UNIVERSITY OF SOUTHAMPTON

GENETIC POLYMORPHISMS OF THE CYTOCHROME P450 2C XENOBIOTIC METABOLISING ENZYMES SUBFAMILY AND PREDISPOSITION TO ADENOMATOUS POLYPS OF THE COLON AND RECTUM

A thesis submitted for D.M. degree examination

to

Southampton University
October 2002

by

Thomas Cecil (FRCS gen surg)

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE

DEPT OF SURGERY

D.M. degree examination

XENOBIOTIC METABOLISING ENZYMES OF THE CYTOCHROME P450 2C SUBFAMILLY AND PREDISPOSITION TO ADENOMATOUS POLYPS OF THE COLON AND RECTUM.

By Thomas Desmond Cecil

Aim: To study cytochrome P4502C subfamily gene polymorphisms in the polyp and non-polyp population of the Imperial Cancer Research Fund flexible sigmoidoscopy screening trial and establish any association with adenomatous polyps of the colon.

Background: Colorectal cancer is the second most common cause of cancer deaths in the United Kingdom (1). In view of this the ICRF has set up a multicentre trial to assess the impact of screening with a once-only flexible sigmoidoscopy. It is thought that colorectal cancer and adenomatous polyps arise due to an interaction between environmental and genetic factors (2). The vast majority of environmental compounds are inert requiring activation to become carcinogens by xenobiotic metabolising enzymes. Individuals exhibiting different expression patterns of these enzymes, possibly due to genetic polymorphisms, vary in their susceptibility to the effects of environmental factors. The CYP2C subfamily enzymes are known to be involved in the metabolism of several commonly used drugs, notably, omeprazole, warfarin, non-steroidal anti-inflammatories, tolbutamide & diazepam, as well as a number of environmental mutagens such as benz[a]pyrene (3).

Methods: DNA based polymerase chain reaction and restriction fragment length polymorphism methods were used to determine the frequency of CYP2C8, 9, 18 and 19 polymorphisms (Table 17) in individuals attending the ICRF screening trial.

Results: For CYP2C8 there is a reported base pair change a to c at position 390. The c base is the wild type (WT) and a allele is very rare and may not exist at all. For CYP2C18 a reported t to c base pair change at position 1154 did not appear to exist. Allele frequencies in a UK population for CYP2C9, CYP2C18 and CYP2C19 polymorphisms have been established. There was no statistically significant difference for any of the polymorphisms studied between those with adenomas and controls. There were more heterozygotes and homozygotes combined in the adenoma group for the polymorphism termed **CYP2C9.** This did not reach significance (p=0.064). There is evidence that this polymorphism has a dominant effect and is involved in benz[a]pyrene metabolism, a smoking carcinogen. Smoking has been shown to predispose to colorectal adenomas.

Conclusions: None of the CYP2C subfamily polymorphism studied were shown to predispose to adenomatous polyps of the distal colon and rectum. **CYP2C9** warrants further investigation.

List of contents **Page**

Title 1

Abstract 2

List of Contents 4

List of tables 9

Dedication 12

Acknowledgements 13

List of abbreviations 14

Section 1 Introduction, historical review and background to studies 18

Chapter 1: Overview of Xenobiotic metabolising enzymes 19

1.1 Environmental influences in cancer 19

1.2 Polymorphisms in xenobiotic metabolising enzymes 21

1.3 The cytochrome P450s 26

1.3.1 CYP1 family 28

1.3.2 CYP2 family 30

1.3.3 CYP3 family 33

Chapter 2: Colorectal adenomas and cancer 34

2.1 Colorectal cancer 34

2.2 Adenoma-carcinoma sequence 35

2.3 Genetic basis of the adenoma-carcinoma sequence 37

2.4 Environmental factors and xenobiotics in colorectal adenomas and cancer 43

Chapter 3: Xenobiotic metabolising enzyme polymorphisms and predisposition to colorectal cancer 47

3.1 Acetylation NAT1 and NAT2 47

3.2 Glutathione S-transferases 54

3.3 Microsomal epoxide hydrolase 56

3.4 Cytochrome P450 (CYP1A1, CYP1A2 and CYP2D6) 59

3.5 Apolipoprotein E 61

Chapter 4: Cytochrome P450 2C subfamily 63

4.1 Introduction 63

4.2 Purification from human liver tissue and distribution 64

4.3 Identification of CYP2C subfamily genes and gene structure 65

4.4 CYP2C8 genetic variation, substrates and catalytic activity 66

4.5 CYP2C9 genetic variation, substrates and catalytic activity 66

4.6 CYP2C18 genetic variation, substrates and catalytic activity 71

4.7 CYP2C19 genetic variation, substrates and catalytic activity 72

4.8 Hypothesis for CYP2C subfamily and colorectal tumorogenesis 74

Chapter 5: Screening for colorectal cancer 75

5.1 Rationale for screening for colorectal cancer 75

5.2 Screening methods for average risk individuals 78

5.2.1 Faecal occult blood testing 78

5.2.3 Sigmoidoscopy 81

5.3 Once only flexible sigmoidoscopy trial 83

5.4 Conclusions 84

Section 2 Subjects and Methods 87

Chapter 6 Subjects and Methodology 87

6.1 Subject recruitment and Food Frequency Questionnaire 87

6.2 Materials 89

6.3 Methods 90

6.3.1 Extraction procedure for the isolation of DNA from blood 90

6.3.2 Polymerase Chain Reaction (PCR) 91

6.3.3 Primer design 94

6.3.4 Standardisation of the PCR reaction 95

6.3.5 Restriction and DNA methylation enzymes 96

6.3.6 Polyacrylamide gel electrophoresis 98

6.3.7 Confirmation of PCR specificity by cloning and sequencing 100

Section 3 Results 105

Chapter 7 Flexiscope trial results 105

7.1 Recruitment in the Portsmouth centre 105

7.2 Outcome for flexible sigmoidoscopy procedures in the Portsmouth centre 106

7.3 Sample collection in the Portsmouth centre 107

7.4 Results of food frequency questionnaire 108

7.5 Results of the smoking data collection 110

Chapter 8 Laboratory Results 111

8.1 Selection of CYP2C mutations for analysis 111

8.2 Primer design and optimisation 112

8.3 Selection and optimisation of restriction endonucleases 115

8.4 Results of cloning and sequencing 116

8.5 Results of PAGE analysis of DNA polymerase chain reaction 116

8.6 Results of PCR restriction endonucleases digestion 118

Chapter 9 Results of genotyping for CYP2C subfamily 121

9.1 CYP 2C subfamily mutations and allelic frequencies. 121

9.2 CYP 2C subfamily genotypes and predisposition to colorectal adenomas. 124

9.3 CYP2C subfamily genotypes, environmental exposure and predisposition to colorectal adenomas. 128

Section 4 Discussion and conclusions 131

Chapter 10 Flexiscope trial and sample collection 131

10.1 Flexiscope trial 131

10.2 Sample collection 133

Chapter 11 Cytochrome P450 2C subfamily mutations 135

11.1 Cytochrome P4502C8 135

11.2 Cytochrome P4502C9 136

11.3 Cytochrome P4502C18 137

11.4 Cytochrome P4502C19 138

Chapter 12 Cytochrome P450 2C subfamily mutations, predisposition to colonic adenomas and environmental exposure 139

12.1 Cytochrome P4502C mutations and predisposition to colonic adenomas 139

12.2 Cytochrome P4502C mutations, colonic adenomas and environmental exposure 140

Chapter 13 Summary and conclusions 143

Appendices 145

Bibliography 154

List of tables and figures **Page**

Table 1	Compounds associated with carcinogenesis	21
Table 2	Reactions of xenobiotic metabolism	23
Fig 1	Biotransformation	25
Table 3	Cytochrome P450 families	28
Table 4	NAT acetylator phenotype studies	49
Table 5	NAT 2 genotype studies	51
Table 6	NAT 1 genotype studies	53
Table 7	GSTM1 genotype studies	57
Table 8	GSTT1 and GSTP1 studies	60
Table 9	Percentage amino acid homology for the CYP2C subfamily	65
Table 10	Identified genetic mutations for CYP2C8	69
Table 11	Identified genetic mutations for CYP2C9	70
Table 12	Identified genetic mutations for CYP2C18	73
Table 13	Estimated CRC prevented each year	85
Table 14	Conditions for optimisation of primers	95
Table 15	Preparation of terminator reaction mix	103
Table 16	Histology of polyps removed	108
Table 17	Results of food frequency questionnaire	109
Table 18	Mutations and primers for selected CYP2C subfamily polymorphisms	112
Fig 2	2C8 primer optimisation	113

Fig 3	2C9 primer optimisation	113
Fig 4	2C9 primer optimisation	113
Fig 5	2C18 primer optimisation	114
Fig 6	2C18 primer optimisation	114
Fig 7	2C19 primer optimisation	114
Table 19	PCR conditions	114
Table 20	Restriction endonucleases	115
Table 21	Restriction endonucleases conditions	116
Fig 8	CYP2C8 PCR	116
Fig 9	CYP2C9 PCR	117
Fig 10	CYP2C9 PCR	117
Fig 11	CYP2C18 PCR	117
Fig 12	CYP2C18 PCR	118
Fig 13	CYP2C19 PCR	118
Fig 14	CYP2C8 digest	119
Fig 15	CYP2C9 digest	119
Fig 16	CYP2C9 digest	119
Fig 17	CYP2C18 digest	120
Fig 18	CYP2C18 digest	120
Fig 19	CYP2C19 digest	120
Table 22	Number of people genotyped for each gene mutation according to centre	121
Table 23	CYP2C8 mutation allele frequency	122
Table 24	CYP2C9 mutation allele frequency	122

Table 25	CYP2C9 mutation allele frequency	123
Table 26	CYP2C18 mutation allele frequency	123
Table 27	CYP2C18 mutation allele frequency	123
Table 28	CYP2C19 mutation allele frequency	124
Table 29	CYP2C9 cases vs controls	125
Table 30	CYP2C9 cases vs controls	125
Table 31	CYP2C18 cases vs controls	125
Table 32	CYP2C19 cases vs controls	126
Table 33	CYP2C9 cases vs controls for phenotype	126
Table 34	CYP2C9 cases vs controls for phenotype	127
Table 35	CYP2C18 cases vs controls for phenotype	127
Table 36	CYP2C19 cases vs controls for phenotype	127
Table 37	CYP2C9 cases and smoking	129
Table 38	CYP2C9 controls and smoking	129
Fig 20	CYP2C8 sequence	136
Table 39	Percentage of CYP2C9 heterozygotes and homozygotes	141

This thesis is dedicated to my family

My wife Alison

And my two children

Tobias and Jemima

Declaration

I declare that the data contained in this thesis is original and the product of my own work.

Acknowledgements

I would like to take this opportunity to thank Dr Alan Gough for his ideas, Professor John Primrose, Mr Mike Thompson and Miss Asha Senapati for their support for this project.

I would also like to thank Karan Palmer and Matt Darley for their tireless support in the laboratory. I would also like to thank all the flexiscope staff in the Portsmouth office who supported me throughout.

A special thanks goes to the British Journal of Surgery who supported me with this work with a BJS scholarship and to the St Mary's League of Friends for its financial support

I would like to acknowledge the cooperation with Mark Watson (Cambridge and Norwich) and Chris Macklin (Leeds) and thank them for the exchange of DNA samples.

Finally, I am grateful to Wendy Atkin for her support and encouragement throughout and Dr Rob Edwards for his patient help with statistical analysis.

List of abbreviations

DNA	Deoxyribonucleic acid
cDNA	Complementary deoxyribonucleic acid
CYP	Cytochrome P450
XME	xenobiotic metabolising enzyme
RFLP	Restriction fragment length polymorphism
t	Thymine
c	Cytosine
a	Adenine
g	Guanine
PM	Poor metabolisers
EM	Extensive metabolisers
OR	Odds ratio
DAG	Diacylglycerol
FAP	Familial adenomatous polyposis
HNPCC	Hereditary non polyposis colorectal cancer
APC	Adenomatous polyposis coli
LOH	Loss of heterozygosity
DDC	Deleted in colorectal cancer
RER	Replicative errors
MIN	Microsatellite instability
RER+	Replication error positive
MMR	Mis match repair

NAT1	N-acetyltransferase 1
NAT2	N-acetyltransferase2
CRC	Colorectal cancer
HAA	Heterocyclic aromatic amines
PHIP	2-amino-1-methyl-6-phenylimidazo-[4,5-b]-pyridine
R	Arginine
C	Cysteine
L	Leucine
I	Isoleucine
A	Alanine
N	Asparagine
D	Aspartic acid
Q	Glutamine
E	Glutamic acid
G	Glycine
H	Histidine
K	Lysine
M	Methionine
F	Phenyalanine
P	Proline
S	Serine
T	Threonine
W	Trytophan
Y	Tyrosine

V	Valine
PCR	Polymerase chain reaction
GST	Glutathione S-transferase
MEPH	Microsomal epoxide hydrolase
Apo E	Apolipoprotein E
°C	Degrees Celsius
Tm	Annealing temperature
PCR	Polymerase chain reaction
$MgCl_2$	magnesium chloride
DMSO	dimethyl sulfoxide
APS	ammonium persulfate
TEMED	N, N, N', N'-tetramethlyethylene-diamine
µl	micro litre
PAGE	Polyacrylamide gel electrophoresis
WT	Wild type
Hom	Homozygote
Het	Heterozygote
CYP2C8	CYP2C8 mutation a → c position 390
CYP2C9	CYP2C9 mutation a → c position 1075
CYP2C9	CYP2C9 mutation c → t position 430
CYP2C18	CYP2C18 mutation t → c position 1154
CYP2C18	CYP2C18 mutation t → c position 655

CYP2C19	CYP2C19 mutation g → c position 681

Section 1

Introduction, historical review and background to studies

General introduction

A tumour is a mass of tissue formed as a result of abnormal, excessive and inappropriate proliferation of cells, the growth of which continues indefinitely and regardless of the mechanisms which control cellular proliferation. At the hub of cellular control is deoxyribonucleic acid (DNA). It is the molecule that stores the information required to produce and control all higher organisms. The multi-step or multi-hit theory of carcinogenesis states that tumour formation is due to the consequences of multiple mutagenic events. Indeed all agents known to cause cancer have one feature in common, they cause genetic damage. The study of familial cancers has identified a number of important candidate genes that contribute directly to the high cancer risk in these families. The majority of cancers though do not appear to have a strong familial link. It is now widely accepted that the initiation and development of tumours is determined by a delicate balance of environmental and host factors (4).

The purpose of this introduction is to address genetic susceptibility to environmental factors with specific regard to enzymes involved in carcinogen metabolism, to examine the pathways and aetiology in the development of colorectal adenomas and cancer and discuss the evidence to date linking these two fields. The last two chapters provide an overview of the CYP2C subfamily and address the issue of colorectal cancer screening which is an important part of the methodology and patient and sample collection for this thesis.

Chapter 1 Overview of xenobiotic metabolising enzymes

1.1 Environmental influences in cancer

Epidemiological studies suggest that the majority of all cancers are attributable to environmental factors (5). Human cancer can be induced by a variety of aetiological agents such as chemicals, viruses and radiation. An example of such an agent is tobacco smoke which is unquestionably the most important causative factor in lung cancer, currently the most common malignancy in the world. The cellular response to environmental challenges is pivotal for the preservation of cellular integrity and protection from cytotoxic insults that can lead to cell damage and cancer development. Inter-individual variation in the genes involved in these cellular defences plays a role in determining genetic susceptibility to disease. Epidemiological evidence suggests that both exposure profiles to environmental factors and inherited susceptibility to the action of carcinogenic and mutagenic stimuli are important in the development of tumours. It has been estimated that up to 80% of human tumours may be due to the action of environmental carcinogens (6). Carcinogenesis has been demonstrated in animals in response to single doses of chemical carcinogens, with the development of many different types of tumours. This is often the result of administration of very large quantities of chemicals. These models, although of use in demonstrating the carcinogenic potential of these compounds, do not mimic human exposure to carcinogen except perhaps in catastrophic industrial or occupational exposure. In these situations there is probably a direct toxic effect from the carcinogen insult that

overwhelms any body defences. Epidemiological studies suggest that human exposure to lower levels of environmental carcinogens, particularly those originating in the diet and from cigarette smoke, occurring on a more chronic basis may have an aetiological role in human cancer (7).

Every day humans come into contact with a variety of environmental carcinogens and their metabolism must play a vital role in protection against their potentially damaging effect. Mammals have evolved a complex host of metabolic enzymes to defend themselves against attack from these compounds.

A xenobiotic is a substance foreign to the body or to an ecological system and hence the term xenobiotic metabolising enzymes (XMEs) has been coined to refer to enzymes involved in cellular defence against foreign carcinogens. These defence mechanisms have evolved primarily to combat against xenobiotics such as toxins and carcinogens in the diet, but their importance has gained prominence because of the inter-individual variation in the metabolism of therapeutic drugs (4). Many compounds have been associated with cancer initiation and progression (Table 1), and exposure to a wide variety of both naturally occurring and synthetic genotoxins happens on a daily basis through diet. Furthermore carcinogen exposure can be greatly increased through lifestyle exposure be it through choice i.e. smoking, or occupational exposure or exposure to environmental pollutants, all of which can drastically alter cancer risk.

Table 1: Compounds associated with carcinogenesis

Dietary	Occupational Industrial	Endogenous	Lifestyle Drugs
Aflatoxins	Benz[a]pyrene	Hormones	Cigarette smoke
Ochratoxins	Benzenes	Growth factors	Alcohol
Benzidines	Phenols	Prostaglandins	Cyclophosphamide
Phenacetin	Aromatic amines	Leukotrienes	
Plant toxins	Nitrosamines		
Gut fauna toxins	Epoxides		
	PAHs		
	Anilines		
	Naphthylamines		
	Amino biphenyls		
	Solvents		

1.2 Polymorphisms in xenobiotic metabolising enzymes

Most pharmacologically active molecules and the majority of chemicals associated with cancer are lipophilic. After glomerular filtration in the kidney they would be reabsorbed and remain in the organism. In order to excrete these molecules efficiently they need to undergo conversion into polar water soluble metabolites. This process is known as biotransformation. Metabolites of biotransformation can be less active than their parent compound or in some case may have enhanced activity or toxic effects such as mutagenesis or carcinogenesis (8). Biotransformation therefore has the potential to perform both detoxification and toxification reactions. In fact the majority of carcinogens exist in the environment in inert forms as procarcinogens and require metabolic conversion to carcinogens which can react with cellular macro-molecules (9).

All tissues have some ability to metabolise xenobiotics but the principal organ for biotransformation is the liver. Within hepatocytes, at a cellular level, XMEs are either

attached to the endoplasmic reticulum (cytochrome P450 enzymes) or floating as cytosolic enzymes (eg acetyltransferases). The majority of xenobiotics are therefore metabolised in the liver, but the level of exposure as well as the target site of exposure and the rate of metabolism will all alter the tumorogenic potential of procarcinogen xenobiotics. Inter-individual variation in the expression of XMEs in the liver, or potential target organs or tissues will therefore lead to a difference in the host defence's ability to protect from xenobiotic insult. This in turn may result in susceptibility to disease.

There is a continual exposure to xenobiotics some of which may be beneficial such as pharmacological drugs or some that have the potential to cause harm such as carcinogens. Efficient metabolism of both xenobiotics and endogenous toxins is essential for the preservation of cellular integrity and prevention of cytotoxic insults that can lead to cell damage and disease including cancer (4).

Through the study of drug metabolism, genetically determined enzyme deficiencies have been identified that lead to altered drug activity (10). This has lead to the discovery of large families of enzymes that are responsible for drug and xenobiotic metabolism. A number of altered drug responses observed within populations have now been shown to be due to genetic polymorphisms leading to amino acid changes within the protein, affecting enzyme activity (11) (12).

Large families of enzymes have now been studied and the genetic sequence of many XMEs has now been established. Many XMEs have been shown to be polymorphic in that multiple allelic variants of the XME genetic sequence exist.

Table 2: Reactions of xenobiotic metabolism

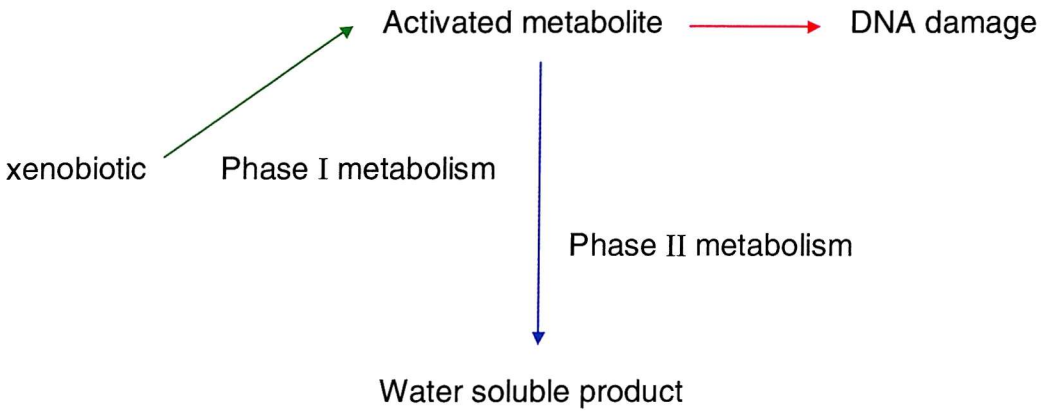
Phase I		Phase II	
Oxidation	Cytochromes P450	Glucuronidation	UDP glucronyl transferases
	Alcohol and aldehyde dehydrogenase	Glucosidation	UDP glycosyl transferases
		Sulphation	Sulphotransferases
	Aromatases	Methylation	O-,N-,S-methyl transferases
Reduction	P450 reductase	Conjugation	Glutathione-S-transferases
	N-reductases		N-acyl transferases
Hydrolysis	Epoxide hydrolase		N-acetyl transferases
	Esterases	Esterification	Fatty acid transferases
	Amidases	Condensation	Non enzymic
Summarisation	Isomerases		
Miscellaneous			
Peroxidation	Glutathione peroxidase		
Radical scavenger	Superoxide dimutase		

Genetic polymorphism can be defined as the occurrence together in the same population of two or more discontinuous traits at a frequency where the rarest could not be maintained by recurrent mutation alone. In general, if 1 in 50 or more of the general population has the rare allele then the condition is polymorphic. Genetic polymorphisms affecting the coding region of a gene can lead to expression of an altered protein that can affect enzyme activity. Similarly, a polymorphism in the non-coding region of a gene may affect the gene regulation leading to altered amounts of the XME being expressed. Thus due to genetic polymorphism individuals may express different variants of a specific XME with altered activity, or express greater or lesser amounts of a specific XME. This may account for inter-individual variation in xenobiotic metabolism.

Xenobiotic metabolising enzymes can be divided into two groups, phase I and phase II enzymes, based on their functional properties (Table 2).

Phase I enzymes, which include the cytochrome P450s, metabolise xenobiotics by the addition of functional groups and "reactive-centres" on substrates (eg -OH,-NH_2,-SH,-COOH) (4). This generates xenobiotic products that are highly reactive electrophiles. In contrast phase II enzymes catalyse the conversion of the electrophiles to inactive conjugates which are more water soluble and therefore more readily excreted from the cell. This process of biotransformation, whereby a lipid soluble xenobiotic is enzymatically transformed by phase I and phase II reactions to create water soluble metabolites that can be excreted, is simply illustrated below (Fig 1).

Fig 1: Biotransformation



As mentioned exposure to many diverse xenobiotics can occur and similarly there are many different families of XMEs with many individual enzymes involved in xenobiotic metabolism. There is crossover in substrate specificity so that several enzymes may be involved in metabolising a xenobiotic via different pathways. Furthermore some XMEs can be involved in both phase I and phase II metabolism. Therefore, the ultimate fate of a xenobiotic and hence its potential for carcinogenicity will depend on the type and activity of XMEs available for its metabolism. As previously mentioned, most carcinogens require activation from procarcinogens to their active mutagenic form. Thus, the initial amount and type of activated metabolite will depend on the phase I reactions with phase II reactions becoming the rate limiting step with regard to excretion of the activated metabolite (Fig 1). Genetic polymorphisms of XMEs by altering these reactions can affect the degree and type of exposure to carcinogenic xenobiotics and predispose individuals to disease.

The major XMEs, which have been implicated in susceptibility to disease, include families such as the cytochrome P450s, N-acetyl transferases, glutathione-S-transferases and epoxide hydrolases. The following discussion of the cytochrome P450 family attempts in no way to be comprehensive but is simply to provide a background to their nomenclature and to outline the diversity of these enzymes and the diseases in which they may be implicated.

1.3 The cytochrome P450s

The genes of the P450 superfamily code for a group of enzymes that share common characteristics:

1. They all contain a noncovalently bound haem.
2. They are intrinsic membrane proteins firmly bound to intracellular membranes.
3. They use reducing equivalents from NADPH and an atom of oxygen derived from atmospheric oxygen to oxygenate substrates.

A nomenclature system for the P450 was devised in 1987 to categorise the ever-expanding field of newly discovered P450 proteins. It is based on the amino acid sequence of the individual P450. The superfamily is divided into families, subfamilies and individual P450s. The family is traditionally denoted by Roman numerals and the subfamily by a capital letter. Individual P450s are represented by Arabic numbers (13). If two cytochrome P450s have less than 40% amino acid homology they represent members of a different family. P450s that display greater than 59% amino acid homology are assigned to the same subfamily. Although these rules for nomenclature are arbitrary they have proved useful as members of the same family often have almost

identical intron/exon gene structures. Furthermore genes for members of the same subfamily, that have been examined to date, have been found to lie within the same" gene cluster " (14).

The cytochrome P450s are found throughout the animal and plant kingdom. They have evolved over million of years with the emergence of different families of cytochrome P450 enzymes. The first cytochrome P450s thought to have emerged are those that are now involved in steroid and fatty acid metabolism. The fatty acid metabolising CYP4 family and steroid inducible CYP3 family diverged over one billion years ago. The CYP1 and CYP2 families formed 800 million years ago and with the CYP3 family are those primarily involved in drug and carcinogen metabolism. The CYP2 family further divided into eight families 400 to 600 million years ago. It has been proposed that the divergence and increase in the number of cytochrome P450 genes occurred as a result of the emergence of mammals onto land several million years after plants were established. Plants had therefore had time to develop a multitude of compounds that were foreign and potentially toxic to the " predatory animal species". The development of detoxifying enzymes allowed animals to survive in this hostile environment (15).

The mammalian P450s are divided into 10 families of proteins (Table 3).

Table 3: Cytochrome P450 families

Gene family	sub-families*	substrate †	regulated by †
CYP1	2	Xenobiotics	Substrates
CYP2	6	Xenobiotics	Substrates, hormones and cytokines
CYP3	1	Xenobiotics	Substrates, hormones
CYP4	2	Fatty acids, prostaglandins, leukotrienes	Peroxisome proliferators, hormones, fatty acids
CYP7		Cholesterol	Cholic acid
CYP11	2	Cholesterol,	Hormones
CYP17		Sterols	Hormones
CYP19		Sterols	Hormones
CYP21		Sterols	Hormones
CYP27		Cholesterol, bile acids	Hormones

P450 nomenclature used according to Nebert (see above). CYP represents cytochrome P450. † only general substrates and regulatory examples are given.

In humans it has been estimated that there are at least 50 different P450 genes (16). Of these as previously mentioned CYP1, CYP2 and CYP3 families are those primarily responsible for the metabolism of drug and carcinogen xenobiotics. A number of cytochrome P450s from these families are known to be polymorphically expressed and these genetic variations are thought to predispose to disease in humans.

1.3.1 CYP1 family

In humans, the CYP1 family to date consists of two subfamilies CYP1A and CYP1B. The CYP1A subfamily consists of two highly homologous proteins CYP1A1 and CYP1A2 both of which are involved in the activation of procarcinogens. CYP1A1 is involved in the metabolism of polycyclic aromatic hydrocarbons (PAHS) and CYP1A2 in nitrosamine and arylamine metabolism. Both enzymes are induced by a cytosolic

protein complex consisting of the arylhydrocarbon or Ah (dioxin) receptor bound to an induction agent such as a polycyclic hydrocarbon (PAH) or nitrosamine.

CYP1A1 enzyme is predominantly expressed in extra hepatic tissues including lung (17). It is normally expressed at low levels but it has been demonstrated that it is induced in the bronchial tree of over 80% of smokers (17). Its expression has been shown to be polymorphic with approximately 10% of the population exhibiting a high-inducibility phenotype (in response to inducers such as benz[a]pyrene and 3-methlycholanthrene) (4). At the gene level an Msp1digest restriction fragment length polymorphism (RFLP) has been identified in the 3' non coding region of the CYP1A1 gene as a result of a thymine (t) to cytosine (c) base pair transition at position 6235, 250 base pairs down stream from the polyadenylation site (18). In a Japanese population with lung cancer 21.2% were found to be homozygous for this mutation compared to only 10.6% of a healthy control population (19). A further point mutation of adenine (a) to guanine (g) at position 4889 in exon 7 of CYP1A1 has been linked to the *Msp*1 polymorphism. This results in a isoleucine (I) to valine (V) amino acid change within the haem binding region of the protein and this has since been demonstrated to increase the catalytic activity of the enzyme up to seven fold (20). This mutation has also been linked with lung cancer groups in a Japanese population (21). This link with lung cancer has not been demonstrated in a Caucasian population but the higher activity CYP1A1 genotype is rare in European Caucasians (1% or less) (22)

CYP1A2 is also thought to be polymorphic and exhibits a wide inter-individual variation in CYP1A2 mediated caffeine metabolism that appears trimodal in distribution and is inducible by cigarette smoke and other host factors (23). The genetic basis for this polymorphism is not entirely clear. A recent report suggests that it may be due to a

single nucleotide mutation in intron 1(24). CYP1A2 is involved in the metabolism of dietary heterocyclic amines (25), a potent group of procarcinogens. Variation in CYP1A2 metabolism may be involved in predisposition to bladder, liver and colon cancer (23). The link with colon cancer will be examined later in chapter 3.

CYP1B1 human cytochrome is located on Chromosome 2 at 2p23-22 and contains three exons and two introns (26). CYP1B1 is the predominant CYP1 family member expressed in normal breast tissue and breast cancer. An association with polymorphisms in exon 3 and steroid receptor expression in breast cancer has been reported although no association between exon 3 polymorphism and breast cancer was found (27).

1.3.2 CYP2 family

The CYP2 family is the largest cytochrome P450 family with at least six subfamilies identified in humans. Again many of the CYP2 family genes have been shown to be polymorphic leading to inter-individual variation in metabolism mediated by CYP2C enzymes.

CYP2A6 and CYP2A7 are located on Chromosome 19. CYP2A6 is the main metaboliser of nicotine. Two variants have been reported CYP2A6*v*1 and CYP2A6*v*2. The CYP2A6*v*1 variant encodes an enzyme with a leucine (L) ^{160} to histidine (H) ^{160} amino acid substitution resulting in an inactive enzyme (28). This defective gene has been linked with a decreased risk of becoming a smoker and a reduction in number of cigarettes consumed in those who smoke. Furthermore those with an inactive CYP26 allele may be also less efficient at activating procarcinogens to carcinogens. These three factors could suggest why there may be a reduced risk of tobacco related disease

in those with an inactive CYP2A6 allele (29). CYP2A7 appears to code for an inactive protein (30).

In humans, two CYP2B subfamily genes have been described to date, CYP2B6 and a pseudogene CYP2B7P. CYP2B6 has been implicated in the metabolism of cyclophosphamide and aflatoxin B_1. No genetic polymorphisms have been identified as yet in CYP2B6 but there is 100 fold inter-individual variation in expression within the liver (31).

The CYP2C subfamily consists of at least five different genes in humans located on Chromosome 10. Polymorphisms exist within the CYP2C subfamily, the best characterised is illustrated by differences in the 4-hydroxylation of S-mephenytoin by CYP2C19. A g $\rightarrow$ a mutation at position 681 in exon 5 of CYP2C19 leads to a 40base pair deletion of the CYP2C19 mRNA leading to a truncated inactive protein. This occurs in approximately 5% of Caucasians and results in poor metabolisers (PM) of mephenytoin. Those homozygous for the normal allele or with heterozygous genotypes are extensive metabolisers (EM) of S-mephenytoin. The CYP2C subfamily will be discussed in greater detail in chapter 4.

The CYP2D subfamily genes are located on Chromosome 22 in humans (32). There are three genes at the CYP2D6 locus CYP2D6, CYP2D7 and CYP2D8. CYP2D7 is an inactive gene and CYP2D8 is a pseudogene (4). CYP2D6 has a functional polymorphism of debrisoquine 4-hydroxylase activity in humans (33). As with the CYP2C subfamily humans are split into those with normal enzyme activity that can metabolise debrisoquine, the extensive metabolisers, and those with mutant allele who are poor metabolisers. There are at least 5 mutations that result in no CYP2D6 enzyme

or reduced enzyme activity (4). Between 5-10% of Caucasians are poor metabolisers. In studies using pharmacokinetic assays to determine debrisoquine hydroxylation phenotype the extensive metaboliser phenotype has been linked to the incidence of bladder (34), breast cancer (35) and lung cancer. These findings however have not been as clear cut in studies determining genotype by DNA based assays. A large study looking at many different cancers including lung, breast and bladder cancer as well as overall cancer risk found that the CYP2D6 polymorphism, which gives rise to the PM phenotype, was not associated with reduced cancer risk. In fact they found that PMs had a statistically significantly increased risk of leukaemia (36). In a recent meta-analysis of 18 papers looking at CYP2D6 and lung cancer the authors conclude that "poor metabolisers with respect to CYP2D6 show a small decrease in susceptibility to lung cancer compared with extensive metabolisers and it is hard to justify further studies. The relationship between the CYP2D6 polymorphism and lung cancer, as a determinant of individual susceptibility, is not appreciable (OR = 0.69) compared with that between smoking and lung cancer (OR > 11). Nevertheless, the epidemiological impact on the number of poor metabolisers who are protected from lung cancer may be considerable" (37).

CYP2E1 is the only known member to date of the CYP2E subfamily. The CYP2E1 enzyme is involved in the metabolism of nitrosamines in tobacco smoke. A polymorphism of CYP2E1 detectable by the restriction enzyme Rsa I may be functionally important because it is located in a putative binding site for the transcription factor HNF-1 and has been associated with higher levels of CYP2E1 transcription. It is conceivable that this CYP2E1 Rsa I polymorphism might contribute to differences in susceptibility to cancer. Studies looking at lung oesophageal and renal tract tumours

have found no association with the Rsa I polymorphism (38) (37) (39). The Rsa I polymorphism has however been associated with oral and nasopharyngeal cancers (40) (41).

1.3.3 CYP3 subfamily

In humans the CYP3 family consists of one subfamily CYP3A. Four human CYP3A genes are located on Chromosome 7q, CYP3A3, CYP3A4, CYP3A5 and CYP3A7 (4). They are all involved in the metabolism of aflatoxin and other carcinogens, and there is considerable variation in the hepatic expression of these enzymes. CYP34 enzyme has been shown to be protective against aflatoxin B_1 -induced liver cancer in smokers in China (42). No genetic basis for the inter-individual variation in hepatic expression of the CYP3A subfamily has been established to date.

This brief overview of the CYP1, 2 and 3 families is intended to provide a background to illustrate how genetic polymorphisms of xenobiotic metabolising enzyme genes, including those coding for the cytochrome P450 genes may lead to predisposition and susceptibility to a variety of environmentally induced diseases including cancer.

Chapter 2 Colorectal adenomas and cancer

2.1 Colorectal cancer

In 1993 there were 19,000 deaths from colorectal cancer in England and Wales representing 20% of all cancer deaths (43). It is the second most common malignancy in developed countries (44) and overall the third most frequent cancer in the world except for lung and stomach in men and breast and cervix in women (45). Three distinct clinical features of colorectal cancer have led to research efforts that have improved the understanding of its pathogenesis.

The first feature is the identification of the adenoma-carcinoma sequence as the common pathway for the development of most colorectal cancers. It is now established that in the majority of cases colorectal cancer does not arise de novo, but from pre-existing adenomatous polyps (46) (47) (48). Individuals at high risk for developing colorectal cancer, i.e. patients with adenomas, can readily be identified endoscopically. Secondly, a greater understanding of the genetic basis of colorectal cancer has been revealed through numerous recent breakthrough studies (49). These advances allow for molecular diagnosis of certain high risk groups, specifically patients with familial adenomatous polyposis (FAP) and hereditary nonpolyposis colon cancer (HNPCC) (50) (51). Equally important, the multiple genetic alterations in these syndromes have been noted to occur also in the sporadic colorectal cancers (52). The hope for the future is that these laboratory discoveries will make their way into the clinical arena to improve detection, diagnosis, treatment and eventually prevention of colorectal cancer.

The third feature of large bowel cancer is its environmental and dietary aspects. Epidemiological studies on migrating populations have confirmed the rapid rise in the incidence of colorectal cancer in individuals who move from a low to high incidence country. The risk is noted to occur within one generation suggesting environmental factors acting at a late stage of the carcinogenesis process (53).

2.2 Adenoma-Carcinoma sequence

Evidence for the adenoma-carcinoma sequence comes from both pathological and molecular biological observations. The familial adenomatous polyposes are a group of inherited autosomal syndromes characterised by either the development of at least one hundred to several thousand adenomas or the presence of extra colonic manifestation of FAP in association with APC mutations. Most patients remain asymptomatic until after puberty after which polyps begin to develop. Untreated the polyps increase in both size and number with inevitable progression to adenocarcinoma in the fourth and fifth decades. Treatment by panproctocolectomy eliminates the risk of colorectal cancer. Although these syndromes account for less than 1% of colorectal cancer cases they provide a dramatic example illustrating the progression of adenomas into carcinomas.

Another group in which there is an increased risk of colorectal cancer is those with HNPCC. The following clinical features known as the "Amsterdam criteria" define the diagnosis of HNPCC (54).

(1) Three or more relatives with histologically verified colorectal cancer, one of whom is a first degree relative of the other two.

(2) Colorectal cancer involving at least two generations.

(3) One or more colorectal cancer cases diagnosed before age 50.

Patients with FAP are excluded. Adenomas are more prevalent in HNPCC than in the general population (55) (56) and the adenomas in HNPCC have a greater propensity for malignant change. They tend to be multiple, large, dysplastic and have a greater percentage of villous histology than their sporadic counterparts. They also tend to occur in the proximal colon as does colorectal cancer in HNPCC (56) (57). This phenomenon is known as the Aggressive Adenoma theory (58) and again suggests a progression from adenoma to colorectal cancer.

A large study from St Mark's examined 1,961 malignant tumours and found that 278 (14.2%) had evidence of contiguous benign tumour of tubular or villous histology. Furthermore they found that the frequency with which contiguous benign tumour was found varied with the extent of cancer spread. In tumour with extramucosal invasion only 7% had contiguous benign tumour. However, when the tumour was confined to the bowel wall this rose to 20% and up to 60% when the tumour invasion was limited to the submucosa. This suggests that as the cancer spreads and becomes more invasive it replaces the adenomatous tissue from which it arises (59). This finding is supported by Heald and Bussey (60) who examined histology on 157 surgical specimens all containing synchronous malignant colorectal tumours. They found that 87 (27%) of a total of 323 cancers had benign tumour contiguous with invasive cancer and that 75% of specimens contained associated benign tumours.

As polyps increase in size, their potential for malignancy rises. Muto et al (46) in a large series from St Mark's report a malignancy rate of 1.3% for adenomas under 1cm in size, increasing to 9.5% for adenomas between 1 and 2 cm and a risk of malignancy of 46% for adenomatous polyps greater than 2cm in size.

Further support of the polyp-cancer sequence comes from the study of age distribution for benign and malignant tumours. This shows that the diagnosis of polyps precedes the diagnosis of cancers by an average of 4 years. This is probably an underestimate as polyps are probably diagnosed at a late stage, as the majority remain asymptomatic. In FAP, where individuals are screened from an early age, there is a latent interval of about 12 years between the diagnosis of polyposis and the development of cancer (46).

Several studies have reported a reduction of between 60-85% in incidence of distal colorectal cancer following removal of adenomas at sigmoidoscopy. (48) (61) (62) (63) (64). This suggests that the polyp-cancer sequence can be interrupted by removal of adenomas. Currently large trials are been undertaken to assess the efficacy of secondary prevention by screening for and removing adenomas (1) (65).

These clinical and histopathological observations provide support for the morphology of the polyp-cancer sequence. Further evidence comes from studying the genetic and molecular biology of the " adenoma-carcinoma" sequence.

2.3 Genetic basis of the " adenoma-carcinoma sequence"

In a landmark study Vogelstein examined genetic alterations in colorectal specimens at various different stages of neoplastic development (49). They examined 172 specimens; 40 being early stage adenomas from 7 patients with FAP, 40 adenomas from 33 non FAP patients of which 21 were associated with a focus of adenocarcinoma and 92 carcinomas resected from 89 patients. They looked for ras-gene mutations and allelic deletions of Chromosomes 5, 17 and 18. Ras-gene mutations occurred in 58% of adenomas larger than 1cm and in 47% of carcinomas. They were however only present in 9% of adenomas less than 1cm in size. Deletions of Chromosome 5 were found in 29% and 35 % of adenomas and carcinomas respectively. Chromosome 17p sequences were lost mainly in carcinomas (75%). A specific region of Chromosome 18 was deleted in 73% of carcinomas, 47% of advanced adenomas but only occasionally in earlier stage adenomas (13%). In this study 90% of carcinomas had two or more genetic alterations whilst only 7% of early adenomas had more than two of the four genetic alterations. In a further study Vogelstein studied polymorphic markers from every nonacrocentric autosomal arm in colorectal carcinoma specimens. There was a median of four to five chromosomal arms suffering allelic losses per tumour (66). They concluded that: " These results are consistent with a model of colorectal tumorogenesis in which the steps required for the development of cancer often involve the mutational activation of an oncogene coupled with the loss of several genes that normally suppress tumorogenesis", and furthermore it is the accumulation of genetic abnormalities rather than their order which is most important.

Recognised molecular genetic alterations involved in colonic cancer include oncogene mutations, deletions and mutations of tumour suppressor genes and microsatellite instability with defective mismatch repair genes.

Oncogenes are mutated forms of normal genes termed proto-oncogenes which are involved in cellular growth and differentiation. They produce an abnormal protein with inappropriate function or result in the over-expression of the normal protein. The K-ras oncogene is located on the long arm of Chromosome 12 and is altered by point mutations. It is found in large adenomas and 50% of colorectal cancers. The ras family of proto-oncogenes encodes proteins that are related to the G proteins, these are a class of membrane bound proteins involved in signal transduction. Excitingly, K-ras mutations have been found in the stool of patients whose tumours contain K-ras mutations and this is being investigated as a possible diagnostic modality. K-ras mutations are however not confined to colorectal cancer and are found in a wide variety of tumours. K-ras mutations have also been found in hyperplastic polyps that are generally not regarded as having pre-malignant potential. On the basis of this and histological reports of co-existing adenomatous and hyperplastic areas in the same polyp, the possibility of a hyperplasia-adenoma-carcinoma sequence has been proposed (67).

Tumour suppressor genes function to restrain cell growth and proliferation. They are found in inherited forms of cancer and can also occur by independent mutation of both alleles of a specific gene. Knudson (68) in his hypothesis suggests that one chromosome is inherited with an inactive tumour suppressor gene. The remaining normal chromosome undergoes somatic mutation that predisposes to cancer formation.

In sporadic cancer, two independent somatic mutations of the two alleles of the tumour suppressor gene within a cell are required for tumour progression.

The adenomatous polyposis coli gene (APC), located on the long arm of Chromosome 5, is an example of a tumour suppressor gene which is mutated in the majority of cases of Familial Adenomatous Polyposis and also mutated in sporadic polyps and tumours. It codes for a protein of 2843 amino acids (69) that associates with the cellular protein B catenin. B catenin is complexed within the cytoplasm with the cell adhesion molecule E cadherin. Cadherins are cell surface molecules involved in calcium mediated intercellular interactions, important for morphogenesis. The E cadherin-catenin unit is involved in the normal function of E cadherin, maintaining the adherens junction of epithelial cells. Thus the APC protein is involved in the functional regulation of the E cadherin-catenin unit. The vast majority of APC mutations identified in patients with FAP (greater than 95%) and also those identified in sporadic tumours result in a truncated APC protein (70). The mutated APC protein may disrupt the E cadherin-catenin unit resulting in defective intercellular contact or communication with altered control of epithelial cell growth which may predispose to colorectal tumorogenesis (71) (72).

As previously mentioned loss of heterozygosity (LOH) affecting Chromosome 18q is seen in 70% of colorectal cancers (49). The deleted in colorectal cancer (DDC) tumour suppressor gene has been identified on Chromosome 18q and encodes a 1447 amino acid transmembrane protein with similarity to the neural cell adhesion molecule family of proteins which suggest that it may function through cell-cell and/or cell-extracellular matrix interactions. Patients whose colorectal cancers have 18q LOH have an increased likelihood of distant metastases and death from their disease. The 5 year survival rate in patients with Dukes B colorectal cancer was found to be only 54% in

patients with 18q LOH but 93% in those without 18q LOH (73). Furthermore, evidence for the aggressive nature of colorectal cancers with 18q LOH is shown by the fact that 18q loss occurs in 100% of colorectal cancer liver metastases (74). In more than 90% of cancers with 18q allelic loss, the deleted portion of Chromosome 18 includes the DCC locus. The remaining allele has been shown to be affected by localised somatic mutations in only 10 to 15% of tumours. However, the DCC gene is extremely large (>1350 kb) and only a small subset of the sequences have been examined for mutations(75). Studies have however shown that the expression of DCC is greatly reduced or absent in greater than 50% of primary colorectal tumours and in 100% of colorectal hepatic metastases (75). The DCC gene is thus the likeliest candidate tumour suppressor gene on Chromosome 18q.

Vogelstein also found frequent LOH in Chromosome 17p in colorectal cancer. The P53 tumour suppressor gene is located on Chromosome 17. It codes for a 393 amino acid phosphoprotein that binds to specific DNA sequences leading to inhibition of cell cycle progression (76). This is mediated by the production of a 21kD protein known as Cip1 or WAF1 that inhibits cyclin-dependant kinases. These become active when they associate with other proteins called cyclins and are required for cells to pass through the cell cycle (77). Chromosome 17p LOH can be detected in greater than 75% of carcinomas but is rare in adenomas (49). Wild-type p53 alleles are thought to be targeted by these allelic loses, as the remaining p53 allele is frequently mutated in cases with 17p LOH (78). Relatively few colorectal tumours have a mutant p53 allele without 17p LOH. It is thought that the rate-limiting step in p53 inactivation is a point mutation and that once a mutation occurs, loss of the remaining wild-type allele rapidly

follows. Both mutations and allelic losses generally occur near the transition from benign to malignant growth, and the p53 gene may play a causal role in this progression (79).

As mentioned above HNPCC is a syndrome characterised clinically by the Amsterdam criteria. In 1993 however an unusual form of somatic mutation was reported that was found to be present in 12-15% of all colorectal cancers and virtually all cases with HNPCC (80). These mutations were termed ubiquitous somatic mutations. Others reported similar mutations and called them replicative errors (RER) (81) and microsatellite instability (MIN) (82). Microsatellites are intronic sequences scattered throughout the genome. They are defined as short, tandemly repeated sequences consisting of a core repeated motif of one to six bases. They appear to have no physiological function. The most common di-nucleotide sequence in eukaryotes is the $(CA)_n$ repeat with between 50,000 to 100, 000 microsatellite repeats within the human genome times (83). The number of the repeats and therefore the length of the microsatellite sequence may vary between individuals but should be uniform for every cell of an individual patient. It is the alteration in the length of microsatellite repeats within an individual's cell, which is termed microsatellite instability (MIN), genomic instability or replication error positive phenotype (RER+).

The exact mechanism underlying MIN has been found to be the result of defective mis match repair (MMR) genes. During normal cell replication DNA polymerase corrects most DNA replication errors. Mutations between 1-5 base pairs in length that escape this repair system are repaired by the MMR system. As a result of studies in E. Coli and yeasts MMR genes have been identified in humans (84). Germline mutations in four MMR genes hPMS1, hPMS2, hMLH1and hMSH2 have been clearly linked to HNPCC

(85). Defective mismatch repair leads to a microsatellite mutator phenotype that can lead to accumulation of mutations in known oncogenes and tumour suppressor genes (86). A proposed model for HNPCC development is an inherited germline mutation in the MMR genes followed by a possibly unrelated event such as a mutation in the APC gene. This leads to failure of cell growth regulation with inactivation of the wild-type allele of an MMR gene causing MIN and producing a mutator phenotype (87). This triggers off a rapid development of mutations in other genes such as k-ras, DCC and p53 leading to colorectal cancer progression (88). It has been suggested that MIN is an early event in the adenoma-carcinoma progression. In one study 60% of HNPCC adenomas showed MIN compared with only 3% of sporadic adenomas(89).

2.4 Environmental factors in colorectal adenomas and cancer

Although inherited forms of colorectal cancer such as FAP and HNPCC provide good evidence for the role of genetic pathways in colorectal cancer, they only account for less than 5% of overall colorectal cancer cases (90). The majority of colorectal cancer cases are sporadic with no germline mutations. As previously mentioned however genetic mutations are found within these tumours. What factors account for these mutations? Observations on migrants to the United States from Japan, comparing cause specific mortality rates in 1949 to 1952, with 1959 to 1962 show that the mortality rates of colorectal cancer amongst Japanese males have risen in one decade to almost equal the higher risks seen in United States Caucasians (91). This trend was also observed in Polish and Chinese immigrants to the United States (91), (92). The short time period in which colorectal cancer rates in migrant populations change to approach that of the

host country strongly suggest the involvement of environmental factors in colorectal carcinogenesis. Interestingly, in Southern Europeans immigrating to Australia a less dramatic change to the higher rate of the host country is seen. An association has been made with this group maintaining their original cuisine to a larger extent than other migrant populations (53). It has therefore been suggested that it is the change in diet that is of greater importance in colorectal cancer risk rather than the change in geographical location (53).

Epidemiological data linking colorectal cancer and diet comes from international comparisons linking per capita consumption of dietary components with national incidence and mortality rates of colorectal cancer, case-control studies and a few cohort studies. In a detailed review of nutrition and colorectal cancer, Potter concludes that " there are sufficient empirical data to establish that diets high in meat (and perhaps fat) are associated with increased risk, as are diets low in plant foods - vegetables, fruit and perhaps cereal. Alcohol increases risk; physical activity and lower body size reduce risk "(2).

Various mechanisms have been proposed to link these dietary factors and colon carcinogenesis. The bile acid hypothesis suggests that fat increases the secretion of bile acids, which may be metabolised in the colon to steroids with chemical configurations similar to those of known carcinogens (93). It has been shown that people with colorectal cancer have high faecal concentrations of both bile acids and their metabolites. (94)

The cooked-food hypothesis proposes that diets high in fat contain greater amounts of heterocyclic amines carcinogens from meat proteins as a consequence of cooking at

higher temperatures (95). This arises as cooking in fat generates higher temperatures than cooking in water.

Another theory for the importance of fat in colorectal cancer carcinogenesis is that it is a source of diacylglycerol (DAG). It has been proposed that intracellular DAG is an important part of the cascade that leads from ras activation via a G protein or from growth factors via receptors to protein C activation, protein phosphorylation and cell turnover. Thus the interaction of fat, bile acids and bacteria may produce excess intraluminal DAG which could mimic and amplify these cell replication signals (2).

Burkitt hypothesised that the low rates of colorectal cancer in Africa are due to the fibre-rich diet consumed there (96). It has been suggested that fibre binds bile acids, reduces transit time, increases stool bulk and ferments to volatile fatty acids which may be directly anti-carcinogenic (97) and which by reducing pH may reduce the conversion of primary to secondary bile acids.

Vegetables may act in a similar way to fibre binding bile acids and increasing stool bulk and transit time. They also contain a large number of substances, both micronutrients such as carotenoids and ascorbate, and other bioactive compounds such as phenols, flavenoids, isothiocynates and indoles with a wide variety of potent anti-carcinogenic effects (98).

Calcium has been proposed to act at two levels. Firstly it lowers risk by binding bile acids and fatty acids (99). Secondly it has been suggested that calcium may be involved in the control of cell proliferation (100).

High alcohol intake and deficient intakes of folate has been linked to increased risk of colonic adenomatous polyps (101). This has been linked to methylation of DNA which may have a role in the regulation of gene expression and depends on dietary folate and

methionine. Because aberrant DNA methylation may contribute to the initiation or progression of colon cancer it has been proposed that deficient intakes of folate or methionine and high consumption of alcohol, an antagonist of methyl-group metabolism, increases the risk of colonic neoplasia (102).

These different hypotheses are in no way exclusive and in fact to the contrary complement each other. Fat, cooked meat, vegetables, fibre, calcium and alcohol interact in complex pathways with the balance of carcinogenic and anti carcinogenic effects determining the " internal milieu" to which the colonic cells will be exposed. Colonic carcinogenesis thus depends on exposure to a variety of different xenobiotics and also to the host's response to the exposure. As discussed in the first chapter, at a cellular level, there is an array of xenobiotic metabolising enzymes that are involved in determining the fate of xenobiotics, many of which are polymorphically expressed and therefore may alter the host's susceptibility. What evidence is there for the involvement of xenobiotic metabolising enzymes in colorectal cancer?

Chapter3 Xenobiotic metabolising enzymes polymorphisms and predisposition to colonic adenomas and cancer

As discussed above, colorectal cancer arises as a result of exposure to environmental factors. Both phase I and phase II XMEs are subject to altered enzyme activity as a result of genetic polymorphism so it is therefore plausible that these genetic polymorphisms may alter an individual's susceptibility to environmental and lifestyle factors and hence affect their risk of colorectal cancer (CRC).

3.1 Acetylation phenotype and NAT1 and NAT2 genotype

One of the mechanisms of CRC development discussed above involves the production of heterocyclic aromatic amines (HAA) by pyrolysis of amino acids in meat during cooking. This occurs particularly at high temperatures and with direct contact of meat with the cooking surface. HAA are highly mutagenic and have been demonstrated to induce a variety of tumours in animals (103). DNA adducts formed by PHIP (2-amino-1-methyl-6-phenylimidazo-[4,5-b]-pyridine) and other HAA have been identified in rats and humans (104). PHIP adducts have also been identified in colonic mucosa in humans (105). HAA are metabolised by pathways involving N-acetylation and N-oxidation. N-acetylation is performed by N-acetyltransferase which is produced by the N-acetyltransferase 1 (NAT1) and N-acetyltransferase 2 (NAT2) genes and N-oxidation is mediated by CYP1A2 (106). The activities of both CYP1A2 and NAT1 and NAT2

enzymes have been demonstrated to show inter-individual variation that may predispose to CRC carcinogenesis.

Initial studies looking at acetylation variability and CRC risk used sulfamethazine as a probe drug to determine acetylation phenotype. This phenotyping procedure readily distinguishes fast acetylator phenotypes from slow acetylator phenotypes. Several studies have examined acetylator phenotypes in CRC and/or adenoma patients (Table 4). As can be seen a positive association between fast acetylator phenotype and risk of CRC has been shown in some studies but not all. Two acetylation phenotype studies (107), (108) out of five show a significantly increased risk for the fast acetylator phenotype. A third study from Spain did not confirm this increased risk for fast acetylator phenotype (109). The authors suggest that their finding may be due to a lack of carcinogens in the large bowel reflecting the difference between a Spanish-Mediterranean diet high in fibre and monounsaturated fat (olive oil) and an Anglo-Saxon diet with a higher content of saturated fat and barbecued foods. Two further studies assessed acetylator phenotype in conjunction with CYP1A2 phenotype and dietary exposure (110), (95). Fast acetylator phenotype was not found to be an independent risk factor in either of these studies for CRC. An effect was seen when combining phenotype and dietary risk factors and this will be discussed further below.

In the Caucasian population genetic polymorphisms of the NAT2 gene have been identified which can confer a slow acetylator polymorphism by either reducing the stability of the enzyme or reducing transcription of the gene (111;112). Analysis of these polymorphisms at the NAT2 gene locus, by polymerase chain reaction (PCR) and RFLP, can identify individuals with rapid, intermediate and slow acetylator phenotypes (113). Studies examining NAT2 genotypes, determining

Table 4 Acetylator phenotype studies and colorectal cancer risk.

Author and year	Cases	No	Controls	No	odds ratio (95%CI) fast vs slow acetylators	Exposure assessment	Geographic location
Lang et al 1986(114)	Male CRC cases between 45 to 75.	43	Male surgical patients with no malignant disease	41	2.5 (1.0-6.4)	None	USA, Little Rock, Arkansas
Ilett et al 1987(115)	Patients who had surgery for CRC	49	Patients of similar age, sex and racial origin who are non cancer sufferers.	41	3.8 (1.5-9.7)	Smoking and alcohol: not analysed with acetylator status	Australia, Western
Ladero et al 1991 (109)	Histologicaliy diagnosed CRC cases	109	Healthy Spanish subjects on no medication	96	1.1 (0.7-2.0)	None	Spain
Lang et al 1994 (Study also assessed CYP1A2 phenotype) (110)	New CRC (34) and adenomas (41)	75	Recruited by random digit dialling	205	1.3 (0.9-2.0)	Dietary and smoking	USA, Little Rock, Arkansas
Roberts-Thompson et al 1995 (95)	Adenoma cases Cancer cases	89 110	Outpatients with negative Ba enema or colonoscopy	110	1.1 (0.6-2.1) 1.8 (1.0-3.3) 1.9 (1.0-3.7)*	Dietary questionnaire	Adelaide, Australia

* covariate adjusted odds of diseases over three levels of meat consumption for adenomas and cancers together.

fast, intermediate or slow acetylation, have not found an independent association with CRC or colorectal adenomas (Table 5). In one study where genotype and exposure was assessed a relative risk of 2.25 (95% CI 1.0-5.1) was found for colorectal adenomas in fast acetylators who where current smokers versus slow acetylators who had never smoked (116). Another study showed an increased risk for CRC in patients who were NAT2 rapid acetylator genotype and NAT1 rapid acetylator genotype and consumed greater than 1 portion of red meat per day (OR 5.82 95% CI 1.19-30.6) (117). A Chinese study also found a significant difference in NAT2 allele frequency between CRC cases and controls due to an increase in the NAT2*7 allele in CRC cases (118). There was however no difference in acetylator status between the groups and the authors did not suggest any role for the NAT2*7 allele in CRC development.

These contradictory findings between phenotype and NAT 2 genotype studies may have arisen because phenotype evaluation was influenced by specificity of substrates used and overlapping activity from NAT1 that now is also known to be polymorphic. Furthermore the numbers in many of the studies are small and most failed to assess exposure.

Several studies have looked at NAT1 genotype and CRC and colorectal adenoma risk (Table 6). One study found an increased risk in individuals with the NAT1*10 allele thought to confer rapid acetylator status (119). This study also examined NAT2 genotype and found that rapid acetylation genotypes where not a significant risk factor for CRC but they did find that the risk associated with the NAT1*10 variant allele was most apparent amongst NAT2 rapid acetylators, suggesting a possible gene-gene interaction (119). A further large study, examining colorectal adenomas, however found no increased risk in those with the NAT1*10 variant allele (120). Furthermore they did

Table 5 NAT 2 genotype studies and colorectal cancer risk

Author and year	Cases	No	Controls	No	Mutations analysed	Result	Exposure assessment	Geographic location
Rodriguez et al 1993(121)	Human colon samples from CRC cases	44	non CRC subjects	28	481T, 590A & 857A	NS	None	USA
Shubuta et al, 1994 (122)	Japanese CRC patients	234	Healthy Japanese controls	329	Own classification Genotypes I-X	NS	None	Japan
Oda et al 1994 (123)	Japanese CRC patients undergoing surgery	36	Autopsy subjects without CRC	36	Genotypes I-X	NS	None (Also investigated the occurrence of K-ras gene point mutations, closely linked to rapid acetylator genotype)	Japan
Probst Hensh et al 1995 (116)	Distal colorectal adenoma found on sigmoidoscopy aged 50-74	447	Patients with negative sigmoidoscopy	487	191A, 341C, 481T, 590A & 857A	NS	Smoking assessed and food frequency questionnaire performed. Fast acetylator current smoker Vs slow acetylator never smoked = OR 2.25 (1.00-5.08)	Los Angeles County. USA
Bell et al 1995 (119)	Colorectal cancer cases	202	Non cancer patients in same hospital	112	Nat2*4 Nat2*5A(481), NAT2*6A (590) NAT2*7A(857)	NS	Smoking assessed in cases. Fast acetylator smoking cases relative to non smoking cases= OR 1.3 (0.7-2.5)	UK
Jenkins et al 1997 (124)	CRC cases	1306	Population based controls	1533	Not stated (abstract only)	NS	None	USA
Hubbard et al 1997 (125)	Consecutive series of operable CRC cases	275	Healthy individuals attending occupational heath screening clinics	343	Nat2*4 Nat2*5A,B&C NAT2*6B NAT2*7A	NS	None	UK
Chen et al 1998 (117)	Participants in physicians' health study who subsequently developed CRC	212	Subjects who did not develop CRC	221	Nat2*4 Nat2*5A(481), NAT2*6A (590) NAT2*7A(857)	NS	Dietary assessment. Rapid acetylators for both NAT2 and NAT1 with > 1 serving of red meat per day vs < or =o.5 serving per day =OR 5.82 (1.19-30.6)	USA
Lee et al 1998 (118)	CRC patients	216	Normal individuals	187	Nat2*5A(481), NAT2*6A (590) NAT2*7A(857)	NS*	None	China
Katoh et al 2000 (126)	Japanese CRC patients	103	Healthy Japanese controls	122		NS	Smoking	Japan

NS = No significant difference for rapid acetylator genotype in cases vs controls. * significant difference in allele frequency between CRC and controls (NAT2*7 more frequent on CRC)

not find any evidence of a gene-gene interaction between NAT1 and NAT2 fast acetylators. In this study they did however find that NAT1*10 variant allele conferred an increased risk for incident adenomas (adenomas diagnosed in patients with a negative sigmoidoscopy in the past). This risk was further increased if the negative sigmoidoscopy was in the previous five years. A third study examining NAT1 and NAT2 association with colorectal cancer failed to find any overall association between fast acetylator genotypes and colorectal cancer but concluded that such subjects may be at increased risk with exposure to high meat intake and smoking (124). This was examined in a study already mentioned above were an increased risk for CRC was found in patients who were NAT2 rapid acetylator genotype and NAT1 rapid acetylator genotype and consumed greater than 1 portion of red meat per day (OR 5.82 95% CI 1.19-30.6) (117) (see below). Another NAT1 genotype study found no association with CRC for two further polymorphisms (NAT1*14 and NAT*15) but did not examine the NAT1*10 polymorphism (127). A further study found no increased risk for CRC for the NAT1*10 polymorphism in a Japanese population.

In order to address the question of phenotype and exposure a study from Australia assessed both NAT2 acetylator phenotype and CYP1A2 phenotype in 205 controls and 75 colorectal cancer and polyp patients. They used a method involving caffeine administration and high-pressure liquid chromatographic analysis of urinary metabolites. They also obtained exposure data using a dietary and health habits questionnaire. They found that the rapid CYP1A2 and rapid NAT2 phenotypes were each slightly more prevalent in cases versus controls (57% and 52% versus 41% and 45% respectively). The combined rapid CYP1A2 - rapid NAT2 phenotype was however found in 35% of cases and 16% of controls (OR 2.79 p0.002). In rapid-rapid phenotype well done meat

Table 6 NAT 1 Genotype studies and colorectal cancer risk.

Author and year	Cases	No	Controls	No	Mutations analysed	RR	Exposure assessment	Geographic location
Bell et al 1995 (128)	Colorectal cancer cases	202	Non cancer patients in same hospital	112	NAT1*10 1088 A	1.9(1.2-3.1)	Smoking assessed in cases only	UK
Probst-Hensch et al 1995 (120)	Distal colorectal adenoma found on sigmoidoscopy aged 50-74	441	Patients with negative sigmoidoscopy	484	NAT1*10 1088 A	1.04 (0.79-1.36)	None	Los Angeles County USA
Jenkins et al 1997 (124)	CRC cases	146	Population based controls	183	Not stated (abstract only)	1.2 (0.8-1.8)	None	USA
Hubbard et al 1998 (127)	Patients with CRC	260	Scottish healthy controls	323	Nat1*14 (999T) Nat1*15 (1000A)	NS	None	
Chen et al 1998 (117)	Participants in physicians' health study who subsequently developed CRC	212	Subjects who did not develop CRC	221	Nat1*10	0.93(0.61-1.42)	Dietary assessment. Rapid acetylators for both NAT2 and NAT1 with > 1 serving of red meat per day vs < or =0.5 serving per day =OR 5.82 (1.19-30.6)	USA
Katoh et al 2000 (126)	Japanese CRC patients	103	Healthy Japanese controls	122	Nat1*10	NS	Smoking	Japan

eaters the risk was increased further (OR 6.45) (129). This link between CYP1A2 rapid metabolisers and NAT2 rapid acetylators has been confirmed in a further study where they found a similar increased risk in those with a high meat intake (OR 3.6) (95). A prospective study examining NAT2 and NAT1 genotype and red meat intake in 212 men subsequently diagnosed with colorectal cancer and 221 controls found no independent association of NAT acetylation genotypes and colorectal cancer risk. Among those men, however, who were rapid acetylators for NAT1 and NAT2 and consumed greater than one portion of red meat per day there was a statistically increased risk when compared to those eating less than half a serving of red meat per day (130).

The results of the studies assessing acetylator status with regard to colorectal cancer illustrate the complexity of the relationship between phenotype, genotype and exposure. Although the original phenotype studies suggested a link this was not consistently supported by genotype studies for either NAT1 or NAT2. It may be that other genetic mutations than those being studied may be responsible for altering acetylation phenotype. When combined with exposure to smoking or red meat intake fast acetylator phenotype and genotype have however been consistently related to CRC and adenoma risk emphasising the importance of the gene-exposure interactions with regard to colorectal cancer risk.

3.2 Glutathione S-transferases

The cytosolic glutathione S-transferases (GSTs) catalyse the conjugation of glutathione to a variety of electrophilic compounds including carcinogens such as the polycyclic

aromatic hydrocarbons and cytotoxic drugs (131). In mammals they consist of a superfamily of genes consisting of four distinct families named alpha, mu, pi and theta. In humans, glutathione S-transferase M1 (GSTM1), a member of the mu family, has been shown to be absent in 35-60% of individuals. This is due to a genetic polymorphism resulting in an inherited deletion of the gene termed the null allele (132). This is also found for glutathione S-transferase T1 (GSTT1), a member of the theta family where the null allele is present in between 10-65% of the human population(133). Several studies have reported on colorectal cancer risk for those with the GSTM1 null genotype (table 7). In one study there was a significant excess of the GSTM1 null genotype with this risk being further increased for proximal tumours (134). Another group looking at the GSTM1 null genotype in sporadic and HNPCC CRC cases, interestingly found an increased CRC risk for the sporadic CRC cases only (135). A study in a Japanese population found no overall increase in risk for the GSTM1 null allele and CRC but did report a significantly increased risk for distal tumours (132). They also assessed smoking but found no increased risk for CRC with either GSTM1 or GSTT1 null allele in smokers. The remaining studies have not demonstrated a significantly increased risk for CRC in those with GSTM1 null alleles (136), (137), (138), (139), (140), (141).

Several studies also looked at the association between the GSTT1 null allele and CRC (Table 8). Two of these studies reported a statistically significant increased risk for CRC in those with the GSTT1 null allele (137), (138). One study found that, although the frequency was not significantly different in CRC cases compared to control individuals, GSTT1 null homozygotes were significantly more common in patients who were diagnosed before the age of 70 years, than in those who were diagnosed at a later age

(136). Early age of onset in null GSTT1 and null GSTM1 genotypes has also been reported in studies of lung and breast cancer. (142) (143) (144). The remaining studies showed no statistically significant difference between the frequency of the GSTT1 null genotype in CRC cases compared to the control populations (132), (145), (146). As with the NAT studies no definite consensus as to the role of GSTM1 and GSTT1 null genotypes as CRC susceptibility genes arises. The evidence is strongest and most consistent for an increased CRC risk with the GSTT1 null genotype. The studies however are small and do not assess gene-exposure interactions. No studies to date have looked at adenoma risk in relation to GST genotype.

One study has also looked at GSTP1 polymorphism which results in reduced enzyme activity. They, however, found no association between this polymorphism and CRC (Table 8) (147).

3.3 Microsomal epoxide hydrolase

Epoxides (compounds whose molecule contains an oxygen atom linked to two carbon atoms as part of a ring) may be present in the diet or tobacco smoke and can be generated from a number of sources including benz[a]pyrene, dietary polycyclic hydrocarbons and nitrosamines (148). Microsomal epoxide hydrolase (mEPHX) is expressed in many tissues including the colon and liver. The activity of mEPHX has been shown to vary more than 50 fold in Caucasians (149). Polymorphism's of mEPHX may alter enzyme activity and account for some of this variation. There is a mutation in

Table 7 Studies of GSTM1 genotypes and colorectal cancer risk.

Author and year	Cases	No	Controls	No	Mutations analysed	RR	Exposure assessment	Geographic location
Zhong et al 1993 (134)	Hospital CRC cases	196	Chemical pathology lab patients and volunteers at ICRF	225	GSTM1 null genotype	1.8 (1.2-2.6)	None	UK
Chenevix-Trench et al 1995 (136)	CRC patients	132	100 unselected controls 100 geriatric patients	200	GSTM1 null genotype	0.9 (0.6-1.4)	None	Australia
Katoh et al (132)	Consecutive CRC cases	103	Clinic attendees without GI symptoms	126	GSTM1 null genotype	1.5 (0.9-2.6)	Smoking	Japan
Deakin et al (137)	CRC hospital patients	252	Patients without malignancy in same hospital	577	GSTM1 null genotype	1.0(0.7-1.4)	None	UK
Butler et al (138)(abstract only)	Sporadic CRC patients	219	Blood donors	200	GSTM1 null genotype	1.0(0.7-1.4)	None	Australia
Gertig et al (145)	Male CRC patients	212	Non CRC males matched for DOB and smoking	212	GSTM1 null genotype	1.0(0.7-1.50	Smoking, alcohol and meat intake	USA
Lee et al (146)	CRC	300	Recruits from chemistry dept	183	GSTM1 null genotype	0.8(0.5-1.1)	None	China
Slattery et al (140)	CRC cases	1567	Random selection from social security list	1889	GSTM1 null genotype	0.9(0.8-1.1)	Smoking and diet	USA
Lin et al (141)	First time diagnosis of colorectal adenomas at sigmoidoscopy	446	Subjects matched for age and gender, free from polyps at sigmoidoscopy	488	GSTM1 null genotype	0.9 (0.7-1.1)	Smoking and diet	USA
Gawronska-Szklarz et al 1999 (135)	Sporadic CRC patients HNPCC patients Suspected HNPCC Colonic adenomas	28 17 25 27	Healthy individuals	145	GSTM1 null genotype	2.5 x for sporadic CRC only p= <0.04	None	Poland

exon 3, where a T to C alteration changes a tyrosine amino acid at position 113 to a histidine and is associated with lower enzyme activity when expressed in vitro. A different mutation in exon 4, an A to G transition changing a Histidine residue 139 to Arginine, in contrast results in increased enzyme activity (150).

One published study to date has investigated mEPHX genotype and CRC risk. The frequency of the exon 3 mutation was higher in CRC patients than in controls (OR 3.89 95% CI 1.8 -8.0) suggesting that putative slow epoxide hydrolase activity may be a risk factor for CRC. This risk was present for both right-sided and left-sided tumours but there was increased risk for distal tumours (OR 4.1 95%CI 1.9-9.2) (150). Unpublished data from our laboratory confirms this finding with an increased CRC risk being associated with the slow exon 3 mutation (151).

Epoxide metabolism has been reported to be important in benz[a]pyrene DNA adduct formation in colonic mucosa(152) with mEPHX acting to detoxify carcinogenic benz[a]pyrene metabolites as a phase II XME. Thus those with a slow exon 3 allele would detoxify these carcinogenic metabolites less efficiently enhancing benz[a]pyrene DNA adduct formation.

Furthermore mEPHX is involved in de-epoxidation of reactive lipids generated by oxygenases. This includes substrates such as linoleic acid and arachidonic acids from which prostaglandins are synthesised (153). Prostaglandins are involved in colorectal tumorogenesis and thus efficient removal of precursors such as linoleic acid and arachidonic acid from synthetic pathways will lead to reduced levels of prostaglandins. This is similar to the mechanisms by which NSAIDs and COX2 mutations attenuate tumorogenesis. Patients with the slow exon 3 allele will detoxify the precursors for

prostaglandin synthesis less efficiently, allowing higher levels of prostaglandin to be synthesised favouring tumorogenesis.

These hypotheses need to be confirmed by further studies once again emphasising the importance of assessing exposure as mEPHX may have a role in both diet and smoking induced susceptibility to CRC.

3.4 Cytochrome P450 (CYP1A1, CYP1A2 and CYP2D6)

As discussed in chapter 1, the CYP1A1 enzyme, aryl hydrocarbon hydrolase, is a phase I XME and activates PAHs to DNA-binding carcinogenic metabolites. One study has examined the relationship between *in situ* CRC and homozygosity for the *Msp* I mutant genotype that results in a high inducibility phenotype (154). They found that homozygosity for the *Msp* I mutant genotype was positively associated with *in situ* CRC ($p=0.008$). The odds ratio for the homozygous genotype versus the heterozygous and wild type genotypes was 6.8 (95%CI 1.3-33.1). The numbers in this study were however small, 43 cases and 47 controls, and over half were Japanese who have a higher frequency of the *Msp* I homozygous genotype.

As previously mentioned, two studies assessed CYP1A2 rapid metabolisers using caffeine metabolism for phenotyping and found an association with CRC for rapid CYP1A2 metabolisers combined with NAT2 fast acetylators with high red meat diets. A genetic mutation in intron 3 of CYP1A2 has recently been reported to account for the different CYP1A2 phenotypes. This mutation does not appear to have been investigated with regard to CRC or colorectal adenomas.

Table 8. Studies of GSTT1 and GSTP1 genotypes and colorectal cancer risk

Author and year	Cases	No	Controls	No	Mutations analysed	RR	Exposure assessment	Geographic location
GSTT1								
Chenevix-Trench et al 1995 (136)	CRC patients	125	Unselected subjects	94	GSTT1 null genotype	0.7(0.3-1.4)	None	Australia
			Geriatric patients	54		1.5 (0.6-4.3)		
Deakin et al (137)	CRC hospital patients	211	Hospital patients without malignancy	509	GSTT1 null genotype	1.9(1.3-2.7)	None	UK
Katoh et al (132)	Consecutive CRC cases from two hospitals and one medical centre	103	Clinic attendees without GI symptoms	126	GSTT1 null genotype	1.2(0.7-2.0)	Smoking	Japan
Butler et al 1997 abstract	White adults with CRC	219	White blood donors	200	GSTT1 null genotype	3.4(2.1-5.4)	None	Australia
Gertig et al 1998 (145)	CRC cases in physicians health study	212	Subjects without CRC in Physicians health study	221	GSTT1 null genotype	0.8 (0.5-1.2)	Smoking, alcohol and diet	USA
Lee et al 1998 (139)	CRC cases	300	Recruits from chemistry dept	183	GSTT1 null genotype	N/A	None	China
GSTP1								
Katoh 1999(147)	Consecutive CRC cases	103	Visitors to local medical clinics	122	A/G at 313	1.6 (0.9-2.9)	Smoking	Japan

A genetic polymorphism at the cytochrome P450 CYP2D6 debrisoquine hydrolase gene locus affects 5-10% of the Caucasian population and is responsible for compromised metabolism, the poor metaboliser (PM) phenotype. As mentioned in Chapter 1, CYP2D6 may be involved in the activation of NNK, a carcinogenic component of tobacco smoke and hence the reduced activity of the PM phenotype has been associated with reduced cancer risk. One study has looked at CRC risk in relationship to a genetic polymorphism of CYP2D6 consisting of a G→A mutation at the junction of intron 3 and exon 4, which accounts for 80% of the PM phenotype. They in fact found an increase in the PM genotype in CRC although this did not reach significance and postulate that CYP2D6 may in fact have a detoxification role rather than activation role (155).

3.5 Apolipoprotein E

As previously mentioned, diets high in fat have been associated with CRC possibly by increasing excretion of bile acids. Alterations in plasma lipoprotein levels and bile acid metabolism observed in patients with colorectal adenoma and carcinoma may reflect a genetic background predisposing to altered lipid metabolism and tumours. Apolipoprotein E (Apo E) is one of the key regulatory proteins in cholesterol metabolism. Individuals with the ε4 allele appear to absorb a greater proportion of their luminal cholesterol and have lower faecal bile acid output than individuals with the ε3 or ε2 alleles (156). One study has looked at Apo E in CRC and colorectal adenoma patients. It found that the frequency of the ε4 allele of apolipoprotein E was low (0.075

and 0.073) in patients with proximal adenoma and carcinoma compared with the control subjects (0.181) ($P < 0.05$). In patients with distal tumours, there was no alteration in ε4 frequency. In all subjects with the ε4 allele compared with subjects without ε4, the odds ratio for proximal adenoma was 0.36 (95% confidence interval, 0.14-0.89), and the odds ratio for proximal carcinoma was 0.35 (95% confidence interval, 0.14-0.86). The authors concluded ε4 allele of APO E provides protection from the development of adenoma and carcinoma of the proximal colon and these results support the theory that there are common susceptibility genes modulating the susceptibility to external carcinogenic factors.

Different XMEs' phenotypic activity and genotype have been examined in a variety of studies to determine any link with colorectal cancer. The ideal study would be population based with large numbers, with an appropriate control population, would access exposure and preferably look at genotype rather than phenotype as this can be affected by disease status. Several genetic polymorphisms have been identified which may predispose to colorectal cancer risk but none of the studies fulfil the above criteria and the results of studies remain contradictory.

Chapter 4 Cytochrome P450 2C subfamily

4.1 Introduction

The cytochrome P450 2C (CYP2C) subfamily is the most complex of the CYP subfamilies. Six enzymes have been characterised to date. They are all >80% homologous and their catalytic specificity overlaps. Of the six enzymes, four have been identified in humans, CYP2C8, CYP2C9, CYP2C18 and CYP2C19 (157). The human CYP2C subfamily has primarily been investigated for its role in drug metabolism. CYP2Cs metabolise a number of common therapeutic drugs including the anticonvulsant mephenytoin, the anticoagulant warfarin, the proton pump inhibitor omeprazole, antidepressants such as citalopram and imipramine and a variety of non steroidal anti-inflammatory drugs (3).

Two genetic polymorphisms have been identified in humans within this subfamily to date, by phenotyping using probe drugs. One involves the metabolism of mephenytoin that subdivides into extensive metabolisers (EM) of phenytoin and poor metabolisers of mephenytoin (PM) (158). Family studies have shown that the polymorphism responsible for PM phenotype is inherited as an autosomal recessive trait (158). It is present in approximately 3-5% of the Caucasian population but is much more common in Oriental populations with PM phenotypes in 18-23% (158).

The second genetic polymorphism is much rarer and affects the metabolism of phenytoin and tolbutamide (159) (160). From Hardy -Weinberg calculations it has been estimated that the PM phenotype for phenytoin and tolbutamide occurs in 1 in 500 individuals (161).

The microsomal levels of CYP2C8 in human liver have been found to vary considerably (162). As to whether this occurs as a result of a genetic polymorphism is at present unclear.

4.2 Purification from human liver tissue and distribution

The first CYP2C enzyme protein to be isolated was purified in 1983 and termed $P450_8$ and is now believed to be CYP2C9 (163). Further CYP2C9 proteins have been identified which differ by only a few amino acids (164) (165-167). These will be discussed in further detail later on.

Another CYP protein purified from human liver, called HLx and $P450_{MP-3}$ (162),(164) corresponds to CYP2C8 (168). A further protein has been purified from human liver that corresponds to CYP2C19 (169). The amount of this protein in human liver on immunoblots has been shown to correlate with S-mephenytoin 4'-hydroxylase activity in human liver suggesting CYP2C19 is responsible for S-mephenytoin metabolism (169) (170).

CYP2C enzymes comprise about 18% of total cytochrome P450 enzymes in the liver. The most abundant enzyme in this subfamily is CYP2C9. These enzymes are primarily expressed in the liver although CYP2C9 expression has been reported in the small intestine (171). In a further study looking specifically at human colonic tissue, no evidence of CYP2C expression was found in normal, peritumoral or tumoral colorectal tissue samples (172). CYP2C expression has also been reported in the kidney with possibly CYP2C8 or CYP2C19 protein detected (173).

4.3 Identification of CYP2C subfamily genes and gene structure

The human CYP2C subfamily is encoded by a small family of related genes all of which map to human Chromosome 10q24.1-10q24.3 (166). Southern analysis, by probing genomic restriction patterns with long but non-overlapping 5' and 3' end probes, suggests that there are at least seven CYP2C subfamily genes and/or pseudogenes (164). Four members of the CYP2C subfamily have been cloned from liver cDNA libraries. CYP2C8 has been cloned (168) (164) and encodes the CYP protein corresponding to HLx and $P450_{MP-3}$ previously mentioned. Various cDNA for CYP2C9 which encodes the CYP2C protein originally nominated $P450_8$ and MP-1 (174) (164;166) (167). Further cDNA cloning has identified two more members of the CYP2C subfamily, CYP2C18 and CYP2C19 (167). In line with the nomenclature system devised for CYP enzymes, discussed in Chapter 1, the CYP2C subfamily have all considerably greater than 59% amino acid homology (Table 9).

Table 9. Percentage amino acid homology for the CYP2C subfamily

	2C8	2C9	2C18	2C19
2C8	-	82.8% 89.4%	83.7% 88.7%	82.9% 89.4%
2C9	82.8% 89.4%	-	85.6% 92.7%	93.9% 95.7%
2C19	82.6% 90.6%	93.9% 95.7%	85.7% 92.7%	-

4.4 CYP2C8 genetic variation, substrates and catalytic activity

Okino in 1987, using the rabbit liver progersterone-21-hydroxylase P-450 1cDNA as a probe, identified a human liver cDNA, which he called Hp1-1. The 490 amino acid protein encoded by Hp1-1 corresponds to the protein termed Hlx and $P450_{MP-3}$, the CYP2C8 protein. They also identified a variant cDNA clone termed HP1-2 that was shorter than the Hp1-1 clone but contained an extra 39 bases. When compared to the rat P450e gene, they noticed that the exon 7 and 8 boundaries with intron 7 in the rat gene matched perfectly the exons surrounding the extra 39 base pairs of HP1-2. They proposed that the extra 39 base pairs in Hp1-2 arised as a result of alternative RNA splicing, due to the presence of two 3' acceptor sites at the end of intron 7, allowing formation of either a functional mRNA with complete removal of the intron or a non-functional RNA including 39 bp of the intron (168).

Ged isolated two further cDNA clones MP-12 and MP-20 that correspond to CYP2C8 and differ by four bp (164). MP-20 differs by four bp from HP1-1 and MP-12 differs by three bp from HP1-1. All these bp changes result in amino acid substitutions (Table 10). Kolyada reported two further mutations for CYP2C8 but neither result in an amino acid substitution (175).

4.5 CYP2C9 genetic variation, substrates and catalytic activity

Several allelic variants of CYP2C9 have been cloned from human liver cDNA libraries (Table 11). MP-8 was the first to be cloned in 1987 and differs from what is now considered the wild-type by three bp changes one of which is silent (174). Hum-2 was

also cloned in 1987 and corresponds to the same amino acid composition as the 2C9 wt, having only a single silent bp alteration (176). MP-4 was cloned in 1988 and has the same amino acid change at 358 as MP-8 (164). PB-1 was also cloned in 1988 and has three bp changes resulting in three amino acid changes including an R → C at 144. In 1991 cDNA clones 65 and 25 were identified (167). The cDNA clone 65 corresponds to the wt CYP2C9 gene. Clone 25 varies by two bp, one change being silent the other leading to an I → L amino acid change at 359. CYP2C9 was originally thought to be the main S-mephenytoin 4'- hydroxylase, but cDNA expression studies have since shown that all the allelic variants in fact have low catalytic activity towards S-mephenytoin. They do though all have high turnover numbers for tolbutamide indicating that in fact CYP2C9 is the major tolbutamide hydroxylase (178), (3). Substrate specificity studies using cDNA expression systems have shown that CYP2C9 is the principal human tolbutamide hydroxylase and phenytoin hydroxylase. Recombinant enzyme studies have looked at the effect of the CYP2C9 allelic variants with regard to tolbutamide and phenytoin metabolism. The I^{359} allelic variant has a slightly increased turnover number for tolbutamide and phenytoin than the L^{359} allelic variant (179), (3). The $C^{144} \rightarrow R^{144}$ has also been shown to increase tolbutamide hydroxylase activity (179).

A study looking at tolbutamide hydroxylase activity genotyped two individuals known to be poor metabolisers of tolbutamide and found that one was homozygous for the L^{359}/ L^{359} and one was heterozygous for the L^{359} / I^{359} (180). They also determined the overall frequency of the L^{359} allele in a Caucasian American population and found an allele frequency of 0.06 indicating that it is rare. Furthermore the L^{359} enzyme when expressed in yeast, had a nearly two-fold lower V_{max}, and eight-fold lower intrinsic

clearance and a five times higher K_m than the predominant I^{359} allele. This data thus suggests that the L^{359} variant allele may be responsible for the tolbutamide poor metaboliser phenotype and furthermore that the allele may act in a dominant fashion. These finding are supported by a further study in which the L^{359}/L^{359} genotype of a poor metaboliser of tolbutamide was confirmed and the L^{359} allele in a COS expression system was found to have a raised K_m (181). They also reported that the $I^{359} \rightarrow L^{359}$ transition results in disruption of a β strand in a Cho-Fasman plot model that would alter the protein structure.

CYP2C9 is the principal enzyme involved in the metabolism of warfarin in the human liver (3). It has been shown that heterozygotes for the $R^{144} \rightarrow C^{144}$ allelic variant require significantly lower doses of warfarin than those with the normal wt R^{144}/ R^{144} alleles. This is supported by in vitro expression data in HepG2 cells which have shown a 5.6 fold decrease in V_{max}/K_m estimate for (S)-warfarin 7-hydroxylation catalysed by the C^{144} variant suggesting that individuals with this allelic variant would clear warfarin at a slower rate (182). Studying liver RNA, there appears to be a 5-10 fold preferential expression of the C versus R allele in heterozygous individuals suggesting that the C^{144} allele may exert a dominant effect (183). Once again this amino acid change has been postulated to affect the protein structure using the Chou-Fasman model, with the substitution of R^{144} with C^{144} leading to disruption of the Chou-Fasman α-helix. The reported frequency of the C^{144} variant allele varies between studies. Bhasker found a 22% heterozygote rate for the C^{144}/R^{144} frequency (183). Sullivan-Klose (180) found a

Table 10: Identified genetic mutations for CYP2C8

Gene	Gene mutation	Protein change	Ascension number	Author
2C8 clon3 WT HP1-1		Nil	J03472 M17398	Okino 1987 (168)
2C8 clon1 MP20	Ctccctcacaaccttgcggaatt ....a.......... aaagatcagaaatttctcaccctg ....t.......... acccagaggtgacagctaaagtcc ....c......... gaccctggcctctttctagataa ....a..........	N to T 130 N to K 194 M to I 264 H to L 412	M21941a J02832	Ged 1988 (164)
2C8 clon2 HP1-2	Gagctttgataacaagataatgctggctg cataaaacta intron 7		M17398 J03472a	Okino 1987 (168)
2C8 clon4 MP12	gaggcaattcgccaatatctc ....c......... ctccctcacaaccttgcggaatt ....a.......... accaaggcctcaccct ....t....... gagaaagtaagagaacaccaa ....a......... acccagaggtgacagctaaagtcc ....c.........	Nil N to T 130 E to D 155 Nil M to I 264	M21942a J02382	Ged 1988 (164)
2C8clon5 HPH	catgatgacagagaatttcc ....a......... aatgctagctcatctggctgc ....c..........	Nil Nil	X51535a	Kolyada 1990 (175)

Table 11: Identified genetic mutations for CYP2C9

Gene	Gene mutation	Protein Change	Ascension number	Author
2C9 clon2 clone 25	Cagagataccttgaccttctcccc .....a..... gttgtcaatggttttgcctctgtgc .....a.....	I to L 359 Nil	M61855 J05326 g181301	Romkes 1991 (167)
2C9 clon5 MP-4	Accaaccatcagaatttact .....t..... gtccagagatgcattgac .....a.....	Nil Y to C 358	M21940 J02832 g181365	Ged 1988 (164)
2C9 clon6	aaatggagaagg------acaaccaacca .....aaaagc.....		S46963 g258514	Ohgiya 1992 (177)
2c9 clon7 MP-8	Accaaccatcagaatttact .....t..... gtccagagatgcattgac .....a..... gatgaaggtgacaattttaag .....g.....	Nil Y to C 358 G to d 417	M21939 J02832 g181363	Ged 1991 (164)
2C9 PB-1	attgaggaccgtgttcaagagg .....t..... tccctgcaatatgatctgctcc .....g..... aaacgttgctcttatgaaaagttatat .....t.....	R to C 144 C to Y 175 F to L 239		Meehan (166)

C^{144} allele frequency of 0.08 and a similar allele frequency of 0.107 has been reported by Yasar (184). The L^{359} allele is less frequent with a reported allele frequency of 0.074 (184) and 0.06 (185). Bhasker (183) found one heterozygote in 18 patients genotyped (5.5%).

Other reported substrates for 2C9 include non steroidal anti-inflammatory drugs, including diclofenac, mefenamic acid, the oxicams, piroxicam and tenoxicam, ibuprofen, indomethacin and acetylsalicylic. For all of these 2C9 is thought to be the principal enzyme involved in their metabolism. Benz[a]pyrene, a carcinogenic polycyclic hydrocarbon may also be a substrate for 2C9 metabolism (187).

4.6 CYP2C18 genetic variation, substrates and catalytic activity

Less is known about substrates and function for CYP2C18. Allelic variants have been reported (Table 12). In 1991, two CYP clones were identified named 6b and 29c that differ by a one amino acid. This results from a t → c bp change at 1154 resulting in a M to T amino acid substitution (167). Two further allelic variants have been identified and named CYP2C18 P and Q and differ by a t → c bp change resulting in an F → L amino acid substitution (186). There were two further sites where 6b and 29c differed from P and Q but further analysis and comparison with other 2C subfamily genes suggested that these probably arose as a result of sequence misreading (186). An allelic variant has also been reported with a polymorphic Dde 1 digestion site in the 5' flanking region with an allelic frequency of 21.4%(188). The genotype of this allelic variant of 2C18 was

identical to the CYP2C19M1 mutation (see below,) suggesting that the CYP2C18 and CYP2C19 genes are linked and closely related.

In a study looking at 17 human livers, CYP2C18 mRNA was expressed in all subjects but at 7 to 8 fold lower levels than CYP2C8 and CYP2C9. No substrate specific to CYP2C18 has been identified to date.

4.7 CYP2C19 genetic variation, substrates and catalytic activity

As previously mentioned CYP2C19 is responsible for S-mephenytoin metabolism and a genetic polymorphism of CYP2C19 results in a poor metaboliser phenotype. The metabolism of other drugs such as omeprazole, proguanil and hexobarbitol is affected by this CYP2C19 polymorphism. Inter-subject variability in the metabolism of these drugs segregates with the PM phenotype (3). The genetic basis for the PM phenotype has been shown to be principally due to a single base pair change g → a in exon 5 of the CYP2C91 gene. This mutation leads to the formation of an aberrant splice site which results in the formation of a truncated 234 amino acid protein, which lacks the main binding region and is therefore inactive. This mutation accounts for over 80% of Caucasians with a PM phenotype (12). A further mutation has been identified in exon 4 consisting of a g → a base pair change at position 636 which results in a codon change from tryptophan (W) to a stop codon. This results in a truncated 211 amino acid protein containing only the first four exons. This mutation has only been identified in Japanese PMs (189).

Table 12: Identified genetic mutations for CYP2C18

Gene	Gene mutation	Protein change	Ascension number	Author
C18 clon 1 clone 6b P (Furuya)	Ccaagggcatgaccataataaca .....c.....	M to T 385	M61853 J05326 g177181	Romkes 1991 (167)
2C18 clon 5 clone 29c	Ccaagggcacgaccataataaca .....t.....	T to M 385	M61856 J05326 g181299	Romkes 1991 (167)
Q (Furuya)	ggtctgcaataatctccctgctctcat .....t.....	F to L 219		Furuya 1991 (186)

4.8 Hypothesis for CYP2C subfamily and colorectal tumourogenesis

The CYP2C subfamily is complex with a wide variety of substrates. The majority of compounds to date whose metabolism is known to be mediated by the CYP2C subfamily are drugs. CYP2C8 and CYP2C9 are also believed to be involved in the metabolism of benz[a]pyrene a potent carcinogen (187). There is evidence from the literature that smoking predisposes to colonic adenomas (190), (191). Evidence has also been found for a link between colorectal adenomas and smoking with an OR of 2.00 (CI 1.62, 2.46) for those smoking more than 20 cigarettes a day (Table 17). Benz[a]pyrene is a polyaromatic hydrocarbon and is one of the many carcinogens in cigarette smoke. It has been shown in laryngeal cancer that levels of smoking related DNA adduct appear to be determined by the expression of CYP2C9 which varies >10 fold between individuals (192). There is also evidence of anti-benz[a]pyrene diolepoxide-DNA adduct formation in human colon mucosa. Thus suggests that human colon cells can be damaged by benz[a]pyrene, possibly derived from diet or tobacco smoke. Thus benz[a]pyrene and other PAHs could play a role in the aetiology of human colorectal cancer. Polymorphisms of the CYP2C subfamily may alter the activation of benz[a]pyrene to its carcinogenic moieties and thus lead to susceptibility to colorectal tumourogenesis in certain individuals.

Chapter 5 Screening for colorectal cancer and the "Once only flexible sigmoidoscopy trial"

Introduction

This thesis was conceived on the background of the author's involvement in a screening programme for CRC. All the samples collected for analysis were obtained from individuals invited to undergoing screening.

One of the potential uses of XME genetic polymorphisms is that they may act as biomarkers of disease and highlight individuals at risk who may benefit from screening. To date in CRC none of the genetic mutations studied have been found to reliably predict CRC risk (see chapter 3). This chapter aims to outline the concepts behind screening and explain the rationale and recruitment to the "Once only flexible sigmoidoscopy trial" from which the thesis study population was derived.

5.1 Rationale for screening

Prevention of disease can be divided into primary, secondary and tertiary prevention. Primary prevention is the removal of the causal agent, for instance reducing smoking to prevent lung cancer. Secondary prevention is the identification of presymptomatic disease before significant damage is done. Whilst tertiary prevention is the limitation of complications and disability in patients with established disease, for example trying to prevent diabetic complications by good diabetic control. Screening is a form of secondary prevention and can be defined as "the application of sorting procedures to

populations by doctor initiative with the aim of identifying asymptomatic disease or people at particular risk of it".

Wilson (193) in 1966 proposed the following criteria for a screening programme:

1) The condition must be:

(a) Common

(b) Important

(c) Diagnosable by acceptable methods

2) There must be a latent interval in which effective interventional treatment is possible.

3) Screening must be:

(a) Simple and cheap, if possible, and in any case cost-effective

(b) Continuous

(c) On a group agreed by policy to be at high risk

The disease must also be treatable and the screening test must be sensitive (few false negatives), specific (few false positives), safe, acceptable to the patient and easy to interpret. *How does colorectal cancer fare as a candidate for screening?*

Colorectal cancer fulfils the most important of the above criterion for screening in that it is a major health problem, with 28,000 new registrations and 19,000 deaths per year in the United Kingdom. It is the second most common cancer in England and Wales (43). The prognosis of colorectal cancer is poor, with a 5-year survival rate under 40%. Despite new surgical techniques, radiotherapy and adjuvant chemotherapy, there has only been a slight increase in survival rate in England and Wales during the last decades (194). The reason for this is the late stage at which colorectal cancer is diagnosed, with over half of the cases having locoregional or distal metastatic cancer

spread. In contrast in early cases where the tumour has not spread to breach the muscularis mucosa of the bowel wall (Dukes A), the 5 year survival rate is 80-90%.

As discussed above, there is strong evidence that colonic carcinogenesis is a multi-stage process occurring due to the accumulation of genetic abnormalities resulting in the adenoma-carcinoma sequence and that most colorectal carcinomas arise from pre-existing adenomas. The National Polyp Study, in which 1418 patients were followed up for an average of 5.9 years after removal of all identifiable polyps at colonoscopy, showed a reduction in mortality with no deaths from colorectal cancer and only five cancers, all malignant Dukes A polyps. They calculated a 76 to 90% decrease in the incidence of colorectal cancer (195). Thus in colorectal cancer there does exist a latent interval which provides a window of opportunity during which effective interventional treatment is possible.

Individuals with a family history of colorectal polyps or cancer, chronic colitis or inherited conditions such as FAP are at high risk of colorectal cancer and are groups who merit targeting by screening and surveillance. These groups will not be discussed further. However over 75% of colorectal cancers occur in individuals with no known risk factors except for age. Over 90% of cancers in this group occur after 50 and for this reason asymptomatic people over 50 have been labelled as an average risk group and may benefit from screening. It is in this group of individuals that environmental factors are also thought to play an important role and in whom XME genetic polymorphisms may increase CRC risk.

5.2 Screening methods for average risk individuals

5.2.1 Faecal Occult Blood Testing

Guaiac-based faecal occult blood testing (FOBT) is a commonly used screening method in the average risk population. Five controlled prospective long-term clinical trials and two case-controlled studies have provided extensive data and reported a decrease in colorectal cancer mortality with detection of earlier stage cancers. The rationale for FOBT is that cancers and large polyps bleed but that the bleeding is usually subclinical. Mean blood loss from cancer is approximately 5ml per day and is greater for proximal lesions. Blood loss from large adenomas is less at around 2mls per day (196). Those found to have a positive test are further evaluated with colonoscopy. The first controlled trial to show a reduction in colorectal cancer mortality with FOBT was the Minnesota Colon Cancer Control Study (197). In this trial 46,551 participants between the ages of 50 and 80 were randomised to either annual FOBT, biennial FOBT or a control arm not offered screening. The majority of FOBT slides used were rehydrated (83%). The compliance rate for FOBT was approximately 75%. The overall test positivity rate was initially 2.4% with non-rehydrated slides, with a sensitivity of 80% and specificity of 98%. The test positivity rate, however, rose to 9.8% with rehydrated slides, with an increase in sensitivity to 92% and a decrease in specificity to 90%. The test's positive predictive value for colorectal adenomas and cancer was 31% and during a 13-year follow up the trial demonstrated that annual FOBT resulted in a significant 33% reduction in colorectal cancer mortality.

Memorial Sloan-Kettering in collaboration with the Strang Clinic Preventative Medicine Institute in New York used FOBT in conjunction with rigid sigmoidoscopy. 21,756 participants were randomised to either annual FOBT with non-rehydrated slides and rigid sigmoidoscopy or rigid sigmoidoscopy alone. The compliance rate for stool slide preparation was 75% at enrolment. The overall test positivity rate was 1.7% with a sensitivity of 70% and a specificity of 98%. The test's positive predictive value for colorectal adenoma or cancer was 30% and after a ten year follow up period a 43% reduction in colorectal cancer was demonstrated (198).

The Nottingham study (199) in an unselected population based randomised controlled study of 152,850 participants demonstrated a 15% reduction in colorectal cancer mortality with biennial non-rehydrated FOBT. The test positivity rate was 2.3% with a sensitivity of 72% and a specificity of 98%. 59.6% of participants completed at least one FOBT screening.

A Swedish controlled study (200) on the inhabitants of the city of Goteborg randomised 13,759 participants to the test group. In the initial screen, 50% had non-rehydrated slides whereas in the follow up test at between 16 and 22 months all participants had rehydrated slides. The initial compliance for stool slide preparation was 66% with a test positivity rate of 1.9% for non-rehydrated stool slides and 5.8% for rehydrated slides. The study showed a trend to earlier stage cancers, with 65% of cancers in the FOBT group being A or B compared to 33% in the control group. With a median follow up of 8.75 years they have shown a reduction of colorectal cancer mortality of 12% although at this early stage this did not reach significance.

A large Danish randomised controlled study in Funen (201) screened 30,967 participants aged 45-75 with FOBT using non-rehydrated slides. They had an initial

compliance of 67% with a test positivity rate of 1%. They demonstrated a statistically significant 18% reduction in colorectal cancer mortality.

Two retrospective case-controlled studies, one in Saarland in Germany (202), the other performed by the Kaiser Permanente Medical Care Program of Northern California (203), have suggested a reduction in mortality from colorectal cancer in those having FOBT. The Kaiser Permanante study assessed for a history of stool testing in the 5 years before death in 485 individuals who died from colorectal cancer. They compared these results with 727 matched controls and their results suggested a 25 to 30% reduction in colorectal cancer mortality in those in whom FOBT was performed within a year of diagnosis. By 3 to 4 years from screening to diagnosis the mortality benefit was lost. In Saarland, they demonstrated a protective effect of FOBT, with a 57 % reduction in colorectal cancer mortality but only in women and not men.

In a Cochrane meta-analysis (204) of mortality results from randomised controlled trials of Haemoccult testing, a reduction in colorectal cancer mortality of 16% (RR 0.84, CI: 0.77-0.93) was demonstrated for those allocated to screening. The mortality reduction was 23% (RR 0.77, CI: 0.57-0.89) when adjusted for those who actually attended for screening. Overall if 10,000 people were offered a biennial Haemoccult screening and two thirds attended for at least one Haemoccult test, there would be 8.5 deaths (CI: 3.6-13.5) from colorectal cancer prevented over 10 years. However, they also showed that a screening programme would result in at least 2,800 initial colonoscopies. If screening harms from the Minnesota trial are considered there would be 3.4 colonoscopy complications (perforation or haemorrhage). If screening harms from the Gothenburg trial are considered there would be 1.8 colonoscopy complications (perforations or haemorrhages). They concluded that "screening benefits include, reduction in

colorectal cancer mortality, possible reduction in cancer incidence through detection and removal of colorectal adenomas and that potentially, treatment of early colorectal cancers may involve less invasive surgery. Harmful effects of screening include the physical complications of colonoscopy, disruption to lifestyle, stress and discomfort of testing and investigations, and the anxiety caused by falsely positive screening tests. Although screening benefits are likely to outweigh harms for populations at increased risk of colorectal cancer, more information is needed about the harmful effects of screening, the community's responses to screening and screening costs for different health care systems before widespread screening can be recommended."

Thus, the problem with FOBT for mass screening remains the sensitivity and specificity of the test with non-rehydrated slides having a higher specificity but a lower sensitivity than rehydrated slides. Rehydrated slides therefore lead to a greater test positivity rate, a higher colonoscopy rate and hence increased cost. The possibility has also been raised that a significant amount of early cancers may be chance discoveries resulting from the colonoscopies generated (205). The other problem with FOBT is that it detects early cancers rather than trying to prevent cancer by the detection of precancerous adenomas. Furthermore the late stage at which large adenomas and cancers bleed provides a short lead time and hence there is a need for frequent testing.

5.2.3 Sigmoidoscopy

Sigmoidoscopy can be performed using the traditional rigid 25cm proctosigmoidoscope or using the 65cm flexible sigmoidoscope that causes less discomfort and can be passed higher into the colon. Sigmoidoscopy is more expensive and invasive than

FOBT but is more sensitive for small adenomas. It is also therapeutic as well as diagnostic, in that adenomas and some early cancers can be removed during the procedure. Evidence for the efficacy of sigmoidoscopy for colorectal cancer screening comes from several studies.

Gilbertson (206), in an uncontrolled study of more than 21,000 subjects, using annual rigid sigmoidoscopy with removal of all lesions seen, reduced the expected cancer incidence rate by 85%. A retrospective, case control study by the Kaiser Permanente group (61) compared the use of screening rigid sigmoidoscopy during the 10 year period prior to diagnosis in 261 individuals who died of rectosigmoid cancer with 868 matched controls. They showed that 8.8% of cancer patients had a sigmoidoscopy compared to 24.2% of controls, with an adjusted odds ratio of 0.41, demonstrating a 60% reduction in rectosigmoid cancer mortality. Newcomb (64), in a smaller case control study comparing the records of 66 individuals who died of colorectal cancer with 196 matched controls found that case patients were less likely to have had a screening sigmoidoscopy than the controls (10% versus 30%). This study also demonstrated that a single screening flexible sigmoidoscopy resulted in a reduction in colorectal cancer mortality. Atkin (48), in a study of 1,618 individuals who had adenomas removed at sigmoidoscopy up to 30 years previously, demonstrated a reduction in rectal cancer incidence of 80% and furthermore that if all adenomas are removed at sigmoidoscopy the risk of rectal cancer may be very low for many years thereafter. There is however no published randomised controlled study assessing the efficacy of flexible sigmoidoscopy for colorectal cancer screening. The National Cancer Institute has set up a multicentre, long term, randomised controlled screening trial in the United States (PLCO trial - prostate, lung, colorectal and ovary) (207) of 148,000 individuals including flexible

sigmoidoscopy for colorectal cancer screening. The problem with flexible sigmoidoscopy is that it is expensive and invasive and hence compliance may be low. Compliance rates of 70% (208) have been reported in studies using flexible sigmoidoscopy although others have reported much lower compliance rates of 20% when offered in association with FOBT (209). Mass population screening of average risk individuals for colorectal cancer be it using FOBT or flexible sigmoidoscopy or a combination of both is expensive.

5.3 The "Once only flexible sigmoidoscopy trial"

The Imperial Cancer Research Fund, supported by the Medical Research Council under the guidance of Wendy Atkin and John Northover, has established a multicentre, randomised controlled trial in the United Kingdom assessing the use of "once only flexible sigmoidoscopy" in asymptomatic 55 to 64 year olds. From calculations based on the studies previously mentioned, they have predicted that this screening programme could result in a 25% reduction in colorectal cancer mortality (table13).
The initial recruitment and screening for the trial is now complete. The study was undertaken in 14 centres throughout the UK. A local trial coordinator who was a gastroenterologist or a surgeon assumed responsibility for each centre and performed the flexible sigmoidoscopies. The author of this thesis was the local trial coordinator for the Portsmouth centre. Recruitment of participants was via General Practices. Of 354, 612 people asked about their interest in having flexible sigmoidoscopy screening 194, 836 (55%) responded positively. Attendance amongst individuals invited for screening was 71% (40,674 / 57,259). Those screened underwent a flexible sigmoidoscopy using

a 60cm video flexible sigmoidoscopy (Keymed UK), following self-administration of a phosphate enema (Pharmax Ltd, Bexley, Kent). Small polyps less than 1cm were biopsied and or removed during screening. Individuals found to have either no polyps or "low risk polyps" (metaplastic polyps or < 3 adenomas, all of size < than 1cm with tubular histology and mild or moderate dysplasia) were discharged with no further follow up. Those found to have "high risk polyps" (≥ 3 adenomas, size ≥ 1cm, tubulovillous villous histology or severe dysplasia) were invited to undergo a baseline colonoscopy followed by colonoscopic surveillance.

The rationale behind offering a "once only flexible sigmoidoscopy" is that although it may not save as many lives as more extensive screening regimens, it provides a cost effective strategy for reducing colorectal cancer mortality. Based on a cost of £50 per flexible sigmoidoscopy and £150 per colonoscopy generated (5% of those screened deemed to have high risk polyps) with a compliance of 70%, Atkin has calculated that the cost per cancer prevented would be £5,500 and per cancer death prevented £8,500. This compares favourably with other screening programmes such as breast screening were the estimated cost per cancer death prevented is between £30,000- £50,000.

5.4 Conclusions

Colorectal cancer is a major cause of morbidity and mortality in developed countries. Cuthbert Duke, over 50 years ago, demonstrated the link between pathological stage and mortality from colorectal cancer. The perfect test for screening average risk

Table 13 Estimated CRC prevented each year by the proposed screening regimen.

Total CRC diagnosed per year in the UK between ages 58 and 79	17,781
Potentially preventable assuming 70% compliance	12,447
Preventable distal bowel cancers	
64.2% of all preventable colorectal cancers	7,991
Minus screen detected bowel cancer (1.5/1000)	-622
Potentially preventable	7,369
70% of 5,240 prevented between 58 and 74	3,668
50% of 2,129 prevented between 75 and 79	1,064
Total prevented between 58 and 79 years	4,732
Preventable proximal bowel cancers	
36% of all preventable colorectal cancers	4,481
25% identifiable by high risk adenomas	1,120
80% prevented	896
Total colorectal cancers prevented	5,628
Colorectal cancer deaths prevented	
In cases prevented assuming 60% case fatality rate	3,377
In screen detected cancers assuming a 20% improvement in case fatality rate	122
Total	3,499

individuals does not currently exist but trials have demonstrated that FOBT and sigmoidoscopy can do better than waiting for patients to present due to their symptoms. Screening for average risk individuals is now advocated by many bodies including the American Cancer Society, the US Preventive Services Task Force and the World Health Organisation who recommend that "asymptomatic men and women seeking check-ups who have no risk factors should have a 6-window FOBT annually beginning at age 50 and a digital rectal examination and flexible sigmoidoscopy every 3-5 years beginning at age 50". There is currently no national screening programme for colorectal cancer in the United Kingdom and while the above recommendations would probably be prohibitive in terms of cost for a national screening programme, the result of on going trials should allow planning and implementation of a cost effective regime to reduce colorectal cancer mortality using the methods currently available. In the mean time the search for alternative methods of screening such as biomarkers of disease to highlight those who are susceptible to disease and require screening and development of non invasive methods of screening such as virtual reality colonoscopy continues.

Section 2

Subjects, Materials and Methods

Chapter 6 Subjects and Methodology

6.1 Subject recruitment

Ethical Approval

Ethical approval was obtained from the Portsmouth Hospital NHS Trust ethical committee to allow blood to be taken from volunteers attending the 'once only flexible sigmoidoscopy' screening trial and for its use in the study of genetic polymorphisms.

Patient selection

Blood samples were obtained from volunteers attending the Imperial Cancer Research Fund "once only flexible sigmoidoscopy screening trial" (see Chapter 5). Three centres Portsmouth, Leeds and Cambridge/Norwich were interested in studying xenobiotic metabolising enzyme gene polymorphisms and hence collaborated by exchanging DNA extracted from collected blood. In Portsmouth, volunteers attended the screening programme having self-administered a phosphate enema to clear the distal bowel. They had a medical and smoking history taken on a trial proforma by the unit administrator who is a state registered nurse. They then underwent flexible sigmoidoscopy using a 60cm video endoscope (Keymed Ltd, UK). Those found to have polyps were approached and asked if they would be prepared to give a blood sample for the project. If they agreed they were given a consent form with a brief description of the project and asked for a written signature (appendix A). All polyps were sent for histology. For all

attendees with adenomatous histology a corresponding blood sample was also collected from volunteers with a normal flexible sigmoidoscopy. The controls were matched for sex, General Practitioner and aged matched into those between the ages of 55-59 and those between 60 and 64. Blood samples were not obtained from volunteers with polyps if they declined or if the operator felt that they had undergone a particularly arduous procedure or occasionally if the screening lists were running late. All the flexible sigmoidoscopies and sample collections were done by the author.

Food frequency questionnaire

All volunteers attending the "Once only flexible sigmoidoscopy screening trial" completed a food frequency questionnaire prior to being screened (Appendix B). They could consult with the unit administrator if they had any problems completing the form.

Sample collection and storage

Peripheral venous blood was taken from the antecubital fossa. A total of 20mls was normally taken and 6mls was put in a 15ml apex tube (Alpha Laboratories UK) to be used for DNA extraction. 5mls was put into a clotted sample tube for use in a separate study and the remainder was placed in a universal container (Bibby Sterilin Ltd). All blood was stored at -20°C.

6.2 Materials

General

All solutions, media and glassware were sterilised by autoclaving at 120°C for 20 minutes. For all solutions and methods dH_20 was used as standard unless stated otherwise.

Biochemicals

The general chemicals required were purchased from Sigma, Pharmacia and BDH-Merck. Either Promega or New England BioLabs supplied the restriction enzymes used. The reaction buffers and Taq polythermase gold used for the polymerase chain reactions (PCR) were bought from Biogene.

Bacterial stains and vectors

The pGEM™-T Easy vector was obtained from Promega and contains ampicillin resistance and ß-galactosidase gene as selection methods. Epicurian Coli® XL-1 blue supercompetent cells were used with the above vector system and were purchased from Stratagene.

Solutions

All solutions were sterilised by autoclaving and stored at room temperature unless otherwise indicated. The TAE and TBE buffers used for gel electrophoresis were made with dH_20 and not sterilised.

6.3 Methods

6.3.1 Extraction procedure for the isolation of DNA from blood

DNA was extracted from 6mls of human blood using a phenol/chloroform, ethanol precipitation method (210).

The initial step performed was complete lysis of cells. Each sample was incubated with 0.5% final concentration of N-lauryl sarcosine (Sigma) and 200μg/ml proteinase K (Sigma). The sample was mixed vigorously (vortexed) and incubated at 55°C for 24hrs. A repeat vortex was performed every few hours until colour change was observed and clots and lumps were digested. If necessary, another volume of proteinase K was added and the incubation time increased.

Contaminating proteins were then removed by the following method. An equal volume of water-saturated phenol was added to each sample after which the sample was vortexed thoroughly and then centrifuged at 2,500rpm for 10 minutes (Jouan CR422). Two distinct phases were observed after centrifugation. The top phase was carefully removed without disturbing the bottom phase and aliquoted into a fresh pre-labelled tube. This step was then repeated so that each sample underwent two washes with phenol.

An equal volume of chloroform was then added to the sample and the sample was again vortexed and the centrifugation procedure repeated. The top phase was again aliquoted to a pre-labelled tube and the process repeated with chloroform. The top phase if clear was carefully removed into a fresh pre-labelled tube. If the phase was not clear a further chloroform wash was performed.

Precipitation of DNA was then performed by the addition of two volumes of -20°C 100% ethanol/ 0.3M sodium acetate, mixed by inversion and refrigerated at -80°C for 60 minutes or overnight at -20°C.

The DNA was then pelleted by centrifugation at 2,500rpm for 30 minutes (Jouan CR422). The supernatant was then carefully removed ensuring that the DNA pellet was not dislodged. Each pellet was gently washed with 70% ethanol (100µl or 1ml, volume dependent). If the pellet was dislodged the sample was briefly re-centrifuged at 2,500rpm for 5 minutes (Jouan CR422). The 70% ethanol was carefully aspirated by pipette and the sample air-dried at 37°C until the pellet was observed to be free from residual ethanol.

The DNA pellet was re-suspended in 3ml of T.E elution buffer (pH 8.0). All the samples were stored at -20°C until required for PCR analysis.

Some of the samples at a later stage were sent to Genovar® where they underwent extraction commercially using a similar protocol.

6.3.2 Polymerase Chain Reaction (PCR)

The Principles of PCR

The PCR is an invitro method of amplifying a segment of DNA that lies between two regions of DNA for which the base pair sequence is known. Two manufactured sequences of nucleotides (oligonucleotides) are used as primers for a series of synthetic reactions that are catalysed by DNA polymerase. These primers typically have different sequences and are complementary to sequences that lie on opposite strands

of the template DNA and also between them flank the area to be amplified. The template DNA is first denatured by heating which splits the DNA into single strands. The reaction mixture is then cooled which allows the oligonucleotide primers to anneal to their target sequences. The primers are normally used in pairs that are around 15-30 nucleotides in length. One is designed to anneal and amplify from the 5' end to the 3' end of the sequence and is identical to a 15-30 nucleotide segment upstream of the gene fragment to be amplified. The other primer is designed to anneal and amplify from the 3' end to the 5' end of the sequence and is complementary to a 15-30 nucleotide segment downstream of the gene fragment being amplified. The primers anneal to their complementary regions and in the presence of DNA polymerase nucleotides are added copying the gene fragment specific to the primers. The cycle of denaturing, annealing and DNA synthesis is then repeated many times. Because the products of one round of amplification serve as templates for the next, each successive cycle essentially doubles the amount of the desired DNA product. The final result in this exponential reaction is a segment of double stranded DNA defined by the 5' termini of the oligonucleotide primer and whose length the distance between the primers defines.

The PCR reaction

A typical PCR reaction consists of the nucleic acid target (DNA or cDNA) and paired oligonucleotides (primers) specific to a certain gene, which function as described above. The specific working conditions of the primers are dependant upon concentrations of magnesium chloride ($MgCl_2$) buffer and dimethyl sulfoxide (DMSO) which are titrated against each other, and an annealing temperature (Tm) generated from the following formulae:

Tm = 2° (A+T) + 4° (G+C) -2°C

2° C for every a and t

4° C for every g and c

The four deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP) are added to the reaction and form the nucleotide bases a, t, g and c of the sequence during the gene synthesis. A thermostable DNA polymerase (TAQ) is added to extend the nucleotide bases from the primers along the gene. A volume (50-100µl) of the PCR constituents are mixed, adjusted to a final volume (50-100µl) with deionised water and subjected to the PCR process. The PCR reactions were set up as follows.

Each PCR reaction was calculated containing the required amounts of PCR buffer (buffer A), $MgCl_2$ buffer, nucleotides, DMSO, primers and TAQ polymerase. Deionised H_20 was used to make the calculated volume for each PCR reaction up to 49µl. A master mix for all the PCR reactions to be performed was prepared by adding all the above reagents to the master mix in their required volumes multiplied by the number of reaction to be performed with a 10% extra allowance. This served three purposes. It allowed for a degree of pipette and operator error, it removed the need to pipette minute amounts of products and allowed large number of PCR reactions to be set up more efficiently. Then 49µl of the PCR product was pipetted into pre-labelled 0.5 ml eppendorfs including a positive control (to which a known sample of DNA is added) and negative control (to which no DNA is added). 1µl of target DNA was then pipetted into each of the pre-labelled ependorfs. The individual ependorfs were pulse centrifuged at 5000 rpm using a micro-centrifuge (MSE Microcentaur). The ependorfs were then placed in a pre-programmed thermal cycler to undergo the PCR (see below).

The PCR process

The PCR process is fully automated and performed using thermal cyclers that operate by heating and cooling the samples dictated by the working conditions of the primers. The usual PCR proforma consists of a denaturing stage for 3 minutes at 94°C, which causes the hydrogen bonds between the double-strands to break. It is followed by 35 cycles of denaturation for 30 seconds at 94°C then 30 seconds at the annealing temperature of the combined primers (50-65°C), followed by a primer extension for 30 seconds at 72°C to enable the extension of nucleotides along the sequence of interest. The reaction ends with a final extension cycle at 72°C for 2 minutes to complete any synthesis initiated.

6.3.3 Primer design

Primers were designed from sequences retrieved from the Internet and relevant publications. Primers were designed to span the chosen 2C mutations and be specific for the 2C subfamily gene without cross over to related genes. Care was taken to avoid complementarities between the primers to avoid primer-dimer formation with consequent low PCR yield. The g+c content of both primers should preferably be between 40-60% with a/t and g/c residues spaced evenly along the primer sequence.

6.3.4 Standardisation of the PCR reaction

The best condition for each set of primers was standardised. PCRs were performed with variable amounts of $MgCl_2$ and DMSO as in table 14, initially with an annealing temperature 2°C less than Tm and adjusted accordingly. Each set of primers was tested at 35 PCR cycles.

Table 14 Conditions for optimisation of primers

	DMSO		
$MgCl_2$ (mmol)	**0**	**2**(1µl)	**4**(2µl)
1.0(2µl)	1	2	3
1.5(3µl)	4	5	6
2.0(4µl)	7	8	9
2.5(5µl)	10	11	12
3.0(6µl)	13	14	15

The numbers in table 14 refer to PCR tubes in which the primers are tested with a control sample of DNA. The conditions producing the cleanest and brightest band were chosen.

6.3.5 Restriction and DNA methylation enzymes

Restriction enzymes bind specifically to and cleave double stranded DNA at specific sites within or adjacent to a particular sequence known as a recognition site. Restriction enzymes are classified into three different groups. Type I and type III carry modification (methylation) and ATP dependent restriction (cleavage) activities in the same protein. Type I enzymes bind to the recognition site but cleave at random sites when the DNA loops back to the bound enzyme. Type III enzymes cut the DNA at the recognition site and then dissociate from the substrate. Neither type I nor type III restriction enzymes are widely used in molecular cloning.

Type II restriction/modification enzymes are binary systems consisting of a restriction endonuclease that cleaves a specific sequence of nucleotides and a separate methylase that modifies the same recognition sequence. A large number of type II restriction enzymes have been isolated, many of which are routinely used in molecular cloning. The vast majority of type II restriction enzymes recognise specific sequences that are four, five or six nucleotides in length and display twofold symmetry. The location of the cleavage site within the axis of symmetry differs from enzyme to enzyme. Some cleave both strands exactly at the axis of symmetry, generating fragments of DNA with blunt ends, others cleave each strand at similar locations on opposite sides of the axis of symmetry, creating fragments of DNA that carry protruding single-stranded termini.

All restriction enzymes require certain conditions for optional activity. This includes the correct buffer for the enzyme reaction and for some enzymes the addition of BSA. Most

enzymes also work at an optimal temperature for DNA cleavage (digestion) to occur efficiently.

Restriction enzyme digests

All the mutations to be studied consisted of nucleotide substitutions. The relevant segment of double stranded DNA was amplified using PCR as described above. Restriction enzymes were chosen that would cut either the DNA segment containing the mutation or the DNA segment containing the wild type sequence. The restriction enzymes were all stored at the temperature recommended by the manufacturer. Digest reactions were prepared as follows.

A master mix containing the required amount of restriction enzyme, the appropriate amount of Buffer and BSA if required, with dH_20 to correct to the final volume was prepared. A total volume of 10µl per digest reaction was normally used and the master mix was prepared by calculating the total number of digests required with a 10% allowance for pipetting error. 10µl of the master mix was added to prelabelled 0.5 ml ependorfs and 10µl of the PCR product was then added. The individual ependorfs were pulse centrifuged at 5000 rpm using a micro-centrifuge (MSE Microcentaur). The restriction digests were then incubated overnight at the required temperature. The digestion products were then analysed using PAGE (see below) using an uncut product as a control.

6.3.6 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gels are formed by the polymerisation of monomers of acryl amide into long chains. This reaction occurs in the presence of free radicals provided by ammonium persulfate (APS) and stabilised by N, N, N', N'-tetramethlyethylene-diamine (TEMED). With the addition of the bifunctional agent N, N'-methylenebisacrylamide to the polymerisation reaction, the chains become cross-linked to form a gel whose porosity is determined by the length of the chains and the degree of cross-linking. The length of the chains is determined by the concentration of acrylamide in the polymerisation reaction (normally between 3.5% and 20%). The gels are poured between two glass plates that are held apart by glass spacers and sealed on three sides by rubber tubing. This shields most of the acrylamide from exposure to oxygen that inhibits polymerisation except for the top part of the gel plates that remain open. The wells, into which the DNA to be run is placed, are created by inserting a toothed comb into the top end of the gel plates. The gels are run in 1x TBE at a low voltage (1-8V/cm) in a vertical position in specially designed tanks allowing current to be passed through the gel. Most species of double stranded DNA migrate through polyacrylamide gels at a rate that is inversely proportional to the $\log_{10}$ of their size. Their resolving power is so great that they can separate molecules of DNA whose length differs by as little as 0.2% (i.e. 1bp in 500 bp).

Preparation of polyacrylamide gels

The following solutions were prepared in the laboratory. Acrylamide and bisacrylamide were mixed in equal quantities to prepare bisacrylamide-acrylamide 50-50 solution. The solution was stored in dark bottles at room temperature and fresh solution was prepared

every month. 5xTBE (1 litre) was mixed by adding 54g TRIS to a 500ml beaker and adding 400mls dH_20. This was dissolved using a magnetic stirrer and then 27.5g Boric acid and 20mls 0.5 molar EDTA was added. This was then topped up to one litre with dH_20. 25% APS was prepared with 2.5g of ammonium persulfate added to 10mls deionised H_20.

Glass plates were prepared using spacers and rubber tubing and clamps to form a tight seal. The plates took just under 50mls volume of acrylamide solution.

The solution was prepared in a graduated 50ml glass cylinder and 8% gels were made. This was done using 10mls of 50/50 bisacrylamide/acrylamide solution, 10mls of 5x TBE made up to 50mls with 30 mls dH_20. 50µl of TEMED and 200 µl of 25% APS was pipetted into the solution and gently mixed by swirling and inverting. The solution was poured slowly, to prevent air bubbles, into the prepared glass plates which were supported on a decontamination tray with the top end slightly raised. A 30 well, toothed comb was immediately placed into the gap at the top between the gel plates. The gel was allowed to set at room temperature for between 10 and 15 minutes. Once set the clamps and rubber tubing were removed. The gel was then attached to the gel electrophoresis tank and the buffer tank filled up to ensure that the TBE covered the top of the comb teeth. As the comb was gently removed all the wells filled with TBE buffer. The DNA samples to be analysed were mixed with the appropriate amount of gel loading dye. Bromophenyl blue was used for all the PAGE analysis. Each DNA sample to be analysed was drawn up using long fine disposable micropipette tips, which were inserted into the individual well and the sample pipetted in. The same pipette tip was used for all samples when loading the gel but it was washed in the TBE buffer between

samples. A marker dye was loaded onto the gel. For all the gels a Hinf I marker was used. The tank electrodes were connected to the power pack and the gels were run at 40mA until the bands had run the required distance for analysis.

6.3.7 Confirmation of PCR specificity by cloning and sequencing

Ligation reaction

Ligation of the PCR product into a linearised plasmid vector involves the formation of phosphodiester bonds between adjacent 50-phosphate and 30 hydroxyl residues. This is catalysed by T4 DNA ligase. The pGEM®-T easy vector system was used for all PCR products.

The reactions were performed in a final volume of 10µl using the pGEM®-T easy vector system. A mixture of 3µl of PCR DNA product, 50ng of pGEM®-T easy vector, 1x ligation buffer and 5 units of T4 DNA ligase were prepared, centrifuged and incubated at 4-10°C for 24 hours. The ligations were then stored at -20°C until required.

Transformation of ligation products into Epicurian Coli® supercompetent cells

The required number of vials of Epicurian Coli® XL-Blue subcloning-grade competency cells were thawed on ice. The cells were handled with great care at all times. For each transformation reaction a 15ml Falcon 2059 polypropylene tube was prechilled on ice. 100µl of cells was gently pipetted into each tube and 2.5µl β-mercaptoethanol was

added to each tube and gently mixed by pipetting. The cells were incubated on ice for ten minutes swirling gently every two minutes. Iμl of the appropriate PCR ligation reaction was then added to the cells and gently mixed in with the pipette. As a control 1μl of the PUC18 control plasmid was added to a tube of cells. The tubes were then incubated on ice for 30 minutes. The cells were then subjected to heat shock treatment for precisely 45 seconds at 42°C in a water bath and then immediately placed back on ice for 2 minutes.

Then, 250μl of SOC media (terrific broth) was added to each tube and they were incubated at 37°C in a shaker at 225rpm.

Analysis

LB agar plates were coated in 50μg/ml of ampicillin and 25μl X-gal (20mg/ml). From each transformation, 50μl and 200μl were gently spread to cover a plate. These were incubated at 37°C for at least 18 hours. The plates were then placed in the fridge at 4°C for 2-3 hours to allow for colour development. White colonies were then picked from the plates and put into 4 mls of terrific broth containing 100 μg/ml of ampicillin in a universal container. These were then incubated at 37°C overnight in a shaker at 225rpm.

Plasmid DNA extraction by alkali lysis (Minipreps)

The PCR fragment/vector constructs were extracted from Epicurian Coli® cells and purified using the alkali lysis method (210).

Two sets of 1.5 ml ependorfs were labelled for each colony. 1,400 μl of broth was added to each eppendorfs. This was centrifuged for 3 mins and the broth removed leaving the pellet. This was resuspended in 100μl of TE pH 8. Then, 150μl of lysis buffer (1% Laurylcyclozine in NaOH) was added and the ependorfs inverted until the

solution went clear. Then 250µl of ammonium acetate was added and the ependorf once again inverted and then centrifuged at 13,000 rpm for 10 minutes. The supernatant was then pipetted off leaving the pellet in the pre-labelled ependorfs. To this, twice the volume of -20°C 70% ethanol with sodium acetate was added and the ependorfs inverted and left at -80°C for 60 minutes.

The ependorfs were then centrifuged for 30 minutes at 13,000 rpm. The supernatant (ethanol) was then pipetted off and the pellet was washed in 70% ethanol to remove the sodium acetate with great care being taken not to dislodge the pellet. If the pellet was dislodged the sample was re-centrifuged. The sample was allowed to dry to ensure that all the ethanol had evaporated from the sample. Once dry the pellet was resuspended in 21µl of TE pH 8 and the samples/minipreps were stored at -20°C.

The samples were analysed by 0.8% agrose gel electrophoresis to ascertain the efficiency of the extraction. A mix was made up containing 3µl of miniprep, 3µl of bromophenol loading die and 4µl of TE pH 8 and run on the agrose gel for analysis.

Analysis of purified constructs by restriction enzyme digestion

In order to ascertain that a DNA fragment of the correct size had been successfully incorporated into the plasmid, cloned and extracted, a restriction endonuclease reaction was performed. The pGEM®-T easy vector contains an Eco R I restriction enzyme site on either side of its ligation site therefore ,using Eco RI, the inserted fragment of DNA can be released.

A restriction digest was prepared by adding 3µl of miniprep, 5units of Eco RI, 1x buffer H and adjusting the final volume to 20µl with dH_20 and then mixing and centrifuging in a 0.5 ml ependorf. The digests were then incubated for 24 hrs at 37°C.

5µl of bromophenol loading dye was added to each digest and these were loaded into individual wells and analysed by PAGE electrophoresis. The minipreps containing the DNA fragments of the correct size were kept and stored at –20°C for later analysis by DNA sequencing.

DNA cycle sequencing

In order to ascertain the DNA sequence of the fragment of DNA cloned into the pGEM®-T easy vector commercial Thermo Sequenase radiolabelled terminator cycle sequencing kit and four Redivue™ ^{33}P labelled terminators (Amersham Life Science) were used. The kit was used in accordance with the instructions supplied by the manufacturer.

All reactions were carried out on ice unless otherwise stated. For each sequence reaction, four 0.5ml ependorf tubes were labelled A,C,G and T. To each tube 2µl of dGTP terminator mixture (7.5 µM dATP, cCTP, dGTP and dTTP) was added and O.5µl of the appropriate ^{33}P labelled dNTP was added as shown in table 15.

Table 15: Preparation of terminator reaction mix

	Tube A	Tube C	Tube G	Tube T
^{33}P labelled ddATP (µl)	0.5	–	–	–
^{33}P labelled ddCTP (µl)	–	0.5	–	–
^{33}P labelled ddGTP (µl)	–	–	0.5	–
^{33}P labelled ddTTP (µl)	–	–	–	0.5
Terminator mix (µl)	2	2	2	2

The content of the tube were carefully mixed by pipetting and capped.

The reaction mixture was prepared by combining the following: 2µl of 1 x reaction buffer, 3µl of plasmid DNA (miniprep DNA), 3µl of T7 primer, 2µl of Thermo Sequenase™DNA polymerase and 10µl of ddH_20 to make a final volume of 20µl. The mixture was thoroughly mixed by pipetting and briefly centrifuged. 4.5µl of this solution was then added to each of the four tubes containing the terminator mix and once again mixed by careful pipetting and briefly centrifuged. The reaction mixtures then underwent a PCR reaction under the conditions outlined below:

1x	94°C	3 minutes
35x	94°C	30 seconds
	56°C	30 seconds
	72°C	30 seconds
1x	72°C	2 minutes.

At the end of this reaction 4µl of stop solution was added to the reaction mixture. Each sample was heated at 94°C for 2 minutes and then placed on ice. 3µl of the sample was then analysed by running on a 6% polyacrylamide gel containing 7M urea. The DNA fragments were separated by electrophoresis, under careful supervision, for 2-5 hours at a constant power of 50W. The gel was then placed in a 10% methanol/10% acetic acid solution for 15-30 minutes to fix the DNA bands. The gel was then dried under vacuum onto filter paper.

The DNA sequence for the sample was read after autoradiographic exposure of the gel onto Kodak film over a 24-48 hour period. This then allowed comparison of the DNA sequence with that expected from previously published sequences in the literature in order to confirm the PCR specificity.

Section 3 Results

Chapter 7

Flexiscope trial results

This chapter provides a brief outline of the yield and results of the screening for the Portsmouth centre in the ICRF "Once only flexible sigmoidoscopy trial". This provides a background to the subjects from whom DNA was extracted and who were genotyped in the main study (details for the other centres are not available but the results of the initial findings for the whole trial are about to be published).

7.1 Recruitment in the Portsmouth centre

In total 36 General Practices in Portsmouth were recruited to the trial. All the General Practitioners approached agreed to take part. A total of 29,588 patients were identified as eligible from the practice lists. Of these, 555 patients were deemed unsuitable by their General Practitioners. Initial questionnaires regarding willingness to attend screening were therefore sent to 29,033 patients. The response rate to the questionnaire was 73%. Of the four possible responses 12,788 (44%) said they would definitely take part if invited, 3,855 (13%) indicated they would probably attend, 2,680 (9%) that they probably would not and 2,081 (7%) would definitely not attend the screening programme. Those who expressed a wish to definitely attend became the screening cohort and were randomised for screening. This was done in a ratio of 2:1 for

non-screening versus screening. Thus 12,180 patients were randomised, 4,077 being invited to attend screening and 8,103 being randomised to the control group. Of the 4,077 that were invited to participate in the screening program 2,958 attended a 73% compliance rate for this group.

7.2 Outcome for flexible sigmoidoscopy procedures in the Portsmouth centre

The average duration of the flexible sigmoidoscopy examination, including a digital rectal examination and therapeutic polypectomies if required was 8.5 minutes. This ranged from 1 minute to one procedure lasting 69 minutes. The average length of insertion of the 60cm flexible sigmoidoscope was 57cm, ranging from 10–60 cm. The bowel preparation was judged by the endoscopist to be excellent in 44% of examinations, good in 24%, average in 27% and poor in 2% requiring a repeat enema and procedure. The reported levels of pain for the procedure on the follow up questionnaire was none in 17%, mild pain in 52%, quite a lot of pain in 23% and severe pain in 4%.

The number of patients with polyps detected at flexible sigmoidoscopy was 804 (27%) and 417(14%) were adenomas. A total of 186 (6%) were classified as high risk by the previously outlined criteria and underwent colonoscopy. Of these 186 patients 20 (9%) were found to have more proximal polyps. There were 11(0.3%) cancers detected, 4 were removed endoscopically, 3 were Duke's stage A, 1 Duke's stage B and 3 Duke's stage C.

The following complications arose as a result of the screening trial. There was 1 bowel perforation requiring surgery following biopsy of a proximal polyp at colonoscopy.

Hospitalisation was required for 2 patients because of bleeding post polypectomy. Neither of the patients required transfusion and both settled with conservative treatment. A further 3 patients had rectal bleeding lasting more than 5 days but were not admitted to hospital. A further patient had profuse bleeding and returned to the endosocpy unit the next day and was rescoped and a bleeding polypectomy site was diathermied. Following their pre-procedure phosphate enema, 5 patients fainted.

7.3 Sample collection in the Portsmouth centre

Blood was taken off 583 screening volunteers with polyps and 334 screening volunteers with no polyps detected at flexible sigmoidoscopy. Of the 583 volunteers with polyps 338 had adenomatous histology (table 16). Of the total 417 patients with adenomas detected at screening blood samples were taken from 338 (81%) of patients. Blood was not obtained for several reasons. Firstly, sample collection started 4 months after the screening had started, as ethical approval had not yet been obtained. Secondly, if the screening sessions started to run behind time it was not always possible to collect samples for logistical reasons. Thirdly, volunteers occasionally did not want to donate a blood sample.

DNA that had been extracted from blood was also received and exchanged for analysis with the screening centres in Norwich and in Leeds.

Table 16: Histology of polyps removed on those with blood samples taken.

Histology	No of volunteers
Adenoma	228
Adenoma and Metaplastic	43
Tubulovillous	45
Tubulovillous and metaplastic	11
Metaplastic	188
Carcinoma	11
Carcinoid (all had adenomas as well)	3
Normal	43
Insufficient sample	3
Mucosal prolapse	1
Fibroepithelial	1
Inflammatory	5
Lymphoid	1

7.4 Results of food frequency questionnaire

The data from the food frequency questionnaire was pooled centrally at the Imperial Cancer Research Fund. This allowed analysis of the genotype data with data on exposure. In all five centres that were screening used the food frequency questionnaire

although only the three mentioned previously were doing genotype studies. A broad summary of the food frequency questionnaire data in relation to the risk of developing adenomas or metaplastic polyps is summarised in table 17.

Table 17 Results of food frequency questionnaire

	Odds Ratio (95 % CI) For Adenomas.	Odds Ratio (95 % CI) For Metaplastic Polyps.
Gender		
Females	1	1
Males	2.07 (1.84, 2.34)	1.57 (1.41, 1.75)
Age		
Age in Years	1.05 (1.03, 1.07)	1.03 (1.01, 1.04)
Hospital		
Newcastle	1	1
Leeds	1.19 (0.97, 1.46)	2.05 (1.69, 2.48)
Harrow	1.08 (0.88, 1.34)	1.21 (0.99, 1.48)
Norwich	0.99 (0.81, 1.22)	1.93 (1.59, 2.34)
Portsmouth	1.24 (1.01, 1.51)	1.25 (1.03, 1.53)
Smoking Status (Per day)		
Never Regular	1	1
Ex-Smoker	1.20 (1.06, 1.37)	1.77 (1.57, 1.99)
1-9 Cigarettes	2.30 (1.75, 3.01)	3.14 (2.44, 4.03)
10-19 Cigarettes	1.94 (1.58, 2.39)	4.69 (3.93, 5.59)
20 + Cigarettes	2.00 (1.62, 2.46)	5.15 (4.29, 6.19)
Total Alcohol (Portions per month)		
0-2	1	1
2-10	1.18 (0.99, 1.40)	0.91 (0.77, 1.06)
11-27	1.16 (0.98, 1.37)	1.20 (1.04, 1.38)
28+	1.36 (1.16, 1.59)	1.21 (1.05, 1.39)
Total Red Meat (Portions per month)		
0-5	Not Significant	1
6-13		1.09 (0.95, 1.27)
14-21		1.11 (0.96, 1.29)
22+		1.23 (1.06, 1.42)

7.5 Results of the smoking data collection

Smoking data was collected on the screening questionnaire but not initially from the start of the trial. Data was available on 25,000 out of 40,000 people from all the centres involved in screening. The smoking questionnaire asked about smoking habits, number of cigarettes smoked, years smoked and for ex smokers the year in which they stopped. Some of the results of the smoking data are summarised in table 17. It can be seen that smoking is a risk factor for both adenomas and metaplastic polyps. The more detailed smoking data was difficult to interpret at times, as there was inconsistency in the answers. Furthermore with regard to the genotyping number when already dealing with small subsets, multiple smoking categories resulted in very small numbers in the various groups. Three categories for the analyses were therefore used, current smokers, ex smokers and those that have never smoked. Of these 4,379, 17.61% were current smokers. A further 9,078, 36.52% were ex smokers and 11,403, 45.87% had never smoked.

Chapter 8 Results of laboratory studies for CYP2C subfamily

8.1 Selection of CYP2C mutations for analysis

All the known CYP2C subfamily gene mutations identified (see Chapter 4) were aligned (see Appendix C). Mutations were chosen on the basis of the ability to design specific primers to a mutation, the availability of a suitable restriction digestion enzyme and the likelihood of the base change resulting in an amino acid substitution which might affect protein structure or function.

Of the CYP2C8 mutations (table 10), an a to c base pair change at position 390 resulting in an N to T amino acid substitution was chosen and this was called **CYP2C8**.

Two of the CYP2C9 mutations (table 11) were selected for analysis. A c to t base pair change at position 430 resulting in a R to C amino acid substitution and an a to c base pair change at position 1075 leading to a I to L amino acid substitution. These two mutations were named **CYP2C9** and **CYP2C9** respectively.

Two of the CYP2C18 mutations (table 12) were also selected. A t to c base pair change at position 655 resulting in a F to L amino acid substitution and a t to c base pair change at position 1154 resulting in a T to M amino acid substitution. These two mutations are referred to as **CYP2C18** and **CYP2C18** respectively.

One of the CYP2C19 mutations (chapter 4) was chosen. A g to a base pair change at position 681 leading to the formation of an aberrant splice site, which results in the formation of a truncated 234 amino acid protein which lacks the main binding region and is therefore inactive. This mutation was named **CYP2C19.**

8.2 Primer design and optimisation

The primers were designed according to the methods outlined above and are summarised in table 18.

Table 18 Mutations and primers for selected CYP2C subfamily polymorphisms

Gene Name*	Mutation site	Position	Primer Sequence	Tm°C
2C8	a → c 390	328iD	5'taggaatcatttccagcaa 3'	48°
		410U	5'ccccatcccaaaattccccaa 3'	
2C9	a → c 1075	1051D	5'gtggtgcacgaggtccagatgt 3'	50°
		1138U	5'gatagtttctgaatttaatg 3'	
2C9	c → t 430	278D	5'agttttctggaagaggccattt 3'	58°
		454U	5'ggcagcgggcttcctcttg 3'	
2C18	t → c 1154	i7-77D	5'gataaaagagattggactagg 3'	58°
		1209u	5'gttggggaattctttgtcattgtgc 3'	
2C18	t → c 655	i4-13D	5'aaaaatctttaaggtctgcaat 3'	50°
		740U	5'ctctccaatacataacttttaatg 3'	
2C19	G → a 681	643D	5'atatgcaataatttcccactatc 3'	58°
		750U	5'ttcttttactttctccaaaatatc 3'	

* these codes in bold throughout the thesis refer to these specific mutations

The best conditions for each set of primers were standardised with variable amounts of $MgCl_2$ and DMSO as outlined in the methods. The results for the optimisation of each set of primers are illustrated in figs 2-7 and the conditions summarised in table19. The brightest band was selected and these primer conditions chosen. When the bands were of equal brightness, for ease, similar conditions were chosen, using no DMSO if possible and 4µl of $MgCl_2$ when suitable.

(see table 14 for concentrations of $MgCl_2$ and DMSO for figs 2-7)

Fig 2. **2C8** primer optimisation

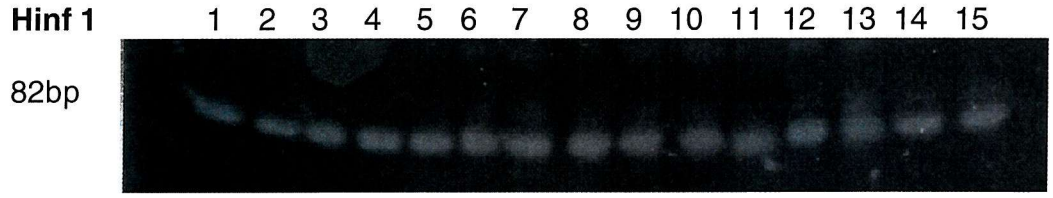


Fig 3. **2C9** primer optimisation

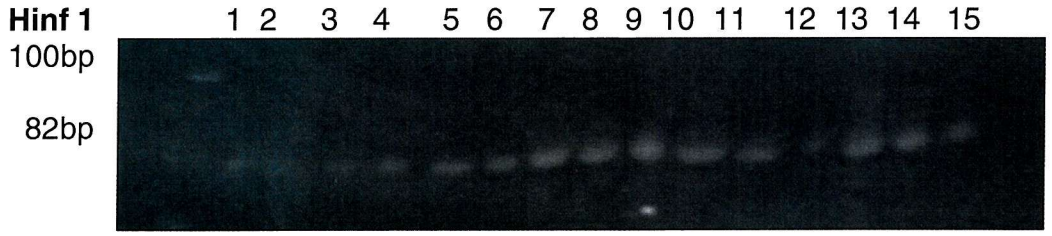


Fig 4. **2C9** primer optimisation

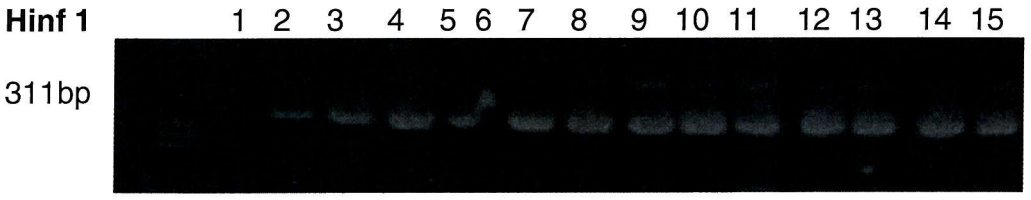


Fig 5. **2C18** primer optimisation

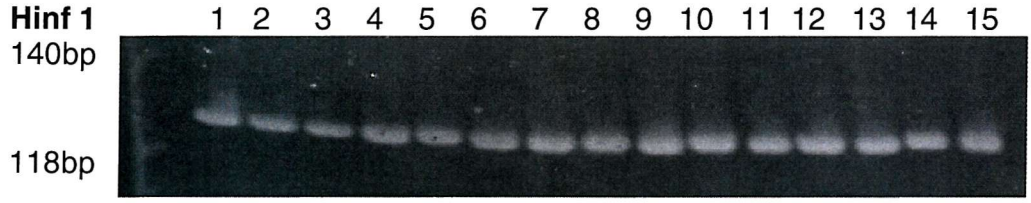


Fig 6. **2C<u>18</u>** primer optimisation

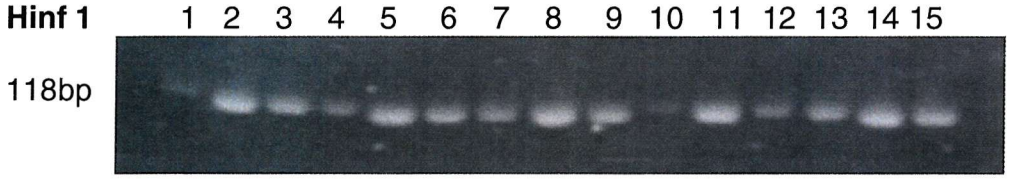


Fig 7. **2C19** primer optimisation

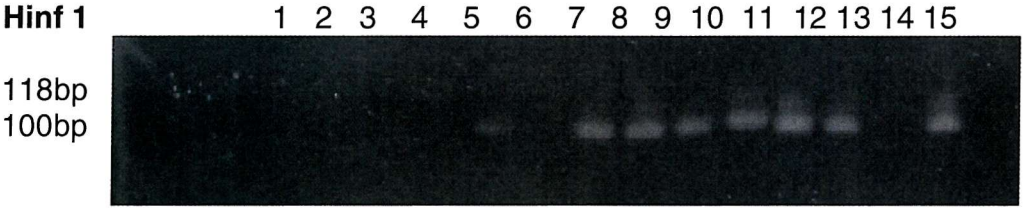


Table 19 PCR Conditions.

	2C8	**2C9**	**2C<u>9</u>**	**2C18**	**2C<u>18</u>**	**2C19**
Buffer A	5µl	5µl	5µl	5µl	5µl	5µl
Buffer B	4µl	7µl	4µl	4µl	4µl	6µl
ATP	1µl	1µl	1µl	1µl	1µl	1µl
CTP	1µl	1µl	1µl	1µl	1µl	1µl
GTP	1µl	1µl	1µl	1µl	1µl	1µl
TTP	1µl	1µl	1µl	1µl	1µl	1µl
DMSO	1µl	0µl	0µl	2µl	0µl	0µl
Primer1	1µl	0.5µl	0.25µl	1µl	1µl	1µl
Primer2	1µl	0.5µl	0.25µl	1µl	1µl	1µl
H2O	33µl	31µl	35.5µl	32µl	34µl	32µl
TAQ	0.1µl	0.1µl	0.1µl	0.1µl	0.1µl	0.1µl
Temp	50°C	50°C	58°C	58°C	54°C	58°C
DNA	1µl	2µl	1µl	1µl	1µl	1µl

8.3 Selection and optimisation of restriction endonucleases

The following restriction endonucleases were chosen and purchased from New England Biolabs, USA.: BSAJ1, BsrG1, AVAII, Nla III, Tsp509I and Sma1. Their recognition sites and digest product sizes are as in table 20.

Table 20 Restriction endonucleases.

Gene	Enzyme	Recognition site	Digest site	Uncut	cut	cuts
CYP2C8	BSAJ1	5'...C↓CNNGG...3' 3'...GGNNC↓C...5'	390	79	57+22	Hom
CYP2C9	BsrG1	5'...T↓GTACA...3' 3'...ACATG↓T...5'	1075	88	68+20	Wt
CYP2C9	AVAII	5'... G↓GA/TCC...3' 3'... CCT/AG↓G...5'	430	350	321+29	Wt
CYP2C18	Nla III	5'... CATG↓...3' 3'... ↓GTAC...5'	1054	137	83+54	Wt
CYP2C18	Tsp509I	5'... ↓AATT...3' 3'... TTAA↓...5'	655	112	86+26	Wt
CYP2C19	Sma1	5'... CCC↓GGG...3' 3'... GGG↓CCC...5'	681	108	70+38	Wt

For **CYP2C8** and **CYP2C9**, base pair alterations were introduced in the primers to allow the enzymes to work. For **CYP2C8** a c was introduced instead of a g, 4 base pairs down from the 3'end of the upstream primer. For **CYP2C9** a t was introduced instead of a g, 3 base pairs down from the 3'end of the downstream primer and a g was introduced instead of an a, 2 base pairs down from the 3' end of the downstream primer.

The concentrations and conditions under which the restriction endonucleases were used are summarised in table 21.

Table 21 Restriction endonucleases conditions

Gene	PCR	Buffer	Enzyme	H2O	BSA	TEMP	Mineral Oil
2C8	10	2µl, 2	2µl BSAJ1	6µl	0	60°C	yes
2C9	10	2µl, 2	0.5µl BsrG1	5.5µl	2µl	37°C	no
2C9	10	2µl, 4	0.5µl AVA II	7.5µl	0	37°C	no
2C18	10	2µl, 4	0.5µl Nla III	5.5µl	2µl	37°C	no
2C18	10	2µl, 1	1µl Tsp509I	7.0µl	0	65°C	yes
2C19	10	2µl, 4	0.5µl SmaI	7.5µl	0	25°C	no

8.4 Results of cloning and sequencing

Using the methods outlined in section 6.3.7 the PCR products were cloned and sequenced. The sequences for all six pcr products corresponded to the published sequences that were expected.

8.5 Results of PAGE analysis of DNA polymerase chain reaction

The PCR products were analysed using PAGE as described in Section 6.3.6. Examples of PAGE for **CYP2C8**, **CYP2C9**, **CYP2C9**, **CYP2C18**, **CYP2C18** and **CYP2C19** PCR products for 20 specimens are illustrated in figs 8-13.

Fig 8 **CYP2C8** PCR

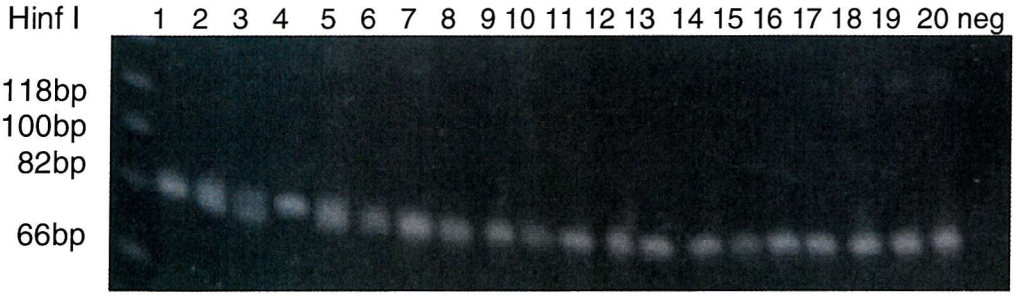


Fig 9 **CYP2C9** PCR

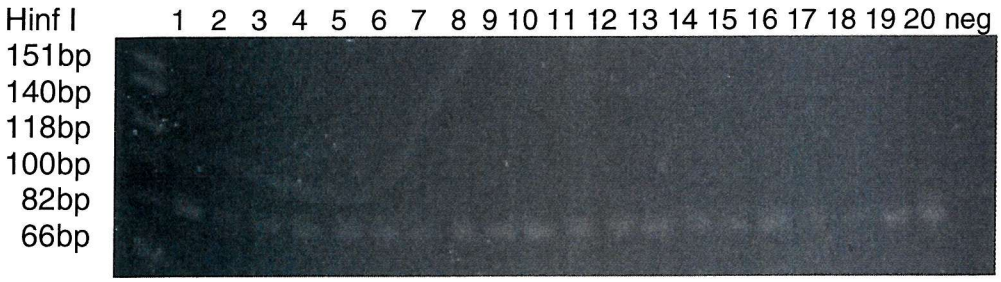


Fig 10 **CYP2C9**

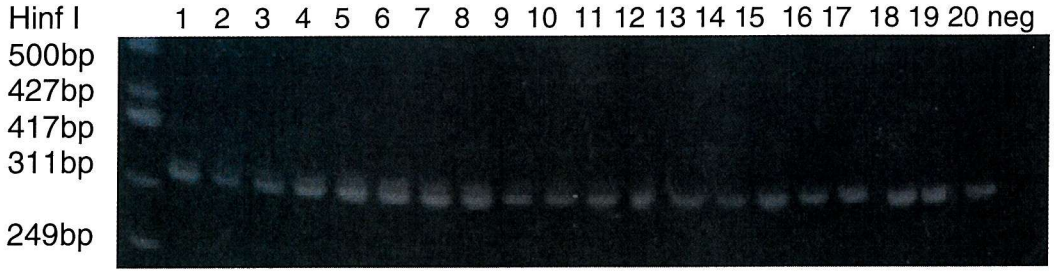


Fig 11 **CYP2C18**

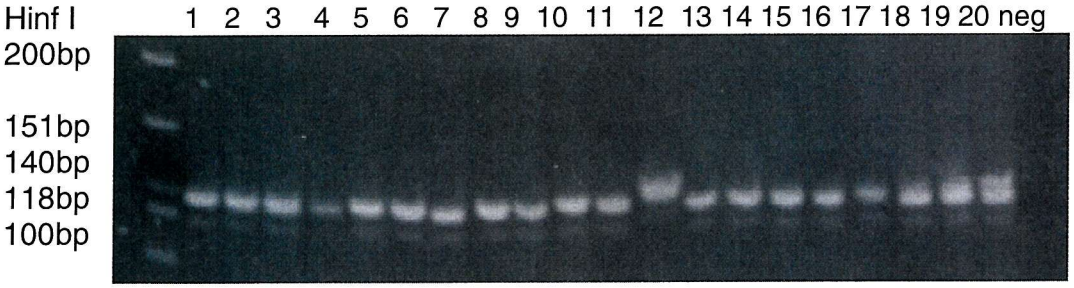


Fig 12 **CYP2C18**

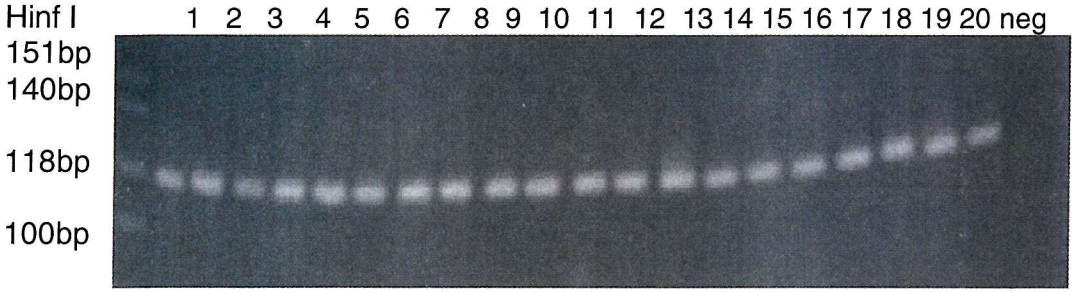
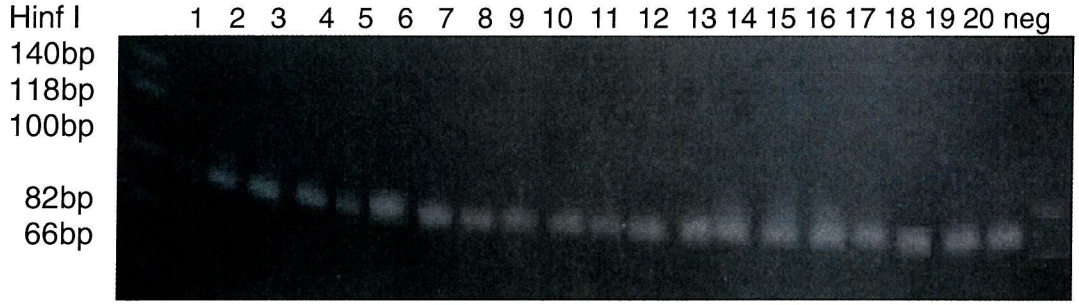


Fig 13 **CYP2C19**



8.6 Results of PCR restriction endonucleases digestion

The PCR products digests were analysed using PAGE as described in Section 6.3.6. Examples of PAGE for **CYP2C8**, **CYP2C9**, **CYP2C9**, **CYP2C18**, **CYP2C18** and **CYP2C19** PCR products digests for 20 specimens are illustrated in Fig 14-19.

Fig 14 **CYP2C8**

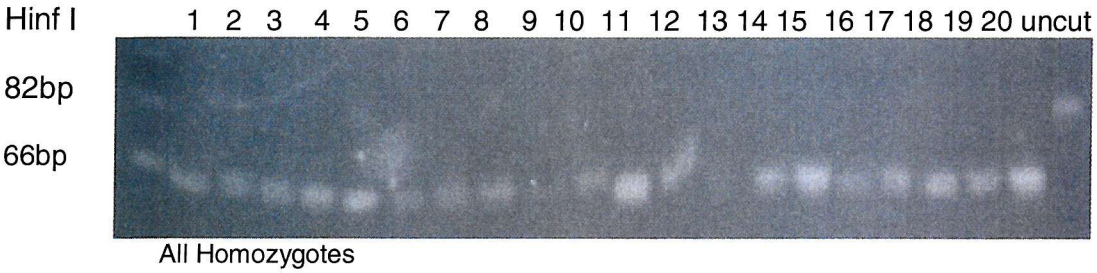


Fig 15 **CYP2C9**

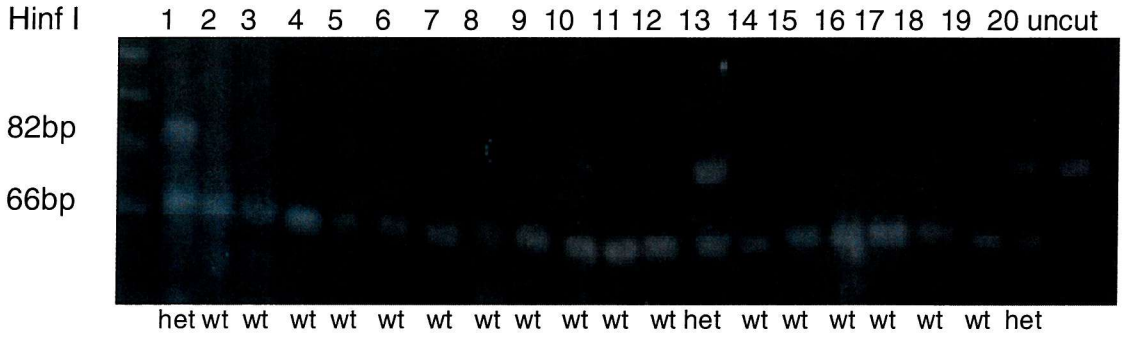


Fig 16 **CYP2C9**

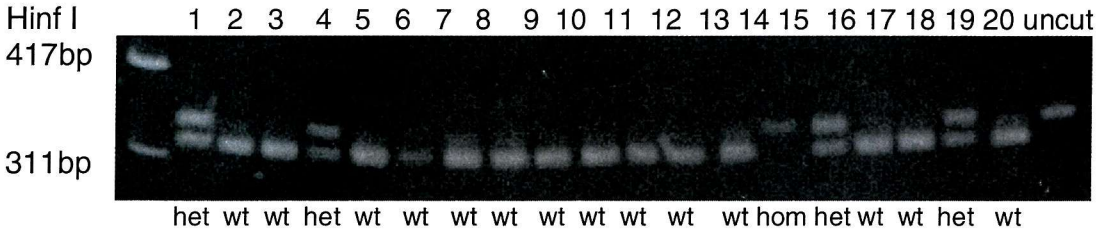


Fig 17 **CYP2C18**

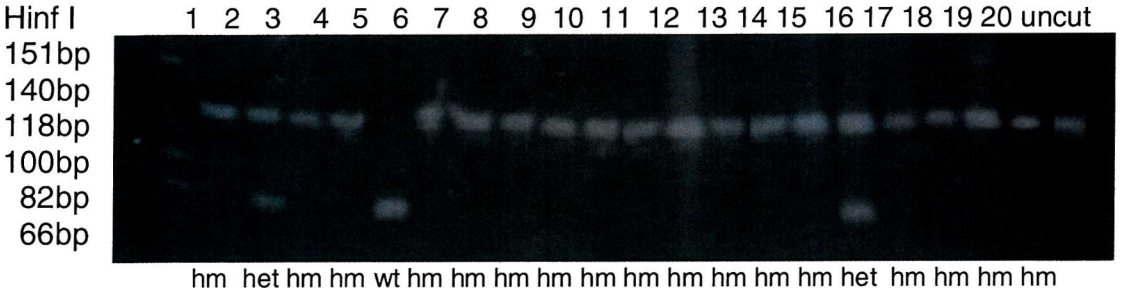


Fig 18 **CYP2C<u>18</u>**

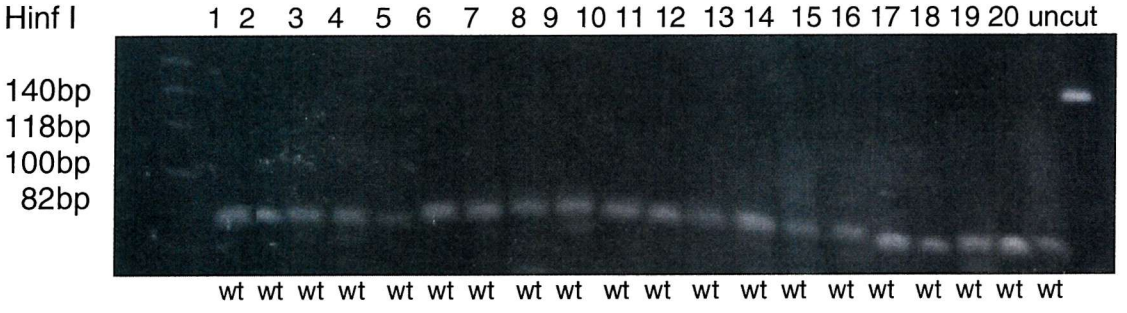
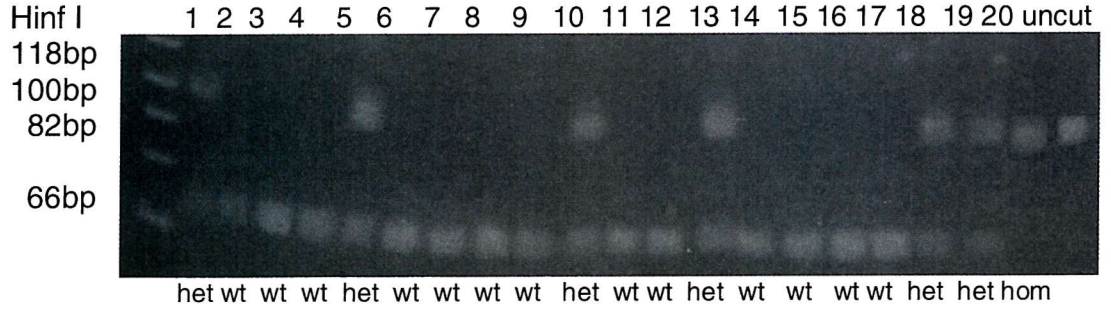


Fig 19 **CYP2C19**



Chapter 9 Results of genotyping for CYP2C subfamily

9.1 CYP2C subfamily mutations and allelic frequencies

As previously mentioned DNA from volunteers undergoing screening was obtained from three centres, Portsmouth, Leeds and Norwich/Cambridge. Table 22 provides a summary of the number of people genotyped for each CYP2C mutation and the number from each centre. The initial aim was to genotype all volunteers attending for screening in the three centres in whom an adenoma was found and an equivalent amount of controls. This proved locally to be too large an undertaking both logistically and financially. Those who were genotyped were selected in chronological order from when they were recruited.

Table 22: Number of people genotyped for each gene mutation according to centre

Mutation	Portsmouth	Leeds	Norwich	Total
CYP2C8	198	102	0	300
CYP2C9	395	190	277	862
CYP2C9	394	188	277	859
CYP2C18	395	191	277	863
CYP2C18	194	0	0	194
CYP2C19	392	190	277	859
Total	1969	862	1108	3939

The allelic frequencies for each mutation analysed, according to centre for the whole population genotyped, including cases and controls, are summarised in tables 23 -28 below.

Table 23 **CYP2C8** mutation allele frequencies

	WT	Hom	Het	Failed
Portsmouth	4 (2%)	187 (94%)	7 (4%)	0
Leeds	0	102 (100%)	0	0
Norwich	0	0	0	0
Total	4 (1%)	289 (97%)	7 (2%)	0

The **CYP2C8** mutation studied was in fact found to be the wild type in that what was thought to be the homozygote sequence was detected in 97% of cases. Because the other allele was so rare after looking at 300 samples no further genotyping for this mutation was performed. This will be looked at further in the discussion.

Table 24 **CYP2C9** mutation allele frequencies

	WT	Hom	Het	Failed
Portsmouth	350(89%)	1	43 (11%)	1
Leeds	167 (88%)	1	22 (12%)	0
Norwich	231 (83%)	0	43 (16%)	3 (1%)
Total	748 (87%)	2	108 (13%)	4

ChiSq = 3.074 df = 2, p = 0.216 (Excluding failed samples, homozygotes and heterozygotes combined)

Table 25 **CYP2C9** mutation allele frequencies

	WT	Hom	Het	Failed
Portsmouth	292 (74%)	9 (2%)	85 (22%)	8 (2%)
Leeds	137 (73%)	2 (1%)	48 (26%)	1
Norwich	203 (73%)	7 (3%)	67 (23%)	0
Total	632 (74%)	18 (2%)	200 (23%)	9 (1%)

ChiSq = 2.220 df = 4, p = 0.695 (Failed column not included)

Table 26 **CYP2C18** mutation allele frequencies

	WT	Hom	Het	Failed
Portsmouth	289 (73%)	12 (3%)	92 (23%)	2 (1%)
Leeds	141 (74%)	5 (3%)	45 (23%)	0
Norwich	209 (75%)	2 (1%)	66 (24%)	0
Total	639 (74%)	19 (2%)	203 (23%)	2 (1%)

ChiSq = 4.411 df = 4, p = 0.354 (Failed column not included)

Table 27 **CYP2C18** mutation allele frequencies

	WT	Hom	Het	Failed
Portsmouth	193 (100%)	0	0	1
Leeds	0	0	0	0
Norwich	0	0	0	0
Total	193 (100%)	0	0	1

As with **CYP2C8** the **CYP2C18** allelic mutation proved to be extremely rare and was not detected in 195 samples.

Table 28 **CYP2C19** mutation allele frequencies

	WT	Hom	Het	Failed
Portsmouth	278 (71%)	17 (4%)	97 (25%)	0
Leeds	134 (71%)	6 (3%)	50 (26%)	0
Norwich	198 (72%)	3 (1%)	76 (27%)	0
Total	610 (71%)	26 (3%)	223 (26%)	0

ChiSq = 6.171 df = 4, p = 0.188 (Failed column not included)

The allelic frequencies for the whole population were very similar, with no statistically significant difference between the centres. Comparison to reported rates in the literature will be made in the discussion.

9.2 CYP2C subfamily genotypes and predisposition to colorectal adenomas

In this section data, comparing the allelic frequency for cases i.e. those with adenomas, is compared with the allelic frequency in controls. As the mutations studied for **CYP2C8** and **CYP2C18** were so rare they will not be considered further. Tables 29-32 show the data for cases versus controls of the different alleles for **CYP2C9**, **CYP2C9**, **CYP2C18** and **CYP2C19**.

Table 29 **CYP2C9** cases vs controls

	WT	Hom	Het	Total
Cases	374 (87%)	0	57 (13%)	431
Control	374 (88%)	2	51 (12%)	427
Total	748	2	108	858

ChiSq = 2.315 df = 2, p = 0.315

Table 30 **CYP2C9** cases vs controls

	WT	Hom	Het	Total
Cases	299 (72%)	9 (2%)	110 (26%)	418
Control	333 (77%)	9 (2%)	90 (21%)	432
Total	632	18	200	850

ChiSq = 3.600 df = 2, p = 0.166

Table 31 **CYP2C18** cases vs controls

	WT	Hom	Het	Total
Cases	321 (74%)	13 (3%)	97 (23%)	431
Control	318 (74%)	6 (1%)	106 (25%)	430
Total	639	19	203	861

ChiSq = 2.991 df = 2, p = 0.225

Table 32 **CYP2C19** cases vs controls

	WT	Hom	Het	Total
Cases	311 (72%)	16 (4%)	104 (24%)	431
Control	299 (70%)	10 (2%)	119 (28%)	428
Total	600	26	223	859

ChiSq = 2.619 df = 2, p = 0.270

It can be seen that there is no statistically significant difference between cases and controls for any of the above CYP2C subfamily mutations. There is however some biological evidence, which will be discussed below, to combine the homozygotes and heterozygotes into one class and statistically it is sensible because of the limited number of homozygote mutants. The following tables 33-36 summarize the data between cases and controls for the mutations above but combining the homozygotes and heterozygotes.

Table 33 **CYP2C9** cases vs controls for phenotype

	WT	Hom/Het	Total
Cases	374 (87%)	57 (13%)	431
Control	374 (88%)	53 (12%)	427
Total	748	110	858

ChiSq = 0.127 df = 1, p = 0.722

Table 34 **CYP2C9** cases vs controls for phenotype

	WT	Hom/Het	Total
Cases	299 (72%)	119 (28%)	418
Control	333 (77%)	99 (23%)	432
Total	632	218	850

ChiSq = 3.434 df = 1, p = 0.064

Table 35 **CYP2C18** cases vs controls for phenotype

	WT	Hom/ Het	Total
Cases	321 (74%)	110 (26%)	431
Control	318 (74%)	112 (26%)	430
Total	639	222	861

ChiSq = 0.031 df = 1, p = 0.860

Table 36 **CYP2C19** cases vs controls for phenotype

	WT	Hom/Het	Total
Cases	311 (72%)	120 (28%)	431
Control	299 (70%)	129 (30%)	428
Total	610	249	859

ChiSq = 0.551 df = 1, p = 0.458

In this analysis the only mutation that comes close to significance is **CYP2C9**, which gives a p-value of 0.064 when the homozygote mutants and heterozygotes are

considered together. There is a plausible hypothesis to suggest that this may be a real effect although it does not reach significance. This will be addressed in the discussion.

9.3 CYP2C subfamily genotypes, environmental exposure and predisposition to colorectal adenomas

Comparing the genotypes of cases and controls, there was no difference between the genotypes for **CYP2C8, CYP2C9, CYP2C18, CYP2C18** and **CYP2C19** mutations studied. As mentioned in the introduction, environmental factors are important in the development of colorectal tumours. Similarly, environmental exposure will be important in determining the impact of xenobiotic metabolising enzyme mutations. Data from the food frequency questionnaire (Appendix B) was available for analysis with the mutations studied. This was performed centrally at the ICRF (Dr R Edwards). There was, however, no dietary interaction with the genetic polymorphism studied when analysed with the food frequency questionnaire data.

Data was also collected on screening attendees' smoking habits. In the hypothesis for the role of the CYP2C subfamily in colorectal tumorogenesis (Chapter 4 section 4.8), a role was postulated based on the altered metabolism of benz[a]pyrene a potent carcinogen found in cigarette smoke. For **CYP2C9** when the heterozygotes and homozygotes were combined, this group was found to be more common amongst the cases, those with adenomas. This did not though reach significance with a p value of 0.064 (table 34). Therefore the frequency of the **CYP2C9** alleles in current, ex and never smokers in cases (table 37) and controls (table 38) was examined.

Table 37 **CYP2C9** cases and smoking

Cases				
CYP2C9	Current	Ex-smoker	Never smoked	Total
Het/hom	26 (7%)	42 (12%)	35 (9%)	103 (28%)
Wt	69 (19%)	107 (30%)	82 (23%)	258 (72%)
Total	95 (26%)	149 (42%)	117 (32%)	361 (100%)

Table 38 **CYP2C9** controls and smoking

Controls				
CYP2C9	Current	Ex-smoker	Never smoked	Total
Het/Hom	11 (3%)	39 (10%)	43 (11%)	93 (24%)
Wt	31 (8%)	116 (29%)	156 (39%)	303 (76%)
Total	42 (11%)	155 (39%)	199 (50%)	396 (100%)

Data on smoking is available on 757 individuals out of 850 of those genotyped for **CYP2C9**, so the study population is smaller. It is clear from these tables that smoking itself is a major risk factor for adenomas. From the data, 26% of cases were current smokers and 32% had never smoked. For the controls, 11% were current smokers versus 50% who had never smoked. This difference is highly significant statistically ($p > 0.0001$). Looking at the impact of **CYP2C9** genotype on adenoma risk in current smokers, 26 out of 95 (27%) of current smokers with adenomas were either het of hom for **CYP2C9**. For the control population 11 out of 42 (26%) of current smokers were either het of hom for **CYP2C9**. There clearly is no statistical difference between these

two groups (p=0.886). The numbers are however small, as the overall population is smaller and smoking is such a strong risk factor for adenomas that there are relatively few controls who are current smokers. The adenoma-carcinoma sequence is thought to occur over 5-10 years so being an ex smoker may be a risk factor for developing adenomas. Looking at the above data 42% of ex smokers developed an adenoma compared to 39% of controls. When compared to non smokers, ex smokers have a significantly higher risk of an adenoma (p=0.03). However, **CYP2C9** genotype has no effect on the risk of adenoma development for ex smokers. Out of 149 ex smokers with adenomas 42 (28%) were either het or hom for **CYP2C9**, whilst out of 155 ex smokers in the control group, 39 (25%) were either het of hom for **CYP2C9** (p=0.551). Finally looking at the effect of **CYP2C9** genotype on adenoma risk in non smokers, 35 out of 117 (30%) of non smokers with adenomas were either het or hom for **CYP2C9**, whilst 43 out of 199 (22%) of non smokers in the control group were either het or hom for **CYP2C9** (p=0.099). Thus in non smokers with adenomas the **CYP2C9** het or hom genotype is more common although this does not reach statistical significance. Contrary to the proposed hypothesis, this could suggest **CYP2C9** may have a role in predisposing to adenomas via alternative pathways than those involved in smoking. The interaction between smoking and **CYP2C9** is examined in greater detail in the discussion.

Section 4 Discussion and conclusions

Chapter 10 Flexiscope trial and sample collection

10.1 Flexiscope trial

The thesis was specifically set up because of the author's participation in the flexiscope screening trial. The project was designed bearing in mind that the samples from which the DNA was obtained would by definition be from an average risk group for colorectal cancer. We could not look at the known colorectal cancer mutations such as APC, DDC and K-Ras as these would not be present in the somatic DNA extracted from the blood of average risk individuals. It was therefore decided to focus on xenobiotic metabolising enzymes whose genes are known to be polymorphic and for whom there is some evidence that they may be involved in predisposition to colorectal cancer and adenomas (Chapter 3).

All the samples were obtained from volunteers attending for screening, taking blood of those with polyps and an equal number of controls. The age group, 55-64, selected for screening were those at average risk for colorectal cancer but at an age when that group would be expected to have a high yield of adenomas but a low pick up for cancer. Thus genotyping was performed on individuals with colorectal adenomas, not cancers. There is however strong evidence that the groups share similar risk factors and good evidence for the adenoma-carcinoma sequence (Chapter 2).

There were several areas that may have introduced degrees of bias into the study population. Firstly, the flexiscope trial used a two-tier recruitment system where

potentially eligible participants were entered into the trial only if they expressed an interest in screening. This strategy was used to minimise costs and to maximise the power to provide an assessment of the benefits of screening. The group from which DNA was acquired may therefore not necessarily have the same disease patterns and exposure to risk factors as the general population. Individuals who present for screening may be of higher social class, may be more health aware and less exposed to CRC risk factors such as smoking alcohol and diets high in meat and lower in fibre. This might dilute the effect of XME polymorphisms within the screened population.

Secondly, not all patients with adenomas had blood taken for analysis (19%). The reasons for this have been listed above and are mainly logistical in that the screening program started before ethical approval had been granted to take blood. Also rarely patients did not wish to give a blood sample and occasionally time restraints did not allow time to take samples. If however, the number of blood samples taken of those with non adenomatous histology from the whole population screened is examined, the following is observed. There were 245 blood samples taken from 387 patients with non adenomatous histology of which the vast majority were metaplastic polyps. Thus, 63% of people with a non adenomatous polyps had a sample taken. In contrast 417 patients had histologically proven adenomas and samples were taken from 338 patients 81%. Metaplastic polyps tend to be smaller and whiter that adenomatous polyps and endoscopically with practice it becomes easier to decide which polyps are most likely to be adenomas rather than metaplastic. Obviously, the interest for the purpose of the study was in adenomas rather than metaplastic polyps. There may be a bias therefore to larger adenomas as samples may not have been taken on polyps thought to be metaplastic at endoscopy that were adenomatous on histology.

Thirdly, a further minor area of contamination may have occurred in the control population as they only underwent a flexible sigmoidoscopy and did not have the rest of their colon examined. In fact as mentioned in chapter 5, colonoscopy was only performed on those with adenomas defined as high risk. This was decided from data suggesting that with a small adenoma less than 1cm in the distal colon the chance of developing a right sided malignancy is 4% (48). This would suggest that the risk of a proximal adenoma in those with a negative flexible sigmoidoscopy is small and unlikely to introduce significant bias. Within the trial, a small study was conducted using a full colonoscopy for screening those with a family history. From this, the approximate prevalence of proximal adenomas can be estimated in the controls with no adenomas from the family history volunteers who underwent colonoscopy. There were 342 such individuals of whom 26 (7.6%) had a proximal adenoma and no distal high risk adenoma. 21 (6.1%) had proximal adenomas and no distal adenomas at all. The latter is the figure that would give the likely prevalence of proximal adenomas in the controls although this was in a group with a family history who may be at increased risk of proximal polyps (personnal communication Dr W Atkin).

10.2 Sample collection

Between the three centres Portsmouth, Leeds and Cambridge/Norwich blood samples have been taken and DNA extracted blood on 1,043 patients with adenomas and an equal number of controls. An aliquot of DNA from all patients has been stored centrally at the ICRF. This resource is available for further work on polymorphisms. All data is also being recorded centrally at the ICRF to allow for gene-gene interaction analysis

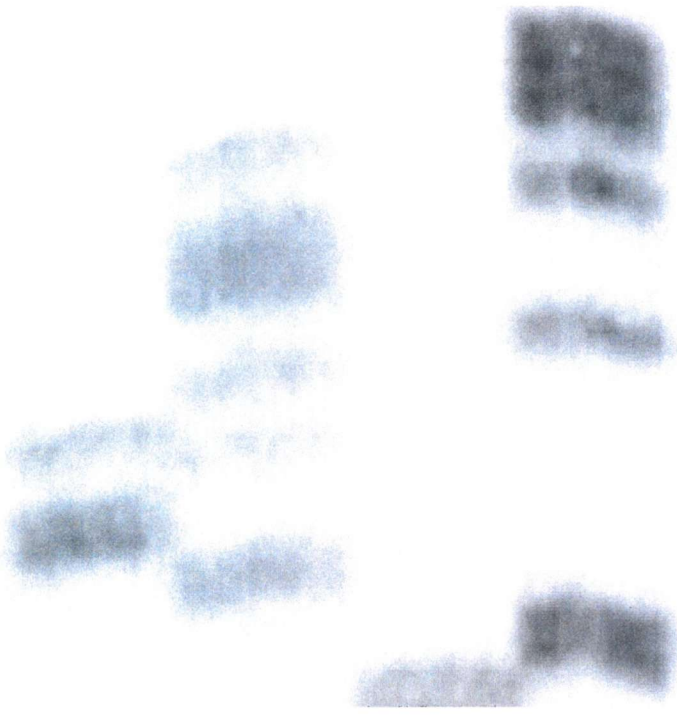
and gene-exposure analysis.

Chapter 11 Cytochrome P4502C subfamily mutations

11.1 Cytochrome P4502C8

A mutation in the cytochrome P4502C8 gene at position 390 was studied. The mutation results from a nucleotide a to c base pair change resulting in an N to T acid substitution. The original reported sequence, which was taken to be the cytochrome P4502C8 wild type, was described by Okino (168). The sequence was discovered using a rabbit liver progesterone –21-hydroxylase P4501 cDNA as a probe to identify a highly homologous liver cDNA. The cDNA was sequenced and found to be 82% homologous to s-mephenytoin 4-hydroxylase (CYP2C9) and called HP1-1. Ged reported two further cDNAs that differed by four base pair changes from HP1-1, MP-12 and MP-20 (164). They both had a c at position 390 and this mutation was studied. Of the genotypes performed, 97% were the same as that described by Ged et al. The DNA product was therefore sequenced and a c base pair was found at position 390 rather than an a. Hence this sequence should be regarded as the wild type (Fig 20). Of the 302 samples genotyped, 3% were homozygote or heterozygote for the a base pair mutation. The digest was slightly unreliable, as it had to be done at 60°C under oil and also required the primer to alter the sequence to allow a cut. Whether the a base pair mutation at 390 truly exists is not clear and none of the heterozygote or homozygote products were sequenced.

Fig 20: **CYP2C8** sequence



Base pairs 375-394 tttctcctcacaa**c**cttg

11.2 Cytochrome P4502C9

Two cytochrome P4502C9 mutations were studied, a c to t base pair change at position 430 resulting in a R to C amino acid substitution, and an a to c base pair change at position 1075 leading to a I to L amino acid substitution.

The reported frequency of the C amino acid allele varies between studies. Bhasker found a 22% heterozygote rate for C variant allele in 18 patients studied with no homozygotes (183). Sullivan-Klose (180) found a C variant allele frequency of 0.08 in 100 Caucasian Americans and a similar allele frequency of 0.107 was reported by Yasar in 430 healthy Swedish volunteers (184). Of the total population, 74% were wild

type for the R variant, 24% were heterozygotes with an R and a C allele and 2% were homozygote with two C alleles. This gave a C allele frequency of 0.1386 slightly higher than that reported by Yasar and a Hardy-Weinberg P value of 0.92.

The L amino acid allele is less frequent with a reported allele frequency of 0.074 (184) and 0.06 (185). Bhasker (183) found one heterozygote in 18 patients genotyped (5.5%). Of the total population genotyped, 87% were wild type for the I allele variant, 12.8% were heterozygotes with an L and an I allele and 0.22% were homozygote with two L alleles. This gave an L allele frequency of 0.0664, similar to that reported above and a Hardy-Weinberg P value of 0.60.

11.3 Cytochrome P4502C18

The two cytochrome P4502C18 mutations selected were a t to c base pair change at position 655 resulting in an F to L amino acid substitution and a t to c base pair change at position 1154 resulting in a T to M amino acid substitution. For the first mutation selected only one heterozygote in 193 samples genotyped was found. This mutation was only reported in one paper and was a clone isolated from a cDNA library (186). No other authors reported this mutation and it may either be a rare mutation rather than a true polymorphism, or may be the result of a sequencing error. This gene was not sequenced.

For the second mutation, 74.1% were wild type with two T alleles, 23.7% were heterozygote with a T and an M allele and 2.2% were homozygotes with two M alleles. This gave an M allele frequency of 0.1404 and a Hardy-Weinberg p value of 0.89. No reported data on the allele frequencies for this mutation was found in the literature.

11.4 Cytochrome P4502C19

The cytochromeP4502C19 mutation studied was a g to a base pair change at position 621 leading to the formation of an aberrant splice site, which results in the formation of a truncated 234 amino acid protein which lacks the main binding region and is therefore inactive. Of the total population, 71% had the wild type with a g base pair on both alleles and that 26% were heterozygotes with a g base pair allele and an a base pair allele and 3% were homozygotes with an a base pair on both alleles. This gave us an allele frequency for the a base pair allele of 0.1621 and a Hardy-Weinberg p value of 0.75. This is similar to that reported in the literature. Sagar reported a heterozygote rate of 22.4% and a homozygote rate of 2.8% in 143 Caucasians (211). A study of the frequency of this mutation in different ethnic groups found an allele frequency of 0.13 for European Americans (212).

No statistically significant differences in genotype frequencies between the populations from the three different centres from which blood samples were received were found (Tables 23-28).

Chapter 12 Cytochrome P4502C subfamily mutations, predisposition to colonic adenomas and environmental exposure

12.1 Cytochrome P4502C mutations and predisposition to colonic adenomas

There was no difference in the frequencies of **CYP2C9, CYP2C18** and **CYP2C19** polymorphisms studied between cases and controls. Over 400 cases and 400 controls for each mutation were studied and therefore it is unlikely that these polymorphisms are important in colorectal tumorogenesis.

For the **CYP2C9** polymorphism the frequency of the wild type with two R amino acids was compared to the frequency of both the heterozygotes with an R and a C allele combined with the homozygotes with two C alleles. The rationale for combining these groups is that there is evidence that the C amino acid allele has a dominant effect and that the enzyme activity of heterozygotes and homozygotes may be similar. Studying liver RNA, there appears to be a 5-10 fold preferential expression of the C allele versus the R allele in heterozygous individuals suggesting that the C allele may exert a dominant effect (183). CYP2C9 is the principal enzyme involved in the metabolism of Warfarin in the human liver (3) and it has been shown that heterozygotes with an R and a C allele require significantly lower doses of Warfarin than those with the normal wild type with two R alleles.

There were more of the combined heterozygote/homozygote group in those with adenomas than in the controls (table 34). This difference however did not reach statistical significance (p=0.064). The odds ratio for the combined het/hom **CYP2C9** group was 1.34 (95% ci 0.98 - 1.82). There is evidence that this polymorphism has a

dominant effect and that it may play a role in benz[a]pyrene metabolism a known smoking carcinogen. Smoking has been demonstrated to predispose to adenomas in the smoking data (table 17) and others have reported similar findings. Thus, there is a hypothesis to suggest a possible role for the **CYP2C9** polymorphism and predisposition to colorectal adenomas. The effect would however appear to be small as it has not reached significance despite looking at 850 samples. If however this is a true effect it may well be important as colorectal adenomas are common having been found in 14% of 55-64 years olds screened in Portsmouth.

12.2 Cytochrome P4502C mutations, colonic adenomas and exposure

The results from the FFQ showed that alcohol was the only dietary risk factor for adenomas (table 17). High consumption of meat (greater than 22 portions a week) was found to be a risk factor for metaplastic polyps but not adenomas. Various dietary factors in relation to all the CYP2C subfamilies mutations studied were examined but as expected no interaction with any of the dietary factors was found. This analysis was done centrally at the ICRF (Dr R Edwards).

From the smoking data collected on the screening forms it has been shown that smoking predispose to distal colorectal adenomas. Furthermore this would appear to be dose dependent as the risk increases with the number of cigarettes smoked (table 17). There was a particular interest in looking at the effect of smoking on adenoma risk in those with the **CYP2C9** polymorphism. This data has been summarised in section 9.3 (tables 37&38). Smoking data on all those genotyped was not available and therefore this reduced the size of the study population from 850 to 757. Furthermore with small

subgroups, the population was divided into three fairly crude categories, smokers, ex smokers and non smokers. There was no statistically significant difference between non smokers and current smokers and no difference between ex smokers and non smokers with regard to **CYP2C9** genotype and predisposition to distal colorectal adenomas. Due to the small numbers in the three smoking categories the smoker and ex smoker groups were combined and this gave an odds ratio of developing an adenoma in a smoker or ex smoker who is a heterozygote or homozygote for the **CYP2C9** polymorphism of 1.39 (ci 0.89-2.15 p=0.14). Thus there is no statistically significant difference for adenoma risk when smoking exposure and **CYP2C9** genotype are combined. In table 39 the percentage of heterozygotes and homozygotes for **CYP2C9** in relation to smoking status for cases and controls are shown. None of the differences reach significance but it can be seen that there is a trend to more of the het/hom group in all three smoking categories for cases and the lowest number of the het/hom **CYP2C9** group was in the non smoking controls. The problem for the smoking analysis is that the

Table 39 Percentage of **CYP2C9** heterozygotes and homozygotes.

	Smokers	Ex smokers	Non smokers	Study population
Cases	27% (26/95)	28% (42/149)	30% (35/117)	28% (119/418)
Controls	26% (11/42)	25% (39/155)	22% (43/199)	23% (99/432)

numbers are small as only 17% of the screened population were current smokers (section 7.5). Smoking is a risk factor for adenomas as discussed above and hence 26% of cases (95/361) were current smokers. In contrast only 11% (42/396) of controls

were current smokers. The numbers in the subgroups are thus small and if the het/hom **CYP2C9** genotype polymorphism does indeed contributes to smoking carcinogen metabolism pathways it would require much larger numbers to reach significance for a small effect. This effect, should it exist, even if small may be important as smoking and adenomas are common in the population as a whole. In table 39, the biggest difference in percentage between the het/hom **CYP2C9** groups is seen in non smokers, 30% vs 22%. As mentioned in section 9.3, this difference does not reach significance (p=0.099). The trend could however suggest that should the **CYP2C9** polymorphism have a role in adenoma predisposition, it may not be via altered metabolism of smoking carcinogens and alternative xenobiotics and pathways could mediate this. As an aside, in pondering smoking interaction it is interesting to remember that enzymes involved in the metabolism of smoking carcinogens can also affect the likelihood and the amount of cigarettes smoked (CYP2A6 see section 1.3.2).

Chapter 13 Summary

The wild type base pair sequence for CYP2C8 at position 390 is a c base pair rather than an a base pair. This was confirmed by sequencing. A few heterozygotes and homozygotes (11/300) were found but this would not be frequent enough to be referred to as a true polymorphism. The digest used required the wild type to cut and had to introduce a base pair change with the primer for the restriction endonuclease to cut. This may have introduced error and possibly account for the 11 out of 300 heterozygotes and homozygotes. In further work it would be worth sequencing some of the heterozygotes and homozygotes to establish whether this base pair change does really exist. The frequencies for the two CYP2C9 polymorphisms studied were found to be similar to those published in the literature. For CYP2C18 one of the mutations did not appear to be a true polymorphism and the other studied, as with the CYP2C19 mutation did not appear to predispose to colorectal adenomas. The addition of the dietary FFQ exposure data did not change the results with regard to the CYP2C subfamily polymorphisms studied and predisposition to distal colorectal adenomas. There is evidence that the alteration in enzyme activity induced by the **CYP2C9** polymorphism has a dominant heterozygote effect similar to that seen in homozygotes. Thus, there is a biological rationale to combine these two groups. There were more colorectal adenomas in the combined heterozygote and homozygote group for the **CYP2C9** polymorphism studied, although this difference did not reach significance (p=0.064). CYP2C9 may have a role in benz[a]pyrene metabolism and hence a possible link between **CYP2C9** and smoking has been postulated. The smoking data did not support this hypothesis but the numbers in the subgroups were small. In further studies

it would be interesting to investigate the **CYP2C9** polymorphism and predisposition to colorectal cancer rather than adenomas. There is also a reported six base pair deletion for CYP2C9 (177) resulting in an enzyme with lower activity. Unfortunately it is at the beginning of an exon and the proceeding intron sequence was unknown and hence not suitable for RFLP work. This polymorphism may warrant further study with regard to colorectal tumourogensis.

This work has contributed to the creation of a DNA database of around 2,000 adenoma cases and controls with exposure data on most of this population. Central pooling of the genotype results from the three centres involved in collecting DNA and from on going studies will allow further analysis of gene-exposure and gene-gene interaction. This is important in that it appears unlikely that single xenobiotic metabolism enzyme polymorphisms on their own will result in a high risk of colorectal tumorogenesis and hence provide a biomarker for this disease (Chapter 3). The combination, however, of different phase I and phase II enzyme polymorphisms within individuals with certain exposures may highlight people at increased risk of colorectal adenomas who may benefit from screening to reduce their risk of developing colorectal cancer.

Appendices

Appendix A

Consent form for a blood test.

We are doing research into genetic aspects of bowel disease and would like blood from people with and without polyps.
We are also looking at whether exposure to certain bacteria in the bowel may increase the risk of polyps.
This is purely for research and the results will have no future bearing on any care you receive and we will not inform you about any of the test results.
We would be very grateful for your cooperation, the blood test can be done today or you can return another day.

Name:

DOB:

I agree to having a blood test which will be used for research only.

Signature:

Appendix B food frequency questionnaire

FlexiScope *trial* **Food Frequency Questionnaire**

Please estimate your average food use as best you can, and please answer every question do not leave ANY lines blank.
PLEASE PUT A CROSS (*x*) ON EVERY LINE

	FOODS	AVERAGE USE LAST YEAR								
		Never or less than once per month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
	Vegetables, salads (all types) **[EXAMPLE]**				✗					
VEGETABLES	**Vegetables, salads (all types)**									
	Broccoli, spring greens, kale									
	Brussels, sprouts (in season)									
	Cabbage, cauliflower									
	Peas									
	Leeks, onions, garlic									
	Brown or wholemeal bread or rolls									
	White bread or rolls									
BEVERAGES	**Tea (cup)**									
	Coffee, instant or ground (cup)									
	Coffee, decaffeinated (cup)									
	Wine (glass)									
	Beer, lager or cider (half pint)									
	Port, sherry, vermouth, liqueurs (glass)									
	Spirits eg. gin, brandy, whisky, vodka									
	Fish (all types)									
MEATS	**Meat (all types)**									
	Beef: roast, steak, mince, stew, casserole									
	Beefburgers									
	Pork: roast, chops, stew, slices									
	Lamb: roast, chops, stew, slices									
	Chicken or other poultry eg. turkey									
	Bacon, ham									
	Corned beef, spam, luncheon meats									
	Sausages									
	Savoury pies eg. meat pie, pork pie									
		Never or less than once per month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Has your consumption of any of the following meats changed since the Beef scare (March 1996)? Yes ☐ No ☐

If **YES**, please complete the next box for **all** the items listed below prior to that time

	FOODS	AVERAGE USE BEFORE BEEF SCARE (March 1996)								
		Never or less than once per month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
MEATS	**Beef: roast, steak, mince, stew, casserole**									
	Beefburgers									
	Chicken or other poultry e.g. turkey									
	Corned beef, spam, luncheon meats									
	Sausages									
	Savoury pies e.g. meat pie, pork pie									

SIG/FFQ/2/198

How often do you eat grilled, fried or barbecued foods? ☐☐ times a week

How well cooked do you usually have grilled, fried or barbecued meats/ chicken/ fish?

Well done/dark brown ☐ Medium ☐ Lightly cooked/rare ☐ Did not eat them ☐

Have you taken any vitamins, minerals, fish oils, fibre or other food supplements during the past year?

Yes ☐ No ☐ Don't know ☐

If YES, please list any vitamins, minerals, fish oils, fibre or other food supplements you have taken during the past year

Vitamins, Minerals, Fish oils, Fibre, Other food supplements	Average frequency Please tick **one** box per line to show how often on average you consumed supplements									
Name and brand Please list full name, **brand** and strength	**Dose** Please state number of pills, capsules or teaspoons consumed	Never or less than once a month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
	Please state number of pills, capsules or teaspoons consumed	Never or less than once a month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Thank you for your help

Please affix addressograph label here

Appendix C

Alignment of CYP2C subfamily mutations

```
C8                                                  tataggatagggtattt
C8     cactgaaccaccaagcaggaaaactgggattctaatacaaaatcttttactggtgcattg
C8     ataatgctctaactcactgagtcaccaattgctcattcctgaaaaacaaagcaaaattaa
C8     attagtagatctcagagatcccgtctgtctttaaattatctatgttccttttattctata
C8     aaaagaaaggtcaaggcaggagctcagctcaggagaagaaacaaggagcagagcaaggac
C8     aactgtttctcaaggaataaaattattgctctaaagagagaagtgaacttatttttatcca
C8     aataaactagttatacctacgtgagtgaggcagcaaattactacttccctttgccctgga
C8     taaagggttcaccaggacctggactcactcacctttaaaggttataaaaccaaacacgt
C8     ctgacccacattttactcaactggtgctagaattattaactaaattaatgtttatttga
C8     aagtcactgattagattaatccacaagtattgaattttagtcaatcttggtggcccggtt
C8     taactggatgttttgcttaaaaggaaggcagcaagatgcaggggttatggtttccagccc
C8     cagcttggtcacttgcattctgtgtgtccttagctaaagtactgaatctccatggtctaa
C8     ctttctcctctctaaactgggaataattttacagtgggcaaagataattgagagaataaa
C8     aagagatgtgatgagtgtgaaaattctctgtaaatttgtcataatgtctataaacataat
C8     cgataaaacattgtataactgggtctaatattttcttaatgaaagagctggaaataactg
C8     tactggtcaatttagaataaaggtaatctttcagagcatgccctttgtatacacactttg
C8     ttattagtgatctagtaatgttcataaatccagttgtatttagatcttcatgaccattga
C8     ctatcagttcccatttcaggtctgcacattgcagtggttctgtgccctgggtccattcag
C8     tgatttccctgtgttccatcttctgttgaatccacaactgttgttctgtgtataatttct
C8     cttccttgctgtgtatgattacattctattatttgtaacaataacagaccaaaaacaata
C8     gaagcagccatgtctggaggtgactggaaggtggagaagccatagattttcaagccctgt
C8     gccataaattatgtgagattggccctttccttaatagtgctgaacaactttcacttgtaa
C8     ggtgatgcagaggggagaactctaatttttatttcttcttttgagcgtctccggtcctct
C8     tatccttataaacaaataacggacttctatttaatgtgaagcctgttgctttctgaacag
C8     agtcaaggtggcgtatcttcagagtaactaatgtctggggtttgttttgtttttctaaaa
C8     ttgttcttgagccagctgtggtgtaagtggtaatgaacccaatgggtatcagaagatctc
29c                                       cctggtgtttccacccctccttccatt
C8     tgctcaaatccggttttaccggcaatgagctgtgtggcactgacaggtgtcctgttctcc
29c    gcgcgtgtgggctcttagtctaagccactccatattgatttatttcccttcctgcgcatg
C8     cagagtttctttcccaatttgaaaaataaaaaatgataatctttatactccagtctcttt
29c    tgttaaaggtaggaatttttcactgtgggcatatttaggcaagctccctgtgcaagttcc
C8     taatgatgaatatacatttatatatatacttttatatatttaatataatatttaatagta
29c    cttatctgcacaaaacatctagtgtaagtacttggggtttttgtggattgggcaatgacc
C8     taaatatgtatttatgttattattatgtaataatgtatgtaacactccctgctaattcag
29c    tggaaggttggttggaggttctctggggacccttcccttactgcctgcctaatgcaagct
C8     tttgtctctttgacatgtaaagtaaataatcacctattattataataatgtaataataac
29c    ggctaactcctctcaataggataaacattatttttatgtacaaggaatataatacacagat
C8     acaaatattattatgtaataactatatttatgtatattgtttatatacatttaaatatat
29c    tgccctcaaagtcatatttccaactgctcatcaatctaaaaatccaaaattttgaataat
C8     ataaatatacatttattagctaataatttgatatatgtatggtaattcaacatgtatgag
29c    tttttgatgaaataatttatttcattgtttctcaattttggctgcacagtggaaccacct
C8     ttatattcactatttcatgtttaggcagctgtattttaagtgaactatactaaatatttg
29c    gggctgtttgaaaaaacctaggcctagccaggcacggtggctcatgcctgtaatcccagc
C8     aaaggcttttgttatcaagggctaagtctcctatttttgatatagcattacaatgtaca
29c    acttagggagaccagcctggccaacatggtgaaaccctgtctctgctaaaaatacaaaat
C8     ttttttatatacaaaatatagaatacactgatttccctcaaggtcataaattcccaactg
29c    cacctgggcgtagttgtgcattcctgtaatcccagccacgccagaagctgaggcagaatc
C8     gtcattaatctgagaatattgaattttgagtatattctaacatagaatcatttacttcag
29c    gttcgaatccaggaagtggaggctgcagtgagttgagatcttgccattgcactccagcct
C8     tgtttctccatcatcacagcacattggaacaaccagggacttttaattaaaaatacctgg
29c    gggcaacaagagcgaaactccatctcaaggaaaaacaacaacaacaacaacaaaatcctg
C8     gctccaatccaatacaattaaaccagaatctcctagattggcactggaaagaaggagtag
29c    ggctctgcttcagactagttaaaccagaatctccagggtggggcaccggaaagaacaaga
C8     gacaaaagaacattttatttctatccatgggccaaagtccactcagaaaaaaagtataaa
29c    aaaaagaacaccttatttttatcttcttcagtgagccaatgttcattcaaaagagagatt
```

```
C8      ttggatctaggtgattgtttactttacatgtcaaagagacacacactaaattagcaggga
29c     aaagtgctttttgctgactagtcacagtcagagtcagaatcacaggtggattagtaggga
C8      gtgttataaaaactttggagtgcaagctcacagctgtcttaataagaagagaaggcttca
29c     gtgttataaaagccttgaagtgaaagcccgcagttgtcttactaagaagagaagccttca
6b                          ........................................
C8      atggaacctttttgtggtcctggtgctgtgtctctcttttatgcttctcttttcactctgg
C18     .....t..agc.....ct.................c.g.t..t.....c..........
C9      .....tt..c.........t.....c........a.g.t........c..........
C19     .....t.............t.....c........a.g.t........c.....a.....
60
C8      agacagagctgtaggagaaggaagctccctcctggccccactcctcttcctattattgga
C18     ..g.......c.g.a...g...g......gt.................c..g.........
C9      ..........c.g.....g.a..a.......................c..ag.g......
C19     ..........c.g.....g.a..a.......................c..ag.g......
120                                                  exon1exon2 i1?
C8      aatatgctacagatagatgttaaggacatctgcaaatctttcaccaatttctcaaaagtc
C18     .....c..g...t................ga.......c..aa..................
C9      .....c..........g.a...........a.......c..a......c.......g...
C19     .....c............a.......tg..a.......c..a......c........a..
180
C8      tatggtcctgtgttcaccgtgtattttggcatgaatcccatagtggtgtttcatggatat
C18     .....c..........t............c....g.....t........g.........
C9      .....c..........tc...........c....a............c.g.........
Hum2          g
C19     .....c..........tc...........c..g.a.g...g......c.g.........
240
C8      gaggcagtgaaggaagccctgattgataatggagaggagttttctggaagaggcaattcc
MP12                                                              g
C18     ..a..........g............c.........................a.g..tt
C9      ..a........................ct...........................t..t.
C19     ..a.tg.....................ct.........................c...t.
300                               exon2exon3 i21.3kb
C8      ccaatatctcaaagaattactaaaggacttggaatcatttccagcaatggaaagagatgg
C17                                                               ..
C18     ...g.gg..g...a.g...ac..............c...t...................
C9      ...c.gg..g.....gc..ac.g....t.......tg...t..............a....
C19     ...c.gg..g.....gc..ac.g....t........g...t...................
360
C8      aaggagatccggcgtttctccctcacaaacttgcggaattttgggatggggaagaggagc
C17     ..................g.....t..ctc.............................
MP12*                               c
MP20*                               c
C18     ..................g.....tg.ctc.............................
clon6                               a
C9      ........................tg.cgc.............................
C19     ........................tg.cgc.............................
liu                                                 c..................
420
C8      attgaggaccgtgttcaagaggaagctcactgccttgtggaggagttgagaaaaaccaag
Liu     ..........................................................
C17     ..c.......................c.g.............................t
C18     ..c.......................c.g.............................t
C9      ..........................c.g.............................
meeh*           t
C19     ..........................c.g.............................
```

```
480xon3exon4 i3 191bp
C8     gcttcaccctgtgatcccactttcatcctgggctgtgctccctgcaatgtgatctgctcc
Liu    ..........................................................
C17    ..c.......................................................
MP12     c
C18    ..c.....................................................t
C9     ..c.......................................................
meeh                                                        a
C19    ..........................................................
540

C8     gttgttttccagaaacgatttgattataaagatcagaattttctcaccctgatgaaaaga
Liu    ..........................................................
C17    a..a.......t........c..............c.a.....t.a.t.........a.
MP20                                      a
C18    ...a.......tg.t.....................gg.....t.a.t.........a.
C9     a..a.......t.....t..................c.a.....t.a.t.a...g...a.
C19    a..a............t..c...............c.a.....t.a.t.....g...a.
600                                       exon4exon5 i4 2.6kb
C8     ttcaatgaaaacttcaggattctgaactccccatggatccaggtctgcaataatttccct
Liu    ..........................................................
C17    ..g.........a..........a.g.a....c.........................tt.c
C18    ............t..........g...t...............................
Q*                                                            c
C9     ..g.........a...a....t...g.ag...c.........a.............tt..
C19    ..g.........a........g.a.g.a....c..a......a.a...........t..c
E4M                                     g
660
C8     ctactcattgattgtttcccaggaactcacaacaaagtgcttaaaaatgttgctcttaca
Liu    ..........................................................
C17    acta...............g....g...t......t.a.........t.gct.acattg
C18    gct.....c....a.c.........g...t..t...a.agc.g.....t.....tac.tt
C9     .cta.........ac.....g..............t.a........c......t...tg
meeh                                                          a
C19    acta.........a......g.....c..t......t.a........cc.....t...tg
E5M*                     a
720
C8     cgaagttacattagggagaaagtaaaagaacaccaagcatcactggatgttaacaatcct
Liu    ..........................................................
C17    aa......tg.att.....g.a..........t....a...c.....ca.g.....c...
MP12                           g
C18    aa......tg.att.....g.a..........t....a...c.....ca.g....g.g..
C9     aa......t...tt...a...................a....a....ca.g.....c...
C19    ga....g.t...tt.......................a...ga....ca.c.....c...
780                                       exon5exon6 i5 12kb
C8     cgggactttatggattgcttcctgatcaaaatggagcaggaaaaggacaaccaaaagtca
Liu    ..........c...............................................
C17    ...........t.....t..................a.........c....t...c....t
C18    ...........t.....t..................a.........c....t...c....t
C9     .a.........t..............g.........a........c.........cca..t
clo6*                                        ------
MP-4                                                          a
MP-8                                                          a
C19    ...........t.........................a........c.a......c....t
840
C8     gaattcaatattgaaaacttggttggcactgtagctgatctatttgttgctggaacagag
Liu    .......c..............aat.....c......ct..c...ga.....g......
```

```
C17    .....t.c.g......g....a.a.c.......a.....a.g....gg.....
C18    .....t.c.g......g....a.a.c.......a.....a.g....gg.....
C9     .....t.c........g.....aaaa.....c..t...ct.g....ga.....g......
C19    .......c..............aat.....c......ct..c...ga.....g......
900
C8     acaacaagcaccactctgagatatggactcctgctcctgctgaagcacccagaggtcaca
Liu    ..........a..c.........ct.....t.....
C17    ..........a..c.........ct.....t.....
MP12*                                                        g
MP20*                                                        g
C18    .....g.....................................t.....
C9     ..g........a..c.........ct.....t.....
C19    ..........a..c.........ct.....t.....
960xon6exon7 i6 9kb
C8     gctaaagtccaggaagagattgatcatgtaattggcagacacaggagcccctgcatgcag
Liu    ......................a.g...cg........a..c.....
C17    ......................a.g.............a..c.....
C18    ......................atg....g........a..c..........t......
clon6                                              a
C9     ......................a.g...g........a..c.............a
C19    ......................a.g...c........a..c.....
1020
```

```
C8     gataggagccacatgccttacactgatgctgtagtgcacgagatccagagatacagtgac
Liu    ..c...g.........c.....a........g.........g...........tc...
C17    ..c.............c.....a........g.........g...........t....
C18    ..c.....t........c.....a........g.....................t....
C9     ..c.............c.....a........g.........g...........t....
c25*                                                          c
MP-8*                                                      g
C19    ..c...g.........c.....a........g.........g...........tc...
1080
C8     cttgtccccaccggtgtgccccatgcagtgaccactgatactaagttcagaaactacctc
Liu    ..ca........a.cc.................tg...t.t...a.....
C17    ..ca........a.cc.................tg...cgt...a.....
C18    ..cc........aacc.................tg....gt...a....a.....
C9     ...c........a.cc.................tg...c.tt..a...........t...
C19    ..ca........a.cc.................tg...cgt...a.....
1140       exon7exon8 i7 9kb
C8     atccccaagggcacaaccataatggcattactgacttccgtgctacatgatgacaaagaa
Liu    ..t.................t.aa.t.cc..c.....t...........ca........
C17    ..t.....................aa.t.cc..c.....t...........ca........
HP1-2  variant splice see 2C8 sheet
HPH                                                            g
C18    .............tg........aa...cc........t.....g..ca...........
29c*                 c
clon6                c
C9     ..t..................t.aatt.cc........t...........ca........
C19    ..t..................t.aa.t.cc..c.....t...........ca........
1200
C8     tttcctaatccaaatatctttgaccctggccactttctagataagaatggcaactttaag
Liu    .....c..c...g.g..g.........c.t........g...g.agg...a..t......
C17    .....c..c...g.g..g.........c.t........g...g.agg...a..t......
MP20                                    t
```

```
C18    ..c..c..c...g.g..g.....................g.......g.............
C9     .....c..c...g.g..g.........cat........g...g.agg......t......
MP-8*                                                        a
C19    .....c..c...g.g..g.........c.t........g...g.agg...a..t......
1260                              exon8exon9 i8 13kb
C8     aaaagtgactacttcatgcctttctcagcaggaaaacgaatttgtgcaggagaaggactt
Liu    ......a................................g.......tg.....g..c..g
C17    ......a................................g.......tg.....g..c..g
C18    ....................................g..g...atg.....g..c..g
C9     ......a.a..............................g.......tg.......cc..g
C19    ......a................................g.......tg.....g..c..g
1320
C8     gcccgcatggagctatttttatttctaaccacaattttacagaactttaacctgaaatct
Liu    ..............g........c..g...ttc.............................
C17    ..............g........c..g...ttc.............................
C18    ..............g........c..g.....c.....g.......................
C9     ...g..........g........c..g...t.c.............................
C19    ..............g........c..g...ttc.............................
1380
C8     gttgatgatttaaagaacctcaatactactgcagttaccaaagggattgtttctctgcca
Liu    c.gat...ccc....g....tg.c..a...c.t...gt...t...t...c....g.c..g
C17    c.gat...ccc....g....tg.c..a...c.t...gt...t..at...c....g.c..g
C18    cag.t...ccc....g.ta.tg.c.tc..cc.ca..g....tgc.t...g.cg.g.....
C9     c.g.t...ccc.........tg.c..c...c.....gt...t..at...cc...g....g
c25                                                  t
C19    c.gat...ccc....g....tgac..a...c.tg..gt...t..at...c....g.c..g
1440
C8     ccctcataccagatctgcttcatccctgtctgaagaatgctagcccatctggctgctgat
Liu    ....tc..t...c.g........t.............gcac..atgg..........cc.
C17    ....tc..t...c.g........t.............gcac..atgg..........cc.
HPH                                                    t
C18    ....tg......c..........t.............,,,,g,g,,,atag,c,,,,,,,,cc,
C9     ....tc......c.g........t.............gagc..atggc..........c.
C19    ....tc..t...c.g........t.............gcac..atgg..........cc.
1500
C8     ctgctatcacctgcaactctttttttatcaaggacattcccactattatgtcttctctga
Liu    g....g..c.tgcagctctc...cc.c.ggtcc.a...t.actatc.g..atgct...tc
C17    g....g..c.tgcagctctc...cc.c.ggtcc.a...t.actatc.g..atgct...tc
C18    g,,,,g,,,,,,,,,,t,,,ccc,,atcagggccat,gg,,tc,ccct,c,,,ctatgag
29c                                                            g
C9     g...ag..c.tgcagctctc...cc.c.ggg.catta...at..t.gcac.a.ctgtaat

C8     cctctcatcaaatcttcccattcactcaatatcccataagcatccaaactccattaagga
Liu    tga.c.g...tc.cacattt.c.ct..ccccaagatct..tgaa.attcag.c.cc.tt.
C17    .ga.c.g...tc..acattt.c.ct..ccccaagatct..tgaa..ttcag.c.cc.tt.
C18    ggatattttctctgacttgtcaat,cac,,c,t,,cattccc,,a,gatc,a,,g,,cat
C9     g.ct.ttctc.cctg..atc.caca.tttcccttccctgaagat.t.gtgaaca.tc.ac
C18    ccaacctccattaaagagagtttcttgggtcacttcctaaatatatctgctattctccat
C18    actctgtatcacttgtattgaccaccacatatgctaatacctatctactgctgagttgtc
C18    agtatgttatcactataaaacaaagaaaaatgattaataaatgacaattcagagccattt
29c                  g
C18    attctctgcatgctctagataaaaatgattattatttactgggtcagttcttagatttct
C18    ttcttttgagtaaaatgaaagtaagaaatgaaagaaaatagaatgtgaagaggctgtgct
C18    ggccctcatagtgttaagcacaaaaagggagaaaggtaagagggtaggaaagctgtttta
C18    gctaaatgccacctagagttattggaggtctgaatttggaaaaaaaaactatgtccagga
C18    gcagctgtaacctgtagggaaataatggaacaatcatccataagagggatgaacattaag
C18    tgtttgaattcatgctctgcttttgtgttactgtaaacacaagatcaagatttggataat
C18    ctttttcctttgtgtttccaacttagatcatgtctaaatatatgctttcatatggc
```

Bibliography:

(1) Atkin WS, Cuzick J, Northover JM, Whynes DK. Prevention of colorectal cancer by once-only sigmoidoscopy [see comments]. Lancet 1993; 341(8847):736-740.

(2) Potter JD. Nutrition and colorectal cancer. [Review] [278 refs]. Cancer Causes & Control 1996; 7(1):127-146.

(3) Goldstein JA, De Morals SM. Biochemistry and molecular biology of the human CYP2C subfamily. [Review] [99 refs]. Pharmacogenetics 1994; 4(6):285-299.

(4) Smith CA, Smith G, Wolf CR. Genetic polymorphisms in xenobiotic metabolism. [Review] [175 refs]. European Journal of Cancer 1994; 30A(13):1921-1935.

(5) Tomatis LE. Cancer: Causes, Occurrence and Control. IARC Scientific Publications (Lyon) . 1990. Ref Type: Report

(6) Doll R, Peto R. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. [Review] [336 refs]. Journal of the National Cancer Institute 1981; 66(6):1191-1308.

(7) Weinstein IB, Santella RM. Molecular Biology and Epidemiology of Cancer. In: Greenwald P, Kramer BS, Weed DL, editors. Cancer prevention and control. New York: Marcel-Dekker, 1995: 83-110.

(8) Pirmohamed M, Kitteringham NR, Park BK. The role of active metabolites in drug toxicity. [Review] [285 refs]. Drug Safety 1994; 11(2):114-144.

(9) Raunio H, Husgafvel-Pursiainen K, Anttila S, Hietanen E, Hirvonen A, Pelkonen O. Diagnosis of polymorphisms in carcinogen-activating and inactivating enzymes and cancer susceptibility--a review. [Review] [65 refs]. Gene 1995; 159(1):113-121.

(10) Meyer UA. Overview of enzymes of drug metabolism. [Review] [21 refs]. Journal of Pharmacokinetics & Biopharmaceutics 1996; 24(5):449-459.

(11) Gough AC, Miles JS, Spurr NK, Moss JE, Gaedigk A, Eichelbaum M et al. Identification of the primary gene defect at the cytochrome P450 CYP2D locus. Nature 1990; 347(6295):773-776.

(12) De Morais SM, Wilkinson GR, Blaisdell J, Nakamura K, Meyer UA, Goldstein JA. The major genetic defect responsible for the polymorphism of S-mephenytoin metabolism in humans. Journal of Biological Chemistry 1994; 269(22):15419-15422.

(13) Nebert DW, Adesnik M, Coon MJ, Estabrook RW, Gonzalez FJ, Guengerich et al. The P450 gene superfamily: recommended nomenclature. DNA 1987; 6(1):1-11.

(14) Nebert DW, Nelson DR. P450 gene nomenclature based on evolution. Methods in Enzymology 1991; 206:3-11.

(15) Gonzalez FJ. Molecular genetics of the P-450 superfamily. [Review] [312 refs]. Pharmacology & Therapeutics 1990; 45(1):1-38.

(16) Nebert DW, Nelson DR, Coon MJ, Estabrook RW, Feyereisen R, Fujii-Kuriyama Y et al. The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature [published erratum appears in DNA Cell Biol 1991 Jun;10(5):397-8]. [Review] [110 refs]. DNA & Cell Biology 1991; 10(1):1-14.

(17) Anttila S, Vainio H, Hietanen E, Camus AM, Malaveille C, Brun G et al. Immunohistochemical detection of pulmonary cytochrome P450IA and metabolic activities associated with P450IA1 and P450IA2 isozymes in lung cancer patients. Environmental Health Perspectives 1992; 98:179-182.

(18) Spurr NK, Gough AC, Stevenson K, Wolf CR. Msp-1 polymorphism detected with a cDNA probe for the P-450 I family on chromosome 15. Nucleic Acids Research 1987; 15(14):5901.

(19) Kawajiri K, Nakachi K, Imai K, Yoshii A, Shinoda N, Watanabe J. Identification of genetically high risk individuals to lung cancer by DNA polymorphisms of the cytochrome P450IA1 gene. FEBS Letters 1990; 263(1):131-133.

(20) Hayashi SI, Watanabe J, Nakachi K, Kawajiri K. PCR detection of an A/G polymorphism within exon 7 of the CYP1A1 gene. Nucleic Acids Research 1991; 19(17):4797.

(21) Hayashi S, Watanabe J, Nakachi K, Kawajiri K. Genetic linkage of lung cancer-associated MspI polymorphisms with amino acid replacement in the heme binding region of the human cytochrome P450IA1 gene. Journal of Biochemistry 1991; 110(3):407-411.

(22) Tefre T, Ryberg D, Haugen A, Nebert DW, Skaug V, Brogger A et al. Human CYP1A1 (cytochrome P(1)450) gene: lack of association between the Msp I restriction fragment length polymorphism and incidence of lung cancer in a Norwegian population. Pharmacogenetics 1991; 1(1):20-25.

(23) Kadlubar FF, Butler MA, Kaderlik KR, Chou HC, Lang NP. Polymorphisms for aromatic amine metabolism in humans: relevance for human carcinogenesis. Environmental Health Perspectives 1992; 98:69-74.

(24) Sachse C, Brockmoller J, Bauer S, Roots I. Functional significance of a C-->A polymorphism in intron 1 of the cytochrome P450 CYP1A2 gene tested with caffeine. British Journal of Clinical Pharmacology 1999; 47(4):445-449.

(25) Boobis AR, Lynch AM, Murray S, de la Torre R, Solans A, Farre M et al. CYP1A2-catalyzed conversion of dietary heterocyclic amines to their proximate carcinogens is their major route of metabolism in humans. Cancer Research 1994; 54(1):89-94.

(26) Tang YM, Wo YYP, Stewart J, Hawkins AL, Griffin CA, Sutter TR et al. Isolation and characterization of the human cytochrome P450 CYP1B1 gene. Journal of Biological Chemistry 1996; 271(45):28324-28330.

(27) Bailey LR, Roodi N, Dupont WD, Parl FF. Association of cytochrome P450 1B1 (CYP1B1) polymorphism with steroid receptor status in breast cancer. Cancer Research 1998; 58(22):5038-5041.

(28) Fernandez-Salguero P, Hoffman SM, Cholerton S, Mohrenweiser H, Raunio, Rautio A et al. A genetic polymorphism in coumarin 7-hydroxylation: sequence of the human CYP2A genes and identification of variant CYP2A6 alleles. American Journal of Human Genetics 1995; 57(3):651-660.

(29) Pianezza ML, Sellers EM, Tyndale RF. Nicotine metabolism defect reduces smoking [letter]. Nature 1998; 393(6687):750.

(30) Ding S, Lake BG, Friedberg T, Wolf CR. Expression and alternative splicing of the cytochrome P-450 CYP2A7. Biochemical Journal 1995; 306(Pt 1):161-166.

(31) Ekins S, Vandenbranden M, Ring BJ, Gillespie JS, Yang TJ, Gelboin HV et al. Further characterization of the expression in liver and catalytic activity of CYP2B6. *Journal of Pharmacology & Experimental Therapeutics* 1998; 286(3):1253-1259.

(32) Gonzalez FJ, Vilbois F, Hardwick JP, McBride OW, Nebert DW, Gelboin et al. Human debrisoquine 4-hydroxylase (P450IID1): cDNA and deduced amino acid

sequence and assignment of the CYP2D locus to chromosome 22. Genomics 1988; 2(2):174-179.

(33) Mahgoub A, Idle JR, Dring LG, Lancaster R, Smith RL, Ladero JM et al. Polymorphic hydroxylation of Debrisoquine in man
Polymorphic oxidation of debrisoquine in women with breast cancer
Genetic predisposition to bladder cancer: ability to hydroxylate debrisoquine and mephenytoin as risk factors. Lancet 1977; 2(8038):584-586.

(34) Kaisary A, Smith P, Jaczq E, McAllister CB, Wilkinson GR, Ray WA et al. Genetic predisposition to bladder cancer: ability to hydroxylate debrisoquine and mephenytoin as risk factors. Cancer Research 1987; 47(20):5488-5493.

(35) Ladero JM, Benitez J, Jara C, Llerena A, Valdivielso MJ, Munoz JJ et al. Polymorphic oxidation of debrisoquine in women with breast cancer. Oncology 1991; 48(2):107-110.

(36) Wolf CR, Smith CA, Gough AC, Moss JE, Vallis KA, Howard G et al. Relationship between the debrisoquine hydroxylase polymorphism and cancer susceptibility. Carcinogenesis 1992; 13(6):1035-1038.

(37) Rostami-Hodjegan A, Lennard MS, Woods HF, Tucker GT. Meta-analysis of studies of the CYP2D6 polymorphism in relation to lung cancer and Parkinson's disease. Pharmacogenetics 1998; 8(3):227-238.

(38) Farker K, Lehmann MH, Kastner R, Hoffmann A, Janitzky V, Schubert J et al. CYP2E1 genotyping in renal cell/urothelial cancer patients in comparison with control populations. International Journal of Clinical Pharmacology & Therapeutics 1998; 36(9):463-468.

(39) London SJ, Daly AK, Cooper J, Carpenter CL, Navidi WC, Ding L et al. Lung cancer risk in relation to the CYP2E1 Rsa I genetic polymorphism among African-Americans and Caucasians in Los Angeles County. Pharmacogenetics 1996; 6(2):151-158.

(40) Hung HC, Chuang J, Chien YC, Chern HD, Chiang CP, Kuo YS et al. Genetic polymorphisms of CYP2E1, GSTM1, and GSTT1; environmental factors and risk of oral cancer. Cancer Epidemiology, Biomarkers & Prevention 1997; 6(11):901-905.

(41) Hildesheim A, Anderson LM, Chen CJ, Cheng YJ, Brinton LA, Daly AK et al. CYP2E1 genetic polymorphisms and risk of nasopharyngeal carcinoma in Taiwan. Journal of the National Cancer Institute 1997; 89(16):1207-1212.

(42) Lin L, Yang F, Ye Z, Xu E, Yang C, Zhang C et al. Case-control study of cigarette smoking and primary hepatoma in an aflatoxin-endemic region of China: a protective effect. Pharmacogenetics 1991; 1(2):79-85.

(43) OPCS. Review of the Registrar General on deaths in England and Wales 1993. HMSO, editor. 1-1-1995. London, HMSO.
Ref Type: Report

(44) Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of eighteen major cancers in 1985. Int J Cancer 1993;(54):594-606.

(45) Coleman M, Esteve J, Damiecky P, Arslan A, Renard H. Trends in cancer incidence and Mortality. IARC 1993.

(46) Muto T, Bussey H, Morson BC. The Evolution of cancer of the colon and rectum. Cancer 1975; 36:2251-2270.

(47) Stryker SJ, Wolff BG, Culp CE, Libbe SD, Ilstrup DM, MacCarty RL. Natural history of untreated colonic polyps. Gastroenterology 1987; 93(5):1009-1013.

(48) Atkin WS, Morson BC, Cuzick J. Long-term risk of colorectal cancer after excision of rectosigmoid adenomas [see comments]. New England Journal of Medicine 1992; 326(10):658-662.

(49) Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert et al. Genetic alterations during colorectal-tumor development. New England Journal of Medicine 1988; 319(9):525-532.

(50) Powell SM, Petersen GM, Krush AJ, Booker S, Jen J, Giardiello FM et al. Molecular diagnosis of familial adenomatous polyposis [see comments]. New England Journal of Medicine 1993; 329(27):1982-1987.

(51) Fishel R, Lescoe MK, Rao MR, Copeland NG, Jenkins NA, Garber J et al. The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer [published erratum appears in Cell 1994 Apr 8;77(1):167]. Cell 1993; 75(5):1027-1038.

(52) Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau et al. APC mutations occur early during colorectal tumorigenesis. Nature 1992; 359(6392):235-237.

(53) Wilmink AB. Overview of the epidemiology of colorectal cancer. [Review] [84 refs]. Diseases of the Colon & Rectum 1997; 40(4):483-493.

(54) Vasen HF, Mecklin JP, Khan PM, Lynch HT. The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC). Diseases of the Colon & Rectum 1991; 34(5):424-425.

(55) Lynch HT, Smyrk TC, Watson P, Lanspa SJ, Lynch JF, Lynch PM et al. Genetics, natural history, tumor spectrum, and pathology of hereditary nonpolyposis colorectal cancer: an updated review. [Review] [123 refs]. Gastroenterology 1993; 104(5):1535-1549.

(56) Lanspa SJ, Lynch HT, Smyrk TC, Strayhorn P, Watson P, Lynch JF et al. Colorectal adenomas in the Lynch syndromes. Results of a colonoscopy screening program. Gastroenterology 1990; 98(5 Pt 1):1117-1122.

(57) Mecklin JP, Sipponen P, Jarvinen HJ. Histopathology of colorectal carcinomas and adenomas in cancer family syndrome. Diseases of the Colon & Rectum 1986; 29(12):849-853.

(58) Lynch HT, Smyrk T, Watson P, Lanspa SJ, Boman BM, Lynch PM et al. Hereditary colorectal cancer. [Review] [128 refs]. Seminars in Oncology 1991; 18(4):337-366.

(59) Morson BC. Factors influencing the prognosis of early cancer of the rectum. Proceedings of the Royal Society of Medicine 1966; 59(7):607-608.

(60) Heald RJ, Bussey HJ. Clinical experiences at St. Mark's Hospital with multiple synchronous cancers of the colon and rectum. Diseases of the Colon & Rectum 1975; 18(1):6-10.

(61) Selby JV, Friedman GD, Quesenberry CPJ, Weiss NS. A case-control study of screening sigmoidoscopy and mortality from colorectal cancer [see comments]. New England Journal of Medicine 1992; 326(10):653-657.

(62) Friedman GD, Collen MF, Fireman BH. Multiphasic Health Checkup Evaluation: a 16-year follow-up. Journal of Chronic Diseases 1986; 39(6):453-463.

(63) Gilbertsen VA, Nelms JM. The prevention of invasive cancer of the rectum. Cancer 1978; 41(3):1137-1139.

(64) Newcomb PA, Norfleet RG, Storer BE, Surawicz TS, Marcus PM. Screening sigmoidoscopy and colorectal cancer mortality [see comments]. Journal of the National Cancer Institute 1992; 84(20):1572-1575.

(65) Gohagan JK, Prorok PC, Kramer BS, Cornett JE. Prostate cancer screening in the prostate, lung, colorectal and ovarian cancer screening trial of the National Cancer Institute [see comments]. Journal of Urology 1994; 152(5 Pt 2):1905-1909.

(66) Vogelstein B, Fearon ER, Kern SE, Hamilton SR, Preisinger AC, Nakamura Y et al. Allelotype of colorectal carcinomas. Science 1989; 244(4901):207-211.

(67) Otori K, Oda Y, Sugiyama K, Hasebe T, Mukai K, Fujii T et al. High frequency of K-ras mutations in human colorectal hyperplastic polyps [see comments]. Gut 1997; 40(5):660-663.

(68) Knudson AG, Jr. Mutation and cancer: statistical study of retinoblastoma. Proceedings of the National Academy of Sciences of the United States of America 1971; 68(4):820-823.

(69) Kinzler KW, Nilbert MC, Su LK, Vogelstein B, Bryan TM, Levy DB et al. Identification of FAP locus genes from chromosome 5q21. Science 1991; 253(5020):661-665.

(70) Nakamura Y. The adenomatous polyposis coli gene and human cancers. [Review] [63 refs]. Journal of Cancer Research & Clinical Oncology 1995; 121(9-10):529-534.

(71) Smith KJ, Johnson KA, Bryan TM, Hill DE, Markowitz S, Willson JK et al. The APC gene product in normal and tumor cells. Proceedings of the National Academy of Sciences of the United States of America 1993; 90(7):2846-2850.

(72) Rubinfeld B, Albert I, Porfiri E, Munemitsu S, Polakis P. Loss of beta-catenin regulation by the APC tumor suppressor protein correlates with loss of structure due to common somatic mutations of the gene. Cancer Research 1997; 57(20):4624-4630.

(73) Jen J, Kim H, Piantadosi S, Liu ZF, Levitt RC, Sistonen P et al. Allelic loss of chromosome 18q and prognosis in colorectal cancer [see comments]. New England Journal of Medicine 1994; 331(4):213-221.

(74) Ookawa K, Sakamoto M, Hirohashi S, Yoshida Y, Sugimura T, Terada M et al. Concordant p53 and DCC alterations and allelic losses on chromosomes 13q and 14q associated with liver metastases of colorectal carcinoma. International Journal of Cancer 1993; 53(3):382-387.

(75) Fearon ER, Pierceall WE. The deleted in colorectal cancer (DCC) gene: a candidate tumour suppressor gene encoding a cell surface protein with similarity to neural cell adhesion molecules. [Review] [58 refs]. Cancer Surveys 1995; 24:3-17.

(76) Marx J. How p53 suppresses cell growth [news]. Science 1993; 262(5140):1644-1645.

(77) Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 1993; 75(4):805-816.

(78) Baker SJ, Fearon ER, Nigro JM, Hamilton SR, Preisinger AC, Jessup JM et al. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science 1989; 244(4901):217-221.

(79) Baker SJ, Preisinger AC, Jessup JM, Paraskeva C, Markowitz S, Willson et al. p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. Cancer Research 1990; 50(23):7717-7722.

(80) Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature 1993; 363(6429):558-561.

(81) Aaltonen LA, Peltomaki P, Leach FS, Sistonen P, Pylkkanen L, Mecklin et al. Clues to the pathogenesis of familial colorectal cancer [see comments]. Science 1993; 260(5109):812-816.

(82) Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon [see comments]. Science 1993; 260(5109):816-819.

(83) Gyapay G, Morissette J, Vignal A, Dib C, Fizames C, Millasseau P et al. The 1993-94 Genethon human genetic linkage map [see comments]. Nature Genetics 1994; 7(2 Spec No):246-339.

(84) Jiricny J. Colon cancer and DNA repair: have mismatches met their match?. [Review] [30 refs]. Trends in Genetics 1994; 10(5):164-168.

(85) Ruschoff J, Bocker T, Schlegel J, Stumm G, Hofstaedter F. Microsatellite instability: new aspects in the carcinogenesis of colorectal carcinoma. [Review] [64 refs]. Virchows Archiv 1995; 426(3):215-222.

(86) Chung DC, Rustgi AK. DNA mismatch repair and cancer. [Review] [109 refs]. Gastroenterology 1995; 109(5):1685-1699.

(87) Lazar V, Grandjouan S, Bognel C, Couturier D, Rougier P, Bellet D et al. Accumulation of multiple mutations in tumour suppressor genes during colorectal tumorigenesis in HNPCC patients. Human Molecular Genetics 1994; 3(12):2257-2260.

(88) Craanen ME, Blok P, Offerhaus GJ, Tytgat GN. Recent developments in hereditary nonpolyposis colorectal cancer. [Review] [59 refs]. Scandinavian Journal of Gastroenterology - Supplement 1996; 218:92-97.

(89) Aaltonen LA, Peltomaki P, Mecklin JP, Jarvinen H, Jass JR, Green JS et al. Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients. Cancer Research 1994; 54(7):1645-1648.

(90) Foulkes WD. A tale of four syndromes: familial adenomatous polyposis, Gardner syndrome, attenuated APC and Turcot syndrome. [Review] [58 refs]. QJM 1995; 88(12):853-863.

(91) Haenszel W KM. Studies of Japanese migrants.I. Mortality from cancer and other diseases among Japanese in the UNited States. Journal of National cancer Institute 1968; 40:43-68.

(92) Whittemore AS, Wu-Williams AH, Lee M, Zheng S, Gallagher RP, Jiao DA et al. Diet, physical activity, and colorectal cancer among Chinese in North America and China. Journal of the National Cancer Institute 1990; 82(11):915-926.

(93) Jensen OM, MacLennan R, Wahrendorf J. Diet, bowel function, fecal characteristics, and large bowel cancer in Denmark and Finland. Nutrition & Cancer 1982; 4(1):5-19.

(94) Hill MJ, Drasar BS, Williams RE, Meade TW, Cox AG, Simpson JE et al. Faecal bile-acids and clostridia in patients with cancer of the large bowel. Lancet 1975; 1(7906):535-539.

(95) Roberts-Thomson IC, Ryan P, Khoo KK, Hart WJ, McMichael AJ, Butler et al. Diet, acetylator phenotype, and risk of colorectal neoplasia. Lancet 1996; 347(9012):1372-1374.

(96) Burkitt D. Fiber as protective against gastrointestinal diseases. American Journal of Gastroenterology 1984; 79(4):249-252.

(97) Hague A, Paraskeva C. The short-chain fatty acid butyrate induces apoptosis in colorectal tumour cell lines. [Review] [36 refs]. European Journal of Cancer Prevention 1995; 4(5):359-364.

(98) Steinmetz KA, Potter JD. Vegetables, fruit, and cancer. II. Mechanisms. [Review] [28 refs]. Cancer Causes & Control 1991; 2(6):427-442.

(99) Newmark HL, Wargovich MJ. Colon cancer and dietary fat, phosphorus, and calcium in Hawaiian-Japanese men [letter]. American Journal of Clinical Nutrition 1986; 44(6):998-1000.

(100) Atillasoy E, Fein B, Weinstein IB, Holt PR. Fecal diacylglycerol concentrations and calcium supplementation. Cancer Epidemiology, Biomarkers & Prevention 1995; 4(7):795-796.

(101) Giovannucci E, Stampfer MJ, Colditz GA, Rimm EB, Trichopoulos D, Rosner BA et al. Folate, methionine, and alcohol intake and risk of colorectal adenoma [see comments]. Journal of the National Cancer Institute 1993; 85(11):875-884.

(102) Giovannucci E, Rimm EB, Ascherio A, Stampfer MJ, Colditz GA, Willett et al. Alcohol, low-methionine--low-folate diets, and risk of colon cancer in men [see comments]. Journal of the National Cancer Institute 1995; 87(4):265-273.

(103) Ohgaki H, Kusama K, Matsukura N, Morino K, Hasegawa H, Sato S et al. Carcinogenicity in mice of a mutagenic compound, 2-amino-3-methylimidazo[4,5-f]quinoline, from broiled sardine, cooked beef and beef extract. Carcinogenesis 1984; 5(7):921-924.

(104) Pfau W, Brockstedt U, Sohren KD, Marquardt H. 32P-post-labelling analysis of DNA adducts formed by food-derived heterocyclic amines: evidence for incomplete hydrolysis and a procedure for adduct pattern simplification. Carcinogenesis 1994; 15(5):877-882.

(105) Friesen MD, Kaderlik K, Lin D, Garren L, Bartsch H, Lang NP et al. Analysis of DNA adducts of 2-amino-1-methyl-6-phenylimidazo[4,5- b]pyridine in rat and human tissues by alkaline hydrolysis and gas chromatography/electron capture mass spectrometry: validation by comparison with 32P-postlabeling. Chemical Research in Toxicology 1994; 7(6):733-739.

(106) Butler MA, Lang NP, Young JF, Caporaso NE, Vineis P, Hayes RB et al. Determination of CYP1A2 and NAT2 phenotypes in human populations by analysis of caffeine urinary metabolites. Pharmacogenetics 1992; 2(3):116-127.

(107) Lang NP, Chu DZ, Hunter CF, Kendall DC, Flammang TJ, Kadlubar FF. Role of aromatic amine acetyltransferase in human colorectal cancer. Archives of Surgery 1986; 121(11):1259-1261.

(108) Ilett KF, David BM, Detchon P, Castleden WM, Kwa R. Acetylation phenotype in colorectal carcinoma. Cancer Research 1987; 47(5):1466-1469.

(109) Ladero JM, Gonzalez JF, Benitez J, Vargas E, Fernandez MJ, Baki W et al. Acetylator polymorphism in human colorectal carcinoma. Cancer Research 1991; 51(8):2098-2100.

(110) Lang NP, Butler MA, Massengill J, Lawson M, Stotts RC, Hauer J et al. Rapid metabolic phenotypes for acetyltransferase and cytochrome P4501A2 and putative exposure to food-borne heterocyclic amines increase the risk for colorectal cancer or polyps. Cancer Epidemiology, Biomarkers & Prevention 1994; 3(8):675-682.

(111) Grant DM, Hughes NC, Janezic SA, Goodfellow GH, Chen HJ, Gaedigk A et al. Human acetyltransferase polymorphisms. Mutation Research 1997; 376(1-2):61-70.

(112) Vatsis KP, Weber WW, Bell DA, Dupret JM, Evans DA, Grant DM et al. Nomenclature for N-acetyltransferases. [Review] [120 refs]. Pharmacogenetics 1995; 5(1):1-17.

(113) Grant DM. Molecular genetics of the N-acetyltransferases. [Review] [35 refs]. Pharmacogenetics 1993; 3(1):45-50.

(114) Lang NP, Chu DZ, Hunter CF, Kendall DC, Flammang TJ, Kadlubar FF. Role of aromatic amine acetyltransferase in human colorectal cancer. Archives of Surgery 1986; 121(11):1259-1261.

(115) Ilett KF, David BM, Detchon P, Castleden WM, Kwa R. Acetylation phenotype in colorectal carcinoma. Cancer Research 1987; 47(5):1466-1469.

(116) Probst-Hensch NM, Haile RW, Ingles SA, Longnecker MP, Han CY, Lin BK et al. Acetylation polymorphism and prevalence of colorectal adenomas. Cancer Research 1995; 55(10):2017-2020.

(117) Chen J, Stampfer MJ, Hough HL, Garcia-Closas M, Willett WC, Hennekens et al. A prospective study of N-acetyltransferase genotype, red meat intake, and risk of colorectal cancer. Cancer Research 1998; 58(15):3307-3311.

(118) Lee EJ, Zhao B, Seow-Choen F. Relationship between polymorphism of N-acetyltransferase gene and susceptibility to colorectal carcinoma in a Chinese population. Pharmacogenetics 1998; 8(6):513-517.

(119) Bell DA, Stephens EA, Castranio T, Umbach DM, Watson M, Deakin M et al. Polyadenylation polymorphism in the acetyltransferase 1 gene (NAT1) increases risk of colorectal cancer. Cancer Research 1995; 55(16):3537-3542.

(120) Probst-Hensch NM, Haile RW, Li DS, Sakamoto GT, Louie AD, Lin BK et al. Lack of association between the polyadenylation polymorphism in the NAT1 (acetyltransferase 1) gene and colorectal adenomas. Carcinogenesis 1996; 17(10):2125-2129.

(121) Rodriguez JW, Kirlin WG, Ferguson RJ, Doll MA, Gray K, Rustan TD et al. Human acetylator genotype: relationship to colorectal cancer incidence and arylamine N-acetyltransferase expression in colon cytosol. Archives of Toxicology 1993; 67(7):445-452.

(122) Shibuta K, Nakashima T, Abe M, Mashimo M, Mori M, Ueo H et al. Molecular genotyping for N-acetylation polymorphism in Japanese patients with colorectal cancer. Cancer 1994; 74(12):3108-3112.

(123) Oda Y, Tanaka M, Nakanishi I. Relation between the occurrence of K-ras gene point mutations and genotypes of polymorphic N-acetyltransferase in human colorectal carcinomas. Carcinogenesis 1994; 15(7):1365-1369.

(124) Jenkins M, Bigler J, Kampman E, Chen C, Slattery M, Leppert J et al. Associations between colon cancer and acetylation (NAT1 and NAT2) genotypes in a large population-based study. American Journal of Epidemiology 145 (suppl), S68. 1997.

Ref Type: Abstract

(125) Hubbard AL, Harrison DJ, Moyes C, Wyllie AH, Cunningham C, Mannion E et al. N-acetyltransferase 2 genotype in colorectal cancer and selective gene retention in cancers with chromosome 8p deletions. Gut 1997; 41(2):229-234.

(126) Katoh T, Boissy R, Nagata N, Kitagawa K, Kuroda Y, Itoh H et al. Inherited polymorphism in the N-acetyltransferase 1 (NAT1) and 2 (NAT2) genes and susceptibility to gastric and colorectal adenocarcinoma. International Journal of Cancer 2000; 85(1):46-49.

(127) Hubbard AL, Moyes C, Wyllie AH, Smith CA, Harrison DJ. N-acetyl transferase 1: two polymorphisms in coding sequence identified in colorectal cancer patients. British Journal of Cancer 1998; 77(6):913-916.

(128) Bell DA, Badawi AF, Lang NP, Ilett KF, Kadlubar FF, Hirvonen A. Polymorphism in the N-acetyltransferase 1 (NAT1) polyadenylation signal: association of NAT1*10 allele with higher N-acetylation activity in bladder and colon tissue. Cancer Research 1995; 55(22):5226-5229.

(129) Lang NP. Thomas G. Orr Memorial Lectureship. Colon cancer from etiology to prevention. [Review] [35 refs]. American Journal of Surgery 1997; 174(6):578-582.

(130) Chen J, Stampfer MJ, Hough HL, Garcia-Closas M, Willett WC, Hennekens et al. A prospective study of N-acetyltransferase genotype, red meat intake, and risk of colorectal cancer. Cancer Research 1998; 58(15):3307-3311.

(131) Mannervik B, Danielson UH. Glutathione transferases--structure and catalytic activity. [Review] [233 refs]. CRC Critical Reviews in Biochemistry 1988; 23(3):283-337.

(132) Katoh T, Nagata N, Kuroda Y, Itoh H, Kawahara A, Kuroki N et al. Glutathione S-transferase M1 (GSTM1) and T1 (GSTT1) genetic polymorphism and susceptibility to gastric and colorectal adenocarcinoma. Carcinogenesis 1996; 17(9):1855-1859.

(133) Pemble S, Schroeder KR, Spencer SR, Meyer DJ, Hallier E, Bolt HM et al. Human glutathione S-transferase theta (GSTT1): cDNA cloning and the characterization of a genetic polymorphism. Biochemical Journal 1994; 300(Pt 1):271-276.

(134) Zhong S, Wyllie AH, Barnes D, Wolf CR, Spurr NK. Relationship between the GSTM1 genetic polymorphism and susceptibility to bladder, breast and colon cancer. Carcinogenesis 1993; 14(9):1821-1824.

(135) Gawronska-Szklarz B, Lubinski J, Kladny J, Kurzawski G, Bielicki D, Wojcicki M et al. Polymorphism of GSTM1 gene in patients with colorectal cancer and colonic polyps. Experimental & Toxicologic Pathology 1999; 51(4-5):321-325.

(136) Chenevix-Trench G, Young J, Coggan M, Board P. Glutathione S-transferase M1 and T1 polymorphisms: susceptibility to colon cancer and age of onset. Carcinogenesis 1995; 16(7):1655-1657.

(137) Deakin M, Elder J, Hendrickse C, Peckham D, Baldwin D, Pantin C et al. Glutathione S-transferase GSTT1 genotypes and susceptibility to cancer: studies of interactions with GSTM1 in lung, oral, gastric and colorectal cancers. Carcinogenesis 1996; 17(4):881-884.

(138) Butler WJ, Ryan P, Roberts-Thomson IC. Metabolic genotypes and risk for colorectal cancer: increased risk in individuals with glutathione theta 1 (GSTT1) gene defect. Gastroenterology 112[4], A542. 1997.
Ref Type: Abstract

(139) Lee E, Huang Y, Zhao B, Seow-Choen F, Balakrishnan A, Chan SH. Genetic polymorphism of conjugating enzymes and cancer risk: GSTM1, GSTT1, NAT1 and NAT2. Journal of Toxicological Sciences 1998; 23 Suppl 2:140-142.

(140) Slattery ML, Potter JD, Samowitz W, Bigler J, Caan B, Leppert M. NAT2, GSTM-1, cigarette smoking, and risk of colon cancer. Cancer Epidemiology, Biomarkers & Prevention 1998; 7(12):1079-1084.

(141) Lin HJ, Probst-Hensch NM, Ingles SA, Han CY, Lin BK, Lee DB et al. Glutathione transferase (GSTM1) null genotype, smoking, and prevalence of colorectal adenomas. Cancer Research 1995; 55(6):1224-1226.

(142) Alexandrie AK, Sundberg MI, Seidegard J, Tornling G, Rannug A. Genetic susceptibility to lung cancer with special emphasis on CYP1A1 and GSTM1: a study on host factors in relation to age at onset, gender and histological cancer types. Carcinogenesis 1994; 15(9):1785-1790.

(143) Ambrosone CB, Freudenheim JL, Graham S, Marshall JR, Vena JE, Brasure et al. Cytochrome P4501A1 and glutathione S-transferase (M1) genetic

polymorphisms and postmenopausal breast cancer risk. Cancer Research 1995; 55(16):3483-3485.

(144) Rebbeck TR, Walker AH, Phelan CM, Godwin AK, Buetow KH, Garber JE et al. Defining etiologic heterogeneity in breast cancer using genetic biomarkers. [Review] [20 refs]. Progress in Clinical & Biological Research 1997; 396:53-61.

(145) Gertig DM, Stampfer M, Haiman C, Hennekens CH, Kelsey K, Hunter DJ. Glutathione S-transferase GSTM1 and GSTT1 polymorphisms and colorectal cancer risk: a prospective study. Cancer Epidemiology, Biomarkers & Prevention 1998; 7(11):1001-1005.

(146) Lee E, Huang Y, Zhao B, Seow-Choen F, Balakrishnan A, Chan SH. Genetic polymorphism of conjugating enzymes and cancer risk: GSTM1, GSTT1, NAT1 and NAT2. Journal of Toxicological Sciences 1998; 23 Suppl 2:140-142.

(147) Katoh T, Kaneko S, Takasawa S, Nagata N, Inatomi H, Ikemura K et al. Human glutathione S-transferase P1 polymorphism and susceptibility to smoking related epithelial cancer; oral, lung, gastric, colorectal and urothelial cancer. Pharmacogenetics 1999; 9(2):165-169.

(148) Yang SK. Stereoselectivity of cytochrome P-450 isozymes and epoxide hydrolase in the metabolism of polycyclic aromatic hydrocarbons. [Review] [54 refs]. Biochemical Pharmacology 1988; 37(1):61-70.

(149) Omiecinski CJ, Aicher L, Holubkov R, Checkoway H. Human peripheral lymphocytes as indicators of microsomal epoxide hydrolase activity in liver and lung. Pharmacogenetics 1993; 3(3):150-158.

(150) Harrison DJ, Hubbard AL, MacMillan J, Wyllie AH, Smith CA. Microsomal epoxide hydrolase gene polymorphism and susceptibility to colon cancer. British Journal of Cancer 1999; 79(1):168-171.

(151) Palmer KJ. 2001. (Personnal communication)

(152) Alexandrov K, Rojas M, Kadlubar FF, Lang NP, Bartsch H. Evidence of anti-benzo[a]pyrene diolepoxide-DNA adduct formation in human colon mucosa. Carcinogenesis 1996; 17(9):2081-2083.

(153) Moghaddam M, Motoba K, Borhan B, Pinot F, Hammock BD. Novel metabolic pathways for linoleic and arachidonic acid metabolism. Biochimica et Biophysica Acta 1996; 1290(3):327-339.

(154) Sivaraman L, Leatham MP, Yee J, Wilkens LR, Lau AF, Le Marchand L. CYP1A1 genetic polymorphisms and in situ colorectal cancer. Cancer Research 1994; 54(14):3692-3695.

(155) Wolf CR, Smith CA, Gough AC, Moss JE, Vallis KA, Howard G et al. Relationship between the debrisoquine hydroxylase polymorphism and cancer susceptibility. Carcinogenesis 1992; 13(6):1035-1038.

(156) Kesaniemi YA, Ehnholm C, Miettinen TA, Davidson NO. Intestinal cholesterol absorption efficiency in man is related to apoprotein E phenotype Apolipoprotein E polymorphism: another player in the genetics of colon cancer susceptibility? [editorial; comment]. [Review] [21 refs]. Journal of Clinical Investigation 1987; 80(2):578-581.

(157) Rendic S, Di Carlo FJ. Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors. [Review] [295 refs]. Drug Metabolism Reviews 1997; 29(1-2):413-580.

(158) Wilkinson GR, Guengerich FP, Branch RA. Genetic polymorphism of S-mephenytoin hydroxylation. [Review] [108 refs]. Pharmacology & Therapeutics 1989; 43(1):53-76.

(159) Scott J, Poffenbarger PL. Pharmacogenetics of tolbutamide metabolism in humans. Diabetes 1979; 28(1):41-51.

(160) Vasko MR, Bell RD, Daly DD, Pippenger CE. Inheritance of phenytoin hypometabolism: a kinetic study of one family. Clinical Pharmacology & Therapeutics 1980; 27(1):96-103.

(161) Vermeij P, Ferrari MD, Buruma OJ, Veenema H, de Wolff FA. Inheritance of poor phenytoin parahydroxylation capacity in a Dutch family. Clinical Pharmacology & Therapeutics 1988; 44(5):588-593.

(162) Wrighton SA, Thomas PE, Willis P, Maines SL, Watkins PB, Levin W et al. Purification of a human liver cytochrome P-450 immunochemically related to several cytochromes P-450 purified from untreated rats. Journal of Clinical Investigation 1987; 80(4):1017-1022.

(163) Wang PP, Beaune P, Kaminsky LS, Dannan GA, Kadlubar FF, Larrey D et al. Purification and characterization of six cytochrome P-450 isozymes from human liver microsomes. Biochemistry 1983; 22(23):5375-5383.

(164) Ged C, Umbenhauer DR, Bellew TM, Bork RW, Srivastava PK, Shinriki N et al. Characterization of cDNAs, mRNAs, and proteins related to human liver microsomal cytochrome P-450 (S)-mephenytoin 4'-hydroxylase. Biochemistry 1988; 27(18):6929-6940.

(165) Kimura S, Pastewka J, Gelboin HV, Gonzalez FJ. cDNA and amino acid sequences of two members of the human P450IIC gene subfamily. Nucleic Acids Research 1987; 15(23):10053-10054.

(166) Meehan RR, Gosden JR, Rout D, Hastie ND, Friedberg T, Adesnik M et al. Human cytochrome P-450 PB-1: a multigene family involved in mephenytoin and steroid oxidations that maps to chromosome 10. American Journal of Human Genetics 1988; 42(1):26-37.

(167) Romkes M, Faletto MB, Blaisdell JA, Raucy JL, Goldstein JA. Cloning and expression of complementary DNAs for multiple members of the human cytochrome P450IIC subfamily [published erratum appears in Biochemistry 1993 Feb 9;32(5):1390]. Biochemistry 1991; 30(13):3247-3255.

(168) Okino ST, Quattrochi LC, Pendurthi UR, McBride OW, Tukey RH. Characterization of multiple human cytochrome P-450 1 cDNAs. The chromosomal localization of the gene and evidence for alternate RNA splicing [published erratum appears in J Biol Chem 1988 Feb 15;263(5):2576]. Journal of Biological Chemistry 1987; 262(33):16072-16079.

(169) Wrighton SA, Stevens JC, Becker GW, Vandenbranden M. Isolation and characterization of human liver cytochrome P450 2C19: correlation between 2C19 and S-mephenytoin 4'-hydroxylation. Archives of Biochemistry & Biophysics 1993; 306(1):240-245.

(170) Goldstein JA, Faletto MB, Romkes-Sparks M, Sullivan T, Kitareewan S, Raucy JL et al. Evidence that CYP2C19 is the major (S)-mephenytoin 4'-hydroxylase in humans. Biochemistry 1994; 33(7):1743-1752.

(171) de W, I, Cugnenc PH, Yang CS, Leroux JP, Beaune PH. Cytochrome P 450 isoenzymes, epoxide hydrolase and glutathione transferases in rat and human hepatic and extrahepatic tissues. Journal of Pharmacology & Experimental Therapeutics 1990; 253(1):387-394.

(172) de W, I, Cugnenc PH, Berger A, Leroux JP, Beaune PH. Drug-metabolizing enzyme expression in human normal, peritumoral and tumoral colorectal tissue samples. Carcinogenesis 1991; 12(5):905-909.

(173) Beaune P, Flinois JP, Kiffel L, Kremers P, Leroux JP. Purification of a new cytochrome P-450 from human liver microsomes. Biochimica et Biophysica Acta 1985; 840(3):364-370.

(174) Umbenhauer DR, Martin MV, Lloyd RS, Guengerich FP. Cloning and sequence determination of a complementary DNA related to human liver microsomal cytochrome P-450 S-mephenytoin 4-hydroxylase. Biochemistry 1987; 26(4):1094-1099.

(175) Kolyada AY. Sequence of a human liver cytochrome P-450 cDNA clone. Nucleic Acids Research 1990; 18(18):5550.

(176) Yasumori T, Kawano S, Nagata K, Shimada M, Yamazoe Y, Kato R. Nucleotide sequence of a human liver cytochrome P-450 related to the rat male specific form. Journal of Biochemistry 1987; 102(5):1075-1082.

(177) Ohgiya S, Komori M, Ohi H, Shiramatsu K, Shinriki N, Kamataki T. Six-base deletion occurring in messages of human cytochrome P-450 in the CYP2C subfamily results in reduction of tolbutamide hydroxylase activity. Biochemistry International 1992; 27(6):1073-1081.

(178) Brian WR, Ged C, Bellew TM, Srivastava PK, Bork RW, Umbenhauer DR et al. Human liver mephenytoin 4'-hydroxylase cytochrome P-450 proteins and genes. [Review] [22 refs]. Drug Metabolism Reviews 1989; 20(2-4):449-465.

(179) Veronese ME, Doecke CJ, Mackenzie PI, McManus ME, Miners JO, Rees DL et al. Site-directed mutation studies of human liver cytochrome P-450 isoenzymes in the CYP2C subfamily. Biochemical Journal 1993; 289(Pt 2):533-538.

(180) Sullivan-Klose TH, Ghanayem BI, Bell DA, Zhang ZY, Kaminsky LS, Shenfield GM et al. The role of the CYP2C9-Leu359 allelic variant in the tolbutamide polymorphism. Pharmacogenetics 1996; 6(4):341-349.

(181) Bhasker CR, Miners JO, Coulter S, Birkett DJ. Allelic and functional variability of cytochrome P4502C9. Pharmacogenetics 1997; 7(1):51-58.

(182) Rettie AE, Wienkers LC, Gonzalez FJ, Trager WF, Korzekwa KR. Impaired (S)-warfarin metabolism catalysed by the R144C allelic variant of CYP2C9. Pharmacogenetics 1994; 4(1):39-42.

(183) Bhasker CR, Miners JO, Coulter S, Birkett DJ. Allelic and functional variability of cytochrome P4502C9. Pharmacogenetics 1997; 7(1):51-58.

(184) Yasar, Eliasson E, Dahl ML, Johansson I, Ingelman-Sundberg M, Sjoqvist F. Validation of methods for CYP2C9 genotyping: frequencies of mutant alleles in a Swedish population. Biochemical & Biophysical Research Communications 1999; 254(3):628-631.

(185) Sullivan-Klose TH, Ghanayem BI, Bell DA, Zhang ZY, Kaminsky LS, Shenfield GM et al. The role of the CYP2C9-Leu359 allelic variant in the tolbutamide polymorphism. Pharmacogenetics 1996; 6(4):341-349.

(186) Furuya H, Meyer UA, Gelboin HV, Gonzalez FJ. Polymerase chain reaction-directed identification, cloning, and quantification of human CYP2C18 mRNA. Molecular Pharmacology 1991; 40(3):375-382.

(187) Yun CH, Shimada T, Guengerich FP. Roles of human liver cytochrome P4502C and 3A enzymes in the 3-hydroxylation of benzo(a)pyrene. Cancer Research 1992; 52(7):1868-1874.

(188) Tsuneoka Y, Fukushima K, Matsuo Y, Ichikawa Y, Watanabe Y. Genotype analysis of the CYP2C19 gene in the Japanese population. Life Sciences 1996; 59(20):1711-1715.

(189) De Morais SM, Wilkinson GR, Blaisdell J, Meyer UA, Nakamura K, Goldstein JA. Identification of a new genetic defect responsible for the polymorphism of (S)-mephenytoin metabolism in Japanese. Molecular Pharmacology 1994; 46(4):594-598.

(190) Giovannucci E, Martinez ME. Tobacco, colorectal cancer, and adenomas: a review of the evidence. [Review] [118 refs]. Journal of the National Cancer Institute 1996; 88(23):1717-1730.

(191) Hoff G, Vatn MH, Larsen S. Relationship between tobacco smoking and colorectal polyps. Scandinavian Journal of Gastroenterology 1987; 22(1):13-16.

(192) Badawi AF, Stern SJ, Lang NP, Kadlubar FF. Cytochrome P-450 and acetyltransferase expression as biomarkers of carcinogen-DNA adduct levels and human cancer susceptibility. [Review] [123 refs]. Progress in Clinical & Biological Research 1996; 395:109-140.

(193) Wilson JMG. Surveillance and early diagnosis in General Practice. In: Teeling-Smith G, editor. Proceedings of Colloquium Office of Health Economics. London: 1966: 5-10.

(194) Sant M, Capocaccia R, Verdecchia A, Gatta G, Micheli A, Mariotto A et al. Comparisons of colon-cancer survival among European countries: The Eurocare Study. International Journal of Cancer 1995; 63(1):43-48.

(195) Winawer SJ, Zauber AG, Ho MN, O'Brien MJ, Gottlieb LS, Sternberg SS et al. Prevention of colorectal cancer by colonoscopic polypectomy. The National Polyp Study Workgroup [see comments]. New England Journal of Medicine 1993; 329(27):1977-1981.

(196) Knight KK, Fielding JE, Battista RN. US Preventive Services Task Force. Occult blood screening for colorectal cancer. [Review] [99 refs]. JAMA 1989; 261(4):586-593.

(197) Mandel JS, Bond JH, Church TR, Snover DC, Bradley GM, Schuman LM et al. Reducing mortality from colorectal cancer by screening for fecal occult blood. Minnesota Colon Cancer Control Study [published erratum appears in N Engl J Med 1993 Aug 26;329(9):672] [see comments]. New England Journal of Medicine 1993; 328(19):1365-1371.

(198) Winawer SJ, Flehinger BJ, Schottenfeld D, Miller DG. Screening for colorectal cancer with fecal occult blood testing and sigmoidoscopy [see comments]. Journal of the National Cancer Institute 1993; 85(16):1311-1318.

(199) Hardcastle JD, Chamberlain JO, Robinson MH, Moss SM, Amar SS, Balfour et al. Randomised controlled trial of faecal-occult-blood screening for colorectal cancer [see comments]. Lancet 1996; 348(9040):1472-1477.

(200) Kewenter J, Bjork S, Haglind E, Smith L, Svanvik J, Ahren C. Screening and rescreening for colorectal cancer. A controlled trial of fecal occult blood testing in 27,700 subjects. Cancer 1988; 62(3):645-651.

(201) Kronborg O, Fenger C, Sondergaard O, Pedersen KM, Olsen J. Initial mass screening for colorectal cancer with fecal occult blood test. A prospective randomized study at Funen in Denmark. Scandinavian Journal of Gastroenterology 1987; 22(6):677-686.

(202) Wahrendorf J, Robra BP, Wiebelt H, Oberhausen R, Weiland M, Dhom G. Effectiveness of colorectal cancer screening: results from a population-based case-control evaluation in Saarland, Germany. European Journal of Cancer Prevention 1993; 2(3):221-227.

(203) Selby JV, Friedman GD, Quesenberry CPJ, Weiss NS. Effect of fecal occult blood testing on mortality from colorectal cancer. A case-control study [see comments]. Annals of Internal Medicine 1993; 118(1):1-6.

(204) Towler BP ILGPWDKJ. Screening for colorectal cancer using the faecal occult blood test, Hemoccult (Cochrane Review). 3. 2001. Oxford. In: *The Cochrane Library*.

Ref Type: Report

(205) Lang CA, Ransohoff DF. Fecal occult blood screening for colorectal cancer. Is mortality reduced by chance selection for screening colonoscopy? [see comments]. JAMA 1994; 271(13):1011-1013.

(206) Gilbertsen VA. Proctosignmoidoscopy and polypectomy in reducing the incidence of rectal cancer. Cancer 1974; 34(3):suppl-9.

(207) Gohagan JK, Prorok PC, Hayes RB, Kramer BS, CollectiveName, Lung CaOCSTP et al. The Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial of the National Cancer Institute: history, organization, and status. Controlled Clinical Trials 2000; 21(6 Suppl):251S-272S.

(208) Hoff G, Vatn M, Gjone E, Larsen S, Sauar J. Epidemiology of polyps in the rectum and sigmoid colon. Design of a population screening study. Scandinavian Journal of Gastroenterology 1985; 20(3):351-355.

(209) Berry DP, Clarke P, Hardcastle JD, Vellacott KD. Randomized trial of the addition of flexible sigmoidoscopy to faecal occult blood testing for colorectal neoplasia population screening. British Journal of Surgery 1997; 84(9):1274-1276.

(210) Sambrook, Fritsch, Manniatis. Molecular Clonning: a Laboratory Manual. Second ed. Cold Spring Harbour Laboratory Press, 1989.

(211) Sagar M, Seensalu R, Tybring G, Dahl ML, Bertilsson L. CYP2C19 genotype and phenotype determined with omeprazole in patients with acid-related disorders with and without Helicobacter pylori infection. Scandinavian Journal of Gastroenterology 1998; 33(10):1034-1038.

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