة باحث

نود أن نعلم سيادتكم بأن الطالب سمير أبو عيد يقوم بـــاجراء البحـــث العملي الخاص به وذلك ضمن برنامج ماجستير العلوم الحياتية تخصص تحاليل طبية، وبحثه بعنوان:-

CYP2C19 Polymorphism in Childhood Hematological Malignancy.

لذا نرجو من سيادتكم تسهيل مهمة الطالب في جمع عينات الدم اللازمة لبحثه.
سيجرى البحث في بداية مايو 2005 على عينات دم تجمع من الأطفال المرضى بسرطانات الدم من مستشفيات النصر والأوروبي.

ولكم منا جزيل الشكر.

- مرفق نسخة من البحث المطروح.

رئيس قسم الأحياء
مدير برنامج الماجستير

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الجامعة الإسلامية-غزة - الرمال ص.ب:108 فلسطين

Appendix B

بسم الله الرحمن الرحيم

إقرار موافقة

أنا الموقع أدناه ولي أمر الطفل .....

أوافق على سحب عينات دم CBC (2.5 مل) وذلك لإجراء بحث يخص الأسباب الجينية لأمراض الدم بعنوان تعدد الأشكال للجين Cyp2c19 في الأطفال المصابين بسرطانات الدم.

الموافقة وعدم الموافقة لا تؤثر على العلاج

وعلى ذلك أوقع

التوقيع

Appendix C

بسم الله الرحمن الرحيم

Palestinian National Authority
Ministry of Health
Helsinki Committee

السلطة الوطنية الفلسطينية
وزارة الصحة
لجنة هلسنكي

Date: 3/5/2005

التاريخ: 2005/5/3

Mr./ Sameer Abu -Eid

السيد: سمير أبو عيد

I would like to inform you that the committee has discussed your application about:

نفيدكم علماً بأن اللجنة قد ناقشت مقترح دراستكم حول:-

CYP2C19 Polymorphism in Childhood Hematological Malignancy.

In its meeting on May 2005

and decided the Following:-

To approve the above mention research study.

و ذلك في جلستها المنعقدة لشهر مايو 2005

و قد قررت ما يلي:-

الموافقة على البحث المذكور عاليه.

مع الموافقة شرط موافقة وزارة الدفاع لاخذ عينات الدم لغرض البحث

Ministry of Health
Helsinki Committee

Signature

توقيع

Member
عضو

Member
عضو

Chairperson

Conditions:-

- Valid for 2 years from the date of approval to start.
- It is necessary to notify the committee in any change in the admitted study protocol.
- The committee appreciate receiving one copy of your final research when it is completed.

Gaza Etwam – Telefax 972-7-2878166

Appendix D

CYP2C19 Polymorphisms in Childhood Hematological Malignancy

Islamic University-Gaza
Biological sciences
Master program

Hospital: _____

No.	Patient no.	Age	Sex m/f	Disease	Onset age	Family History	Area
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							