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THE INFLUENCE OF METABOLIC PHENOTYPES UPON THE DEVELOPMENT OF COLORECTAL NEOPLASIA

by

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Table of Contents	(i)-(ii)
Declaration	(iii)
Acknowledgements	(iv)
Abbreviations	(v)
Definitions	(vi)-(vii)
Abstract	(viii)-(ix)
Appendix	(x)-(xxii)

TABLE OF CONTENTS

Chapter 1 **Background and Literature Review**

1.1 Significance of this Research

1.2 Meat and Colon Cancer

1.3 Xenobiotic Detoxification and Colon Cancer
- 1.3.1 N-Acetyltransferase
- 1.3.2 Glutathione S Transferase

1.4 Cellular Proliferation and Colon Cancer

1.5 Aims and Hyphotheses

Chapter 2 **Patients and Methods**

2.1 Patient criteria and samples

2.2 Questionnaire
- 2.2.1 Clinical
- 2.2.2 Dietary

2.3 N-Acetyl transferase
- 2.3.1 Systemic phenotype
- 2.3.2 Tissue
- 2.3.3 Genotyping

2.4 Glutathione S transferase
- 2.4.1 Tissue GST
- 2.4.2 Tissue GSH
- 2.4.3 GSTμ phenotype

2.5 Cellular Proliferation using Flow cytometry

2.6 Statistical methods

Chapter 3 **N-Acetyl transferase and Colorectal Neoplasia**

3.1 Introduction

3.2 Results
- 3.2.1 Systemic phenotype
- 3.2.2 Tissue NAT
- 3.2.3 NAT Genotype

3.3 Summary

Chapter 4 **Glutathione S Transferase and colorectal neoplasia**

4.1 Introduction

4.2 Results

4.2.1 Tissue GST
4.2.2 Tissue GSH
4.2.3 GSTμ phenotype
4.2.4 Tissue protein

4.3 Summary

Chapter 5 **Cellular proliferation and colon cancer**

5.1 Introduction

5.2 Results

5.3 Summary

Chapter 6 **Meat consumption and acetylator phenotype in colon cancer**

6.1 Introduction

6.2 Results

6.3 Summary

Chapter 7 **Conclusions**

References

Appendix

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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ABBREVIATIONS

CI	Confidence interval
dNTPs	dinucleotide triphosphates
GSH	Glutathione
GST	Glutathione S Transferase
HPLC	High performance liquid chromatography
kDa	kilodalton
LI	labelling index
mw	molecular weight
NAT	N-Acetyltransferase
NS	non-significant (statistically)
OR	odds ratio
PCR	Polymerase chain reaction
PI	proliferative index
r	Correlation coefficient
RFLP	Restriction fragment length polymorphisms
SD	Standard deviation
SE	Standard error
Tm	Melting temperature

DEFINITIONS

Allele:	One of several alternative forms of a gene occupying a given locus on a chromosome.
Annealing:	Pairing of complementary single strands of DNA to form a double helix.
Amine:	Any of a group of chemical compounds formed from ammonia by replacing 1 (primary), 2 (secondary) or 3 (tertiary) of the H atoms by organic (hydrocarbon) radicals.
Amphipathic:	Of or relating to molecules containing groups with characteristically different properties eg. both hydrophilic and hydrophobic properties.
Arylamine:	Any of a group of amines in which one or more of the hydrogen atoms are replaced by aromatic groups.
Arylhydroxamic acid:	N-acetyl N hydroxy arylamines.
Base pair(bp):	Partnership of A with T or of C with G in a DNA double helix; other pairs can be formed in RNA under certain circumstances.
Benzo(a)pyrene:	A polycyclic aromatic hydrocarbon containing five fused benzene rings, mw 25,230. It occurs in coal tar, cigarette smoke and air polution, and is a potent procarcinogen that undergoes metabolic activation to an aryl epoxide, the direct-acting carcinogen.

cDNA:	A single-stranded DNA complementary to an RNA, synthesized from it by reverse transcription *in vitro*.
Gene:	The segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).
Genotype:	Genetic constitution of an organism.
Phenotype:	Appearance or other characteristrics of an organism, resulting from the interaction of its genetic constitution with the environment.
Polymorphism:	Simultaneous occurrence in the population of genomes showing allelic variations (eg. as seen in alleles producing different phenotypes).
Restriction enzymes:	Bacterial enzymes that recognize specific short sequences of DNA and cleave the duplex.
Xenobiotics:	Chemicals foreign to the biological system.

ABSTRACT

Epidemiological studies have clearly demonstrated the influence of environmental factors on the risk for development of colonic adenomas and carcinomas. Other studies have raised the possibility that the risk of cancer is influenced by inherited metabolic phenotypes which may increase or decrease the concentrations of carcinogens. The purpose of this study was to assess the role of acetylator status and glutathione S-transferase μ (GSTμ) null phenotype on the risk for development of colorectal neoplasms in humans and to determine whether this was influenced by the dietary intake of meat. The major achievements of this project were as follows:

1 Acetylator phenotype was determined using high pressure liquid chromatography (HPLC) to detect acetylation metabolites of sulphamethazine. Two groups were identified and classified as slow and fast acetylators.

2. Genotyping for acetylator status was developed and concordance was demonstrated between acetylator status as determined by drug metabolism and acetylator status as determined by genotyping.

3. A method was developed for genotyping for the GSTμ null phenotype.

4. The frequency of fast acetylators in patients with colon cancer was higher than control subjects with an odds ratio of 1.8 (95% C.L. 1-3). This effect appeared to be more prominent in women than in men. Patients with colonic adenomas had a similar frequency of fast acetylators to that in control subjects.

5. Patients with colorectal cancer had a similar frequency of the GSTμ null phenotype to that in control subjects.

6. Fast acetylators had higher polymorphic (NAT2) activity in colonic tissue than slow acetylators. However, the activity of colonic polymorphic NAT is much less than the activity of monomorphic NAT (NAT1).

7. Glutathione (GSH) concentrations in colonic tissue were higher in carcinomas and adenomas than in adjacent "normal" mucosa. Furthermore, concentrations of glutathione S-transferase (GST) were higher in carcinomas than in adjacent "normal" tissue.

8. Cell proliferation was higher in neoplastic tissue than in "normal" mucosa and was higher in aneuploid when compared with diploid neoplasms. There was no apparent correlation between cell proliferation and NAT or GST activity or the GSH content of tissues. Cell proliferation in the rectum was similar in patients with adenomas and carcinomas to that in control subjects and was uninfluenced by acetylator status.

9. The risk for development of colon cancer in fast acetylators apeared to be influenced by the dietary intake of meat. For example, odds ratios higher than 1.8 were found in patients with a moderate or high intake of meat when compared to those with a low intake of meat. This effect appeared to be more prominent in women than in men.

The above observations provide new information on the influence of acetylator status on the risk for colorectal cancer and likely interactions with dietary components, specifically meat. This new area of ecogenetics may shed new light on the pathogenesis of intestinal and other cancers.

CHAPTER 1 BACKGROUND AND LITERATURE REVIEW

1.1 Significance of this Research

Colorectal carcinoma is the second most common cause of death from cancer in Australia, the United States of America and Great Britain (Doll and Peto, 1981) and will affect 4-5% of the population. It is the third most common malignancy in men and women worldwide(Muir, 1990). The incidence and mortality of this extremely common malignant condition has not changed appreciably during the last 40 years. In the United States, the five-year survival rate is only 42%, reflecting the advanced stage of the cancer in most patients when first detected (Fath and Winawer, 1983). In Australia, approximately 7000 new cases of colorectal cancer arise each year, resulting in 3700 deaths annually (Armstrong, 1988). Colorectal carcinoma is, therefore, a major Australian health problem.

The cause of colorectal carcinoma is considered multi-stage and multi-factorial as a result of an interaction between environmental and genetic factors. Most malignant colorectal tumours (carcinomas) arise from pre-existing benign tumours (adenomas) (Morson, 1974; Sugarbaker, Gunderson and Wittes, 1985); a phenomenon which allows tumours in various stages of development to be studied. Epidemiological studies of environmental factors have shown a positive association with diets high in protein, fat and calories and a negative association with diets high in dietary fibre . The latter applies to both colorectal carcinoma (Trock, Lanza and Greenwald, 1990) and adenomas(Giovannucci *et al*, 1992).

At least three observations attest to the importance of environmental factors in the pathogenesis of colorectal carcinoma. Firstly, the wide (at least 15 fold) range in age-standardised incidence rates in different parts of the world. Secondly, the rise in cancer incidence when people migrate

from a low incidence country to a high incidence country. Thirdly, changes in incidence in individual countries such as Japan (Willett, 1989) and Greece (Manousos *et al*, 1983) where higher rates for cancer have been associated with the adoption of a westernized diet.

Within a defined population, however, the relationships between colorectal carcinoma and high intakes of protein or fat and low intakes of fibre are poor, and genetic factors are believed to play an important role in determining individual susceptibility to disease. Although hereditary syndromes(familial adenomatous polyposis and the Lynch syndromes) and inflammatory bowel diseases(ulcerative colitis and Crohn's disease) appear to predispose to colorectal carcinoma, these specific conditions exist in only 2-3% of patients (Watne, 1982). In patients with "sporadic" carcinoma, as many as 25% of patients have a family history of the disease, consistent with the involvement of genetic factors. Indeed, it has been predicted that a gene predisposing to colorectal carcinoma is present in almost half of the population (Cannon-Albright *et al*, 1988).

However, despite two decades of epidemiological research into colorectal carcinoma, conclusive identification of pertinent causative factors remain elusive and there is a need to develop new approaches. One approach is to examine the influence of inherited metabolic phenotypes such as the polymorphism associated with N-acetyltransferase and glutathione S transferase. These two enzymes were chosen because of their genetic polymorphism in the human population and their potential influence on the concentration of carcinogens in the colon and other organs of the body. This project, therefore, sought to define the potential role of inherited changes in metabolism which might influence the risk for development of colonic neoplasia. Results were correlated with an assessment of diet and with an assessment of rates of cellular proliferation in the colon; a marker of risk for colonic neoplasia.

1.2 Meat and Colon Cancer

As noted above, the risk for colon carcinoma is likely to be influenced by both environmental and genetic factors. The environmental factors are considered to predominate in causing the wide inter-country differences in incidence but genetic factors might predominate within a country in determining the relative susceptibility of individuals.

Although diet is acknowledged as a major environmental factor, the role of the various components of the western diet remains controversial. A major problem in the studies of diet in colorectal neoplasia is the inter-relationship of foods which create difficulties in ascribing "risk" to individual components of the diet. There is a positive association between meat and fat, and a negative association between animal protein intake and cereal consumption. Furthermore, several studies are complicated by the frequent finding of a positive association between total energy intake and risk for colon cancer; a finding which often fails to diferentiate between total food consumption and consumption of fat.

The evidence for the association of meat consumption with colon cancer is derived from three types of epidemiological studies; correlational, case-control and prospective. Epidemiologically, the strongest association with colon cancer incidence and any dietary factor is with meat. This association was derived mainly from the striking international correlations between the per capita consumption of meat and national rates of the disease(Doll and Peto, 1981), (Rose, Boyar and Wynder, 1986). In addition, several case-control studies have shown an association between meat intake and colon cancer (Benito *et al*, 1990), (Haenszel *et al*, 1973), (La Vecchia *et al*, 1988), (Manousos *et al.*, 1983), (Tajima and Tominaga, 1985) but not in a study from Japan (Haenszel, Locke and Segi, 1980). A prospective study of 88,751 women followed over a six year period (1980-1986) showed a positive association between consumption of red meat and colon cancer with the relative risk for those who ate beef, pork, or

lamb as a main dish daily as 2.49 (95% confidence interval, 1.24 to 5.03) compared to those reporting meat consumption less than once a month (Willett *et al*, 1990). This positive association of meat and colon cancer was also shown in prospective studies from Norway (Blelke, 1980) and Sweden (Gerhardsson, Floderus and Norell, 1988), the latter being a 14 year follow-up of 16,477 men and women. In a limited study on 43 colorectal cancer and 41 control male patients, an enhanced risk of colorectal cancer was associated with bacon, and with barbecued, smoked or cured meats (Wohlleb *et al*, 1990).

The presence of mutagens in meat would provide a rational explanation for the above association. Since 1980, many heterocylic arylamines had been identified in meat and fish cooked at normal cooking temperatures (Felton *et al*, 1986), (Tomita *et al*, 1984). Formation of these arylamines are, however, increased by frying at high temperatures and cooking for longer periods of time (Knize *et al*, 1985). Kasai and co-workers were the first to isolate and identify aminoimidazoquinoline(IQ) and aminomethylimidazoquinoline(MeIQ) from broiled sun-dried sardines (Kasai *et al*, 1980), and aminomethylimidazoquinoxaline(MeIQx) from dried beef (Kasai *et al*, 1981). Both MeIQx and DiMeIQx can be formed by heating mixtures of creatinine, sugars and amino acids (Jagerstad *et al*, 1984), (Negishi *et al*, 1985). Another food derived mutagen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine(PhIP), was isolated from fried ground beef and it has been (Felton *et al* 1986) found to be the most abundant heterocyclic amine in various cooked meats and fish (Wakabayashi *et al* 1992). The mutagens isolated from fried ground beef fall into three groups, imidazoquinolines, imidazoquinoxalines and imidazopyridines. These compounds all have an imidazo group with an amino moiety in the 2 position. They also have a methyl group on one of the ring nitrogens in the imidazo ring and at least one aromatic ring is fused to the imidazo ring(Fig. 1.1).

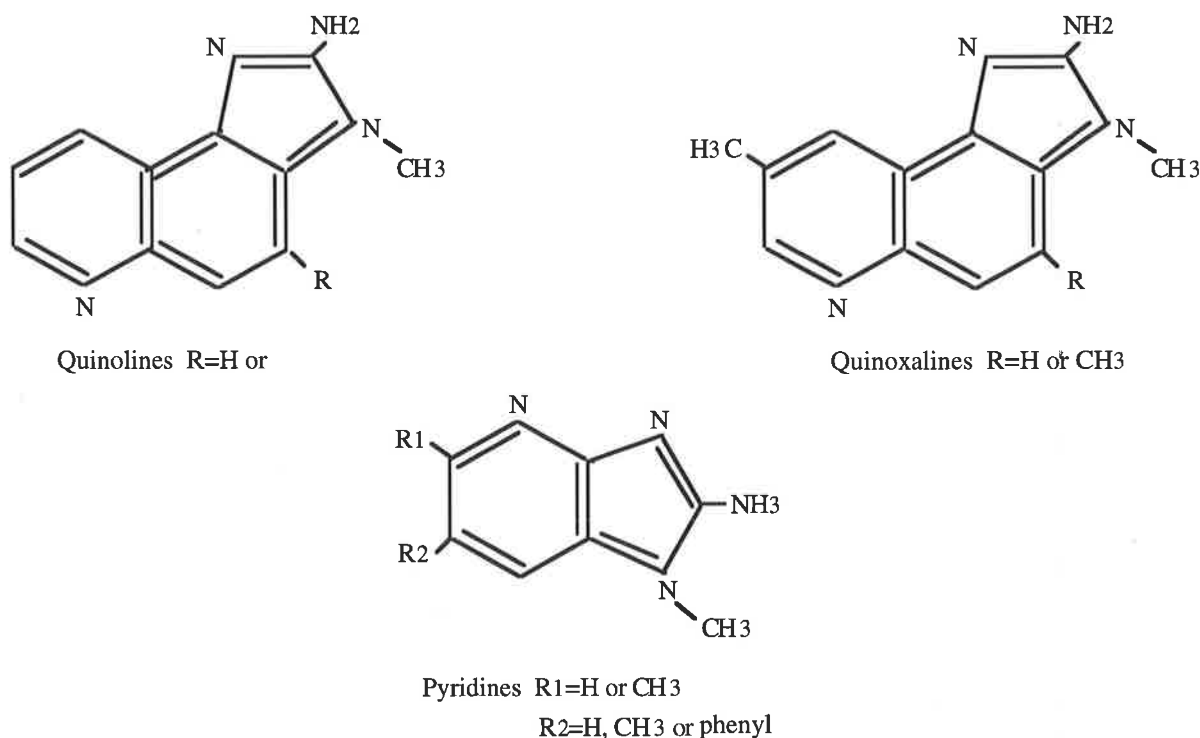


Fig. 1.1 The three classes of arylamines found in cooked ground meat

The carcinogenicity of these heterocyclic arylamines have been shown *in vitro* by the Ames test, and *in vivo*, by the development of tumours in some experimental animals.

In animal studies, Alldrick showed that MeIQx forms covalent links with mouse DNA. He fed mice with radioactive ^{14}C labelled MeIQx intragastrically and then sacrificed the animals after 6 and 24 hours. Analysis of tissues showed radioactive DNA adducts in all organs but particularly in the liver and colon (Alldrick and Lutz, 1989). PhIP has also been shown to induce mutations in the rat *Apc* gene; a phenomenon which may be relevant to PhIP-induced rat colon carcinogenesis (Kakiuchi *et al* 1995). Ohgaki showed that heterocyclic arylamines from cooked meat were carcinogenic when fed to mice and rats. All animals fed arylamines developed tumours, particularly in the liver and, in the case of IQ, in the large intestine (Ohgaki *et al*, 1986). On the other hand, only one of the 100 control rats developed a tumour. It has also been shown that PhIP induces the development of colon carcinoma in F344

rats(Wakabayashi *et al* 1992). The doses that the animals received were much higher than those predicted in humans. We would hypothesise, however, that low doses over long periods of time could result in human tumours, particularly in genetically predisposed individuals. This would be consistent with the observation that colon cancer is a disease of the elderly.

In humans, Hayatsu showed an increase in faecal mutagenicity following a meal of 150 g. of fried beefburgers. Organic compounds of three or more numbers of fused aromatic rings were extracted from fresh faeces using blue cotton, an absorbent cotton bearing covalently linked trisulpho-copper-phthalocyanine residues. The extracted material was subjected to carboxymethyl cellulose chromatography and was positive on Ames tests for mutagenicity. On cessation of meat meals, faecal mutagenicity returned to the original low level. However, standard samples of MeIQx and IQ, on being eluted in cellulose chromatography, were shown not to be compatible with the extract obtained from the cooked ground beef (Hayatsu, Hayatsu and Wataya, 1986).

Other possible procarcinogens in meat are ammonia and phenol (produced from bacterial metabolism of protein), and polycyclic hydrocarbons such as benzopyrene. Studies of these substances in the causation of colorectal carcinoma have been few and inconclusive.

Products of protein fermentation by human intestinal bacteria such as ammonia, branched-chain fatty acids and phenolic compounds progressively increase in concentration from the right to the left colon (MacFarlane, Gibson and Cummings, 1992) and could explain the higher frequency of tumours in the distal colon. Ammonia may also enhance cell proliferation (Visek, 1978). In dietary studies on 4 patients, urinary phenol increased with high meat diets and decreased with diets high in fibre. (Bone, Tamm and Hill, 1976). However, case-control and geographical population studies have shown no association between cancer incidence and urinary phenol excretion (Bone, Tamm and Hill, 1976).

Polycyclic hydrocarbons are environmental carcinogens that produce gastric cancers in mice when administered orally and skin cancers when applied topically (Digiovanni, Slaga and Boutwell, 1980). Smoke and barbecued meat contain benzopyrene although the major source in foods is from vegetables grown in smoky areas (Preussman, 1985).

A polymorphism of a hepatic cytochrome P450 isoenzyme, the P4501A2(CYP1A2) as well as the NAT acetylator phenotype was examined in 205 controls and 75 patients with colorectal adenomas and carcinomas. Both the rapid CYP1A2 and rapid NAT2 phenotype were slightly more prevalent in cases *versus* controls (57% and 52% *versus* 41% and 45% respectively). The combined rapid CYP1A2 and rapid NAT2 phenotype was found in 35% of cases and only 16% of controls, giving an odds ratio of 2.79. However, the consumption of well-done red meat was associated with an increased risk of colorectal neoplasia, such that the odds ratios for cooked meat preference and phenotype combinations ranged from 1.00 for rare/medium preference with slow/slow phenotype to 6.45 for well-done preference with the rapid/rapid NAT2/CYP1A2 phenotype. This shows that given a degree of exposure to food-borne heterocyclic amine carcinogens, the risk of colorectal neoplasia maybe different between slow and fast acetylator phenotypes (Lang *et. al.*, 1994).

1.3 Xenobiotic Detoxification and Colon Cancer

Xenobiotics, or chemicals foreign to the human body, tend to be metabolised by enzymes in the liver or kidney or by enzymes on surfaces exposed to the environment such as the gastrointestinal and respiratory tracts. The reactions which xenobiotics undergo are usually in two phases; the oxidations, reductions and hydrolyses in phase 1 (usually mediated by the P450 cytochrome enzyme system), and conjugations in phase 2 such as N-acetylation and glutathione

conjugation (Diagram 1.1) . The aim of these reactions is to increase the polarity of the compounds to facilitate excretion, either in bile and/or urine.

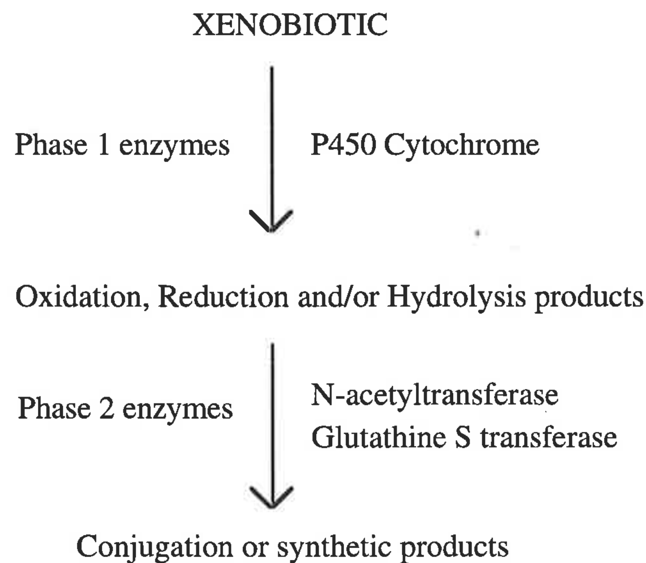


Diagram 1.1 Metabolism of Xenobiotics

1.3.1 N-Acetyl transferase

The N-Acetyl transferases (NAT) are a group of enzymes involved in foreign chemical or xenobiotic (eg. drug and carcinogens) metabolism which are subject to genetic polymorphism. These cytosolic proteins have a molecular weight of approximately 31 kDa and exist as 2 isoenzymes. The monomorphic acetylator genotype-independent form or NAT1 shows little variation between individuals and is present predominantly in extrahepatic tissues. The polymorphic acetylator genotype-dependent form or NAT2 varies between individuals and is found predominantly in the liver (Weber and Hein, 1985). A genetic polymorphism that affects the level of activity of NAT2 is characterised by a reduced capacity to acetylate a variety of arylamine drugs (eg. sulphamethazine, isoniazid, hydralazine, procainamide and dapsone).

These individuals are known as slow acetylators and include about 50% of Caucasians and 10% of Japanese. Those with a greater capacity are known as fast acetylators. The slow acetylators are homozygotes for the recessive allele and the fast acetylators are homo- or heterozygotes for the dominant allele of NAT2 (Weber and Hein, 1985).

It is well known that the acetylator status of individuals influences the frequency of side-effects from some arylamine drugs. The slow acetylator is more prone to develop peripheral neuropathy on conventional doses of isoniazid (Devadatta *et al*, 1960), (Hughes *et al*, 1954), and a systemic lupus erythematosis-like syndrome on hydralazine (Batchelor *et al*, 1980), (Perry *et al*, 1970), (Strandberg *et al*, 1976).

It has also been shown that aromatic chemical carcinogens such as aminofluorene, benzidine and n-naphylamine are acetylated by the same N-acetyltransferase enzymes that acetylate the above mentioned aromatic drugs (Flammang *et al*, 1987). This has led to speculation that susceptibility to certain tumours might be different in the slow and fast acetylator phenotype. This idea has been supported by *in vitro* studies showing that DNA damage by aminofluorene and benzidine was greater in cultured hepatocytes from rabbits showing fast acetylation than from rabbits with slow acetylation (McQueen *et al*, 1982), (McQueen, Maslansky and Williams, 1983).

Epidemiological studies have shown that the slow acetylator phenotype predisposes to the development of bladder carcinoma (Hein, 1988). In contrast, fast acetylators appear to be at increased risk for the development of colorectal carcinoma in Australia and the U.S.A. (Lang, Chu and Hunter, 1986), (Ilett *et al*, 1987) but not in Spain (Ladero, Gonzalez and Benitez, 1991).

Ilett (Ilett *et al.*, 1987) performed a retrospective study of 49 patients with colorectal carcinoma,

41 elderly control subjects and 45 young control subjects. The frequencies of fast acetylators were 44%, 24.4% and 43% in the three groups respectively. There was a statistically significant preponderance of fast acetylators in those with cancer compared to older controls. In this study, the young controls were used to evaluate the influence of age on acetylator status. There were more slow acetylators in the older controls than in young controls but differences were not statistically significant.

The influence of age on acetylator status is controversial. There was a large study of Caucasian Hungarian patients aged 19-91 which showed a significantly increased proportion of slow acetylators in the elderly (Gachalyi, Hajos and Kaldor, 1984). However as Price Evans (Price Evans, 1989) pointed out, they arbitrarily divided patients into those below and above the age of 60 years, and applied the same phenotype separation criteria to both groups. The frequency of slow acetylators was 50% below the age of 60 years (128 subjects) and 66.4% above the age of 60 years (125 subjects). In another study, Paulsen and Nilsson (Paulsen and Nilsson, 1985) showed an increase in the plasma half-life of isoniazid with age in 152 Swedish male patients. Although there was a trend for a longer plasma half-life for isoniazid with increasing age in females, this did not reach statistical significance. However, when they computed the phenotype frequency, they used the dividing antimodal value obtained from the histogram of individuals under 53 years of age and, as a result, found a smaller frequency of fast acetylators in the older group. The phenotype separation between slow and fast tends to be slower with increasing age and the findings of the two studies above might have been different if they had used a different dividing value between slow and fast acetylators for the young and old age groups. A number of smaller studies have shown that age has no significant influence on acetylator status (Cartwright *et al*, 1982), (Desai *et al*, 1973), (Farah *et al*, 1977), (Kergucris, Bourin and Larousse, 1986), (Pontiroli *et al*, 1985). This subject was reviewed by Price Evans (Price Evans, 1989) and Weber (Weber and Hein, 1985) who concluded that the effect of aging on the rate of polymorphic acetylation is slight in comparison with the effect of genotype and is

unlikely to result in misclassification of the individuals' acetylator phenotype.

In Ilett's study, the percentage of slow acetylators was higher in the older control subjects than in other published literature on populations of Caucasian origin. The average frequency of slow acetylators in 20 papers shown below (table 1.1), selected for having the same ethnic groups that are in our sample and probably in Ilett's study (as both were based on the general Australian population), is 58.1% (95 percent confidence interval, 56.6%-59.6%). Conversely, the average frequency of fast acetylators is 41.9% (95% C.L., 40.4%-43.4%)

Reference	Ethnic group.	Location	% Slow acetylator
Evans Series 1(1969)	British White	Liverpool	62.4
Karim & Evans (1976)	British White	Liverpool	68.9
Evans et al.(1972)	British White	Liverpool	65.6
Eze % Evans (1972)	British White	Liverpool	59.3
Shroder & Evans (1972a)	British White	Liverpool	56.3
Shroder & Evans (1972b)	British White	Liverpool	56.3
Gow & Evans (1964)	British White	Liverpool	65.6
Ellard et al. (1975)	British White	London	60.8
Philip et al. (1987)	British White	London	60.8
Lilyin et al. (1984)	Russian	Moscow	51.8
Hoo et al. (1977)	German	Hamburg	51.8
Nanssen et al. (1985)	German	Hamburg	42.3
Bartmann et al. (1960)	German	Berlin	50.4
Iwainsky (1961)	German	Berlin	49
Schmeidel (1960)	German	Leipzig	57.8
Virnerova et al.(1973)	Czech	Prague	59.3
Ellard et al (1973)	Czech	Prague	59.3
Drozdz et al. (1987)	Polish	Katowice	60.8
Pontorili et al. (1985)	Italian	Milan	70.6
Fantoli et al. (1963)	Italian	Rome	54.8

Table 1.1 Percentage of Slow acetylators in Europe

Lang (Lang, Chu and Hunter, 1986) performed a retrospective study, limited to men, on the distribution of acetylator phenotype in patients with colorectal carcinoma and age-matched controls. He showed that there were 28 slow, 2 intermediate and 11 fast acetylators in the controls, and 20 slow, 3 intermediate and 20 fast acetylators in the carcinoma group. There was a significantly higher proportion of fast acetylators in those patients who had colorectal carcinoma compared to controls, when the intermediate acetylators were classsified with the slow acetylators (x^2=3.49, $p<0.03$). However, since the intermediate group is normally composed of heterozygotes (and are usually classified as fast acetylators) statistical significance is lost when the intermediate group is included as fast acetylators in the statistical analysis (x^2=4.07, $0.05<p<0.02$). Morever, if the intermediate group were to be classified as slow acetylators (as was done in Lang's paper), the slow acetylators would have constituted 73.2% of the control sample(a relatively high frequency when compared to other studies above). In both Ilett's and Lang's papers, the excess of slow acetylators in the controls could be due to the small sample size.

Ladero (Ladero, Gonzalez and Benitez, 1991) performed acetylator phenotyping on 109 patients with colorectal carcinoma and 96 age matched controls. Those with colorectal carcinoma consisted of a retrospective group of 74 patients who had previously had surgical resection and a prospective group of 35 who had acetylator phenotyping prior to surgical resection. He found no significant difference in the number of fast acetylators between carcinoma patients (45%) and controls (41.7%). No differences in the proportion of acetylator phenotypes were reported between the retrospective and prospective groups although phenotyping results in subgroups were not given in their paper. Ladero, in discussing the hypothetical rclationship between acetylator status and the risk of colorectal carcinoma, noted that procarcinogens acting as substrates for polymorphic NAT must reach the large intestine and that enzymatic activity must be present in the mucosa before acetylation takes place. The availability of procarcinogens may

be different in Ladero's study when compared to studies by Ilett and Lang since Spanish patients might have been taking a Mediterranean diet rich in fibre and mono-unsaturated fat (olive oil) while patients in Australia and the U.S.A. presumably took a diet with more saturated fat and animal protein. Morever, cooking methods including "barbecuing" which increases pyrolytic changes in food, may be more widely used in the USA and Australia than in Spain.

Ilett's study was done predominantly on male patients (80.5% of controls, 71.4% of carcinoma patients) while Lang's study examined only men. Although no sex differences in acetylator status were known, the proportion of males was smaller in Ladero's paper (43.8% controls, 47.7% carcinoma patients). Ladero reported no differences in the distribution of acetylator phenotype between male and female patients.

If acetylation were to have a role in the aetiology of colorectal carcinoma, the occupational, dietary and drug history of the studied subjects may be important as these would provide information on the exposure of individuals to possible environmental carcinogens. Ilett stated that the distribution of occupations were similar in the older controls and carcinoma patients and that no high-risk occupations were identified. The basis for these conclusions were not displayed in the paper. This issue was not addressed in studies by Lang and Ladero.

Hein , from 12 published studies (comprising 981 bladder carcinoma patients and 1244 control subjects) on the associtation of slow acetylators and bladder carcinoma, estimated an odds ratio of 1.46 slow to fast acetylators. Among the five studies with a documented arylamine exposure (comprising 98 bladder carcinoma patients and 823 control subjects), the odds ratio was 2.24. In addition, analysis of three studies which stratified their bladder carcinoma patients showed an association between tumour invasiveness and the frequency of the slow acetylator phenotype. The application of these methods to the published data on acetylator phenotype and colorectal cancer is as follows:

	Controls (no.)		Carcinoma (no.)	
Acetylator status:	Slow	Fast	Slow	Fast
Ilett et al. 1987	30	11	23	20
Lang et al. 1986	31	10	22	27
Ladero et al. 1991	56	40	60	49

Total Controls; Fast/Slow=61/117 (1) Carcinoma; Fast/Slow=96/105(2)

Odd's ratio =(2)/(1)

$$= \frac{96 \times 117}{105 \times 61}$$

$$= 1.75$$

It thus appears that the association of fast acetylation with colorectal carcinoma is stronger than that of slow acetylation with bladder carcinoma (OR 1.75 cf. 1.46) but less than bladder carcinoma with a history of exposure to arylamines(OR 2.25).

The reasons for the higher risk of colon carcinoma in fast acetylators are unclear, but a consideration of the metabolic pathways that lead to the formation of mutagens provides a plausible explanation. Human populations are chronically exposed to aromatic amines through environmental contamination such as cigarette smoke (Patrianakos and Hoffman, 1979), diesel emissions (Wei and Shu, 1983), (Scheutzle, 1983), complex dyestuffs (Cerniglia *et al*, 1982) and cooked foods. The latter consisted of meat and fish cooked at normal cooking temperatures which have been shown to contain many mutagenic heterocyclic arylamines (Felton *et al.*, 1986), (Tomita *et al.*, 1984). In the tissues(shown in fig. 1.2), the possible metabolic pathways for these arylamines include acetylation by NAT followed by hydroxylation by the P450 cytochrome enzyme system to arylhydroxamic acid. Alternatively, it can be hydroxylated, then acetylated to arylhydroxamic acid. Arylhydroxamic acid is then converted by the action of N,O acyltransferase to N-acetoxyarylamine which is a DNA reactive electrophile capable of promoting carcinogenesis. Hydroxyarylamine can also be converted to N-acetoxyarylamine by

O-acetyltransferase (OAT). Studies done on human liver and colon cytosol indicate much greater activity of O-acetyltransferase than of N,O acyltransferase activity (Turesky *et al*, 1991), (Flammang *et al.*, 1987).

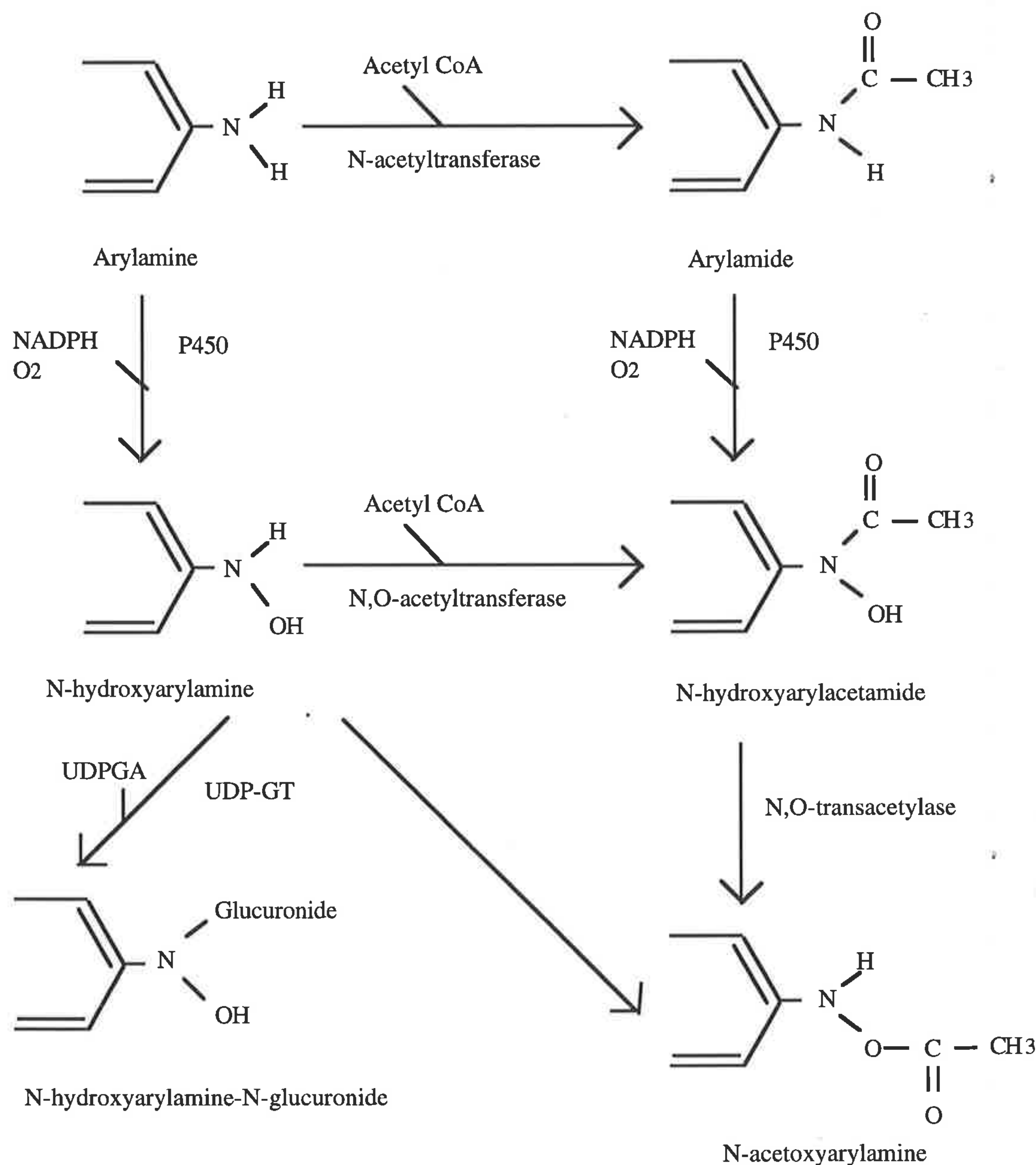


Fig. 1.2 Potential pathways for the enzymatic modification of putative carcinogenic arylamines. Acetyl CoA: acetyl-coenzyme A, NADPH: nicotinamide adenine dinucleotide phosphate, reduced, P450: cytochrome P450 hydroxylase, UDPGA: uridine diphosphoglucuronic acid, UDP-GT: UDP-glucuronyltransferase (adapted from Weber & Hein 1985)

Flammang et al. have shown that sulphamethazine N-acetyltransferase, 2-aminofluorene N-acetyltransferase and acetyl CoA-dependent O-acetylation of N-hydoxy-2-aminofluorene (to form DNA adducts) are all highly correlated with each other and seem to be catalyzed by the same enzyme in human liver cytosols (Flammang *et al.*, 1987). Kirlin showed that NAT and OAT activity in human bladder cytosols vary concordantly with the acetylator phenotype(Kirlin *et al*, 1989). The OAT activity in colonic cytosol was found by Turesky (Turesky *et al.*, 1991) to be lower than fast acetylator liver cytosols and could not be readily segregated into slow-fast phenotypes, probably because of the small numbers(n=8) and the varied contribution of monomorphic NAT which would be expected to participate in the enzymatic acetylation of hydroxyarylamines. Kirlin et al., however, found a polymorphic distribution of NAT activity in 46 human colons for p-aminobenzoic acid(PABA), 4-aminobiphenyl, 2-aminofluorene and β-naphthylamine which segregated into 3 distinct phenotypes consistent with the distribution of patients into rapid, intermediate and slow acetylators(Kirlin *et al*, 1991). This is in contrast to other studies which showed a monomorphic distribution of NAT for PABA and may represent a tissue-specific response of NAT activity towards PABA. It thus appears that OAT and NAT activity are mediated by the same enzyme.

The explanation for the association of slow acetylators with bladder carcinoma, and fast acetylators with colorectal carcinoma may be in the differences in acetyltransferase levels in the different target tissues. Kaderlick (Kaderlik *et al*, 1991) showed that hydroxyarylamines are substrates for human hepatic glucuronyl-transferases and not hepatic N-acetyltransferases, suggesting that the hydoxyarylamines maybe conjugated with glucuronide and excreted in bile into the intestine. Biliary excretion of arylamine conjugates in bile has been shown in animal models but not in humans. An acid labile N-hydroxy 3,2'-dimethyl-4-aminobiphenyl (DMABP,an arylamine carcinogen) N-glucuronide was identified as a major metabolite in the bile and urine of DMABP treated rats and hamsters (Flammang *et al*, 1985). Moriya showed

that bile from DMABP treated rats was mutagenic to Salmonella typhimurium TA100, indicating that biliary DMABP metabolites can react with DNA. In another study on rats, Luks found the glucuronic acid and sulphate conjugates of the 5-hydroxy derivative of IQ in bile, after the administration of 2-amino-3-methylimidazo{ 4,5-f} quinoline (IQ), a carcinogenic arylamine from cooked food. The identification of N-hydroxy PhIP N3-glucuronide as a major rat biliary metabolite , as well as its susceptibility to hydrolysis by β-glucuronidase to reform N-OH-PhIP suggested that N-glucuronidation of N-OH-PhIP could play a similar role in the bioactivation pathway for PhIP (Kaderlik *et al* 1994).

The hydroxyarylamine glucuronide is likely to undergo hydolysis by β glucuronidase in the large intestine. As the human colon has appreciable hydoxyarylamine OAT activity, it is hypothesised that fast acetylators with higher OAT activity have a greater likelihood of N-acetoxyarylamine production leading to carcinoma formation.

However, on ligating the bile duct of rats prior to the oral administration of PhIP, no effect was found on the formation of PhIP-DNA adducts in the colon. This indicated that biliary transport of N-OH-PhIP N3 glucuronide was not required for PhIP -DNA adduct formation in this carcinogen target tissue. In the bile-ligated rats, the urinary concentration of N-OH-PhIP N3 glucuronide was increased 70%, indicating that higher levels of this metabolite were circulating through the blood. These conjugates could be delivered via the systemic blood circulation as part of a bioacativation/deconjugation pathway similar to that described in the colonic lumen, and be another mechanism of causing colonic carcinogenesis(Kaderlik *et al,* 1994).

In the case of bladder carcinoma, the hydroxyarylamine glucuronide is excreted into the urine and is hydrolyzed into the unconjugated hydroxyarylamine in the acid environment of the bladder . In this environment, the N-hydoxyarylamine has been found to spontaneously react with DNA. Turesky showed higher radiolabelled N-hydroxyarylamine metabolites reacting

with DNA at an acidic pH (5.0) than at neutral pH (7.0) in hepatic and colonic cytosols (Turesky *et al.*, 1991). In an animal model, Flamming showed that incubating DMABP with calf thymus DNA formed 370 arylamine residues per 106 nucleotides at pH 4.6 but only 2 residues per 106 nucleotides at pH 7.4 (Flammang *et al.*, 1985). On analysis, the DNA was found to contain major adducts of the same type as those detected in the liver and colon of rats administered DMABP or its hydroxamic acid (Westra *et al*, 1985). It is hypothesised that in slow acetylators, lower levels of hepatic NAT activity shunt greater quantities of hydoxyarylamine glucuronide into the bladder causing a higher level of mutagenic metabolites which result in carcinoma formation. The levels of DNA adducts in exfoliated bladder cells isolated from urine, and of 4-aminobiphenyl-haemoglobin adducts were found to be higher in slow than in fast acetylators (Vinels *et. al.*, 1994).

The human NAT gene has been sequenced from human liver cDNA (Ohsako and Deguchi, 1990) and from human genomic DNA obtained from leucocytes (Blum *et al*, 1990). The coding region of the NAT gene is 870 bases long and has no introns. Two different types of human NAT have been transiently expressed in a mammalian expression system (Chinese hamster ovary cells) (Ohsako and Deguchi, 1990).

Identification of the substrate specificities of the expressed proteins has demonstrated two distinct loci for NAT. One locus encodes the polymorphic and the other, the monomorphic NAT. Both loci have been located on human chromosome 8 (Blum *et al.*, 1990).

The fast acetylator allele(named F1) is inherited in a dominant autosomal manner while the slow acetylator characteristic (due to multiplc allclcs, S1,S2 and S3) is inherited in a recessive fashion.

In Ohsako's study, total RNA was extracted from human livers obtained from autopsy using the

guanidine thiocyanate method, and poly A RNA was selected by oligo(dt) cellulose chromatography. Human cDNA were prepared from the selected mRNA by using viral reverse transcriptase and ligated into bacteriophages. These cDNA libraries were screened with ^{32}P labelled cDNA of rabbit liver NAT on nitrocellulose filters. Positive phages were cloned and sequenced by the dideoxy method.

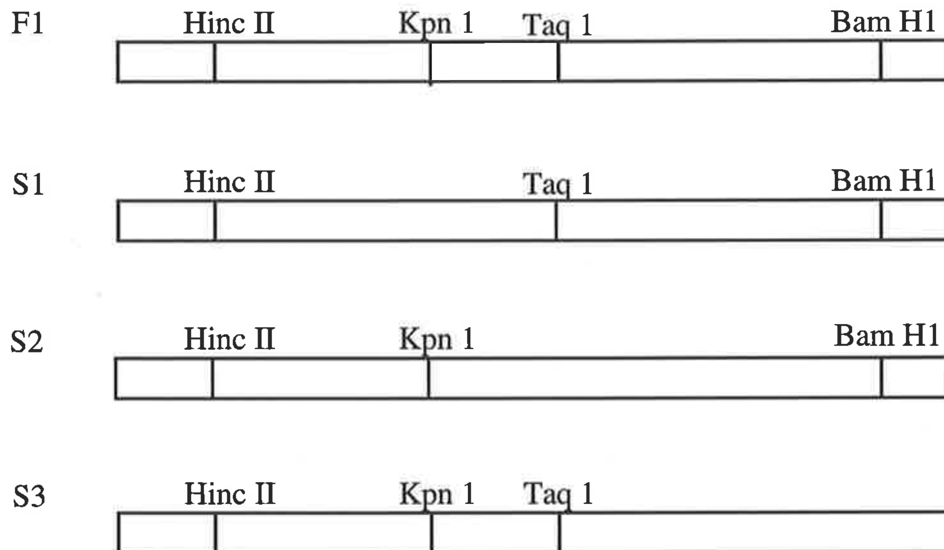
These human cDNAs were inserted into expression vectors and transfected into Chinese hamster ovary cells. Expressed NAT activity was determined using different substrates and led to the assignment of the F1, S3 alleles of the polymorphic NAT as well as the monomorphic NAT allele. Our NAT sequence is based on their polymorphic NAT sequence. This same Japanese group further described the S2, S3 and F1 alleles of the polymorphic NAT as a result of Restriction Fragment Length Polymorphism studies of DNA from individuals of known acetylator phenotype (Deguchi, Mashimo and Suzuki, 1990).

Blum (Blum *et al.*, 1990) derived the NAT sequence from human genomic DNA obtained from leucocytes. The human genomic library were cloned in a bacteriophage and screened with ^{32}P labelled rabbit cDNA as a probe. Positive clones were sequenced using the dideoxy method. They demonstrated the sequences of monomorphic and polymorphic NAT(S1, S2, S3, F1) on chromosome 8, as well as an NAT pseudogene. These NAT genes were found to have no introns.

The deduced amino acid sequences of the NAT genes show about 80% homology with the deduced sequences of cDNA clones for the rabbit and chicken NAT genes.

The alleles of the NAT gene contain different patterns of restriction enzyme sites, shown in fig. 1.3.

Polymorphic NAT



Monomorpnic NAT

Fig. 1.3 On the NAT sequence are marked the enzyme restriction sites that differentiate between the different NAT alleles.The Hinc II site is present in all polymorphic NAT alleles but is missing from the monomorphic allele. The monomorphic allele contains a HindIII site which is not present in any of the polymorphic NAT alleles.

Two small studies have shown an approximate distribution of the polymorphic alleles in Caucasian(Hickman and Sim, 1991) and Japanese (Deguchi, Mashimo and Suzuki, 1990)people as follows:

	Allelic frequency (%)	
	Caucasian	Japanese
F1	26	68
S1	45	0
S2	27	25
S3	2	7

The Caucasians were mostly slow acetylators with the most frequent slow acetylator allele being S1, whereas the Japanese were mostly fast acetylators and their most common slow acetylator allele was S2.

In addition to the three NAT2 slow acetylator alleles described above which are the most common and important, there are also two other rare slow alleles which are mutations within the S1 slow allele (Dickman *et. al.*, 1992).

Although it was suggested that fast acetylators were more likely to develop colorectal carcinoma, the Japanese (who are mostly fast acetylators) have a national incidence of colorectal carcinoma which is lower than Western nations (Doll and Peto, 1981). An explanation for this may be that meat consumption and the preparation of meat are different in the two cultures; changes which substantially lower the concentrations of procarcinogens in the diet of the Japanese.

1.3.2 Glutathione S Transferase

The glutathione S transferases (GST) are a group of isoenzymes which act as mediators of glutathione (GSH) conjugation to xenobiotics and as intracellular binding proteins. They also have organic peroxidase activity. They are dimeric proteins with a molecular weight of approximately 25 kDa and are found in the cytosol of all animal cells, with the exception of a microsomal form that has been identified in rodents which is a trimer (Morgenstein, De Pierre and Jornvall, 1985). As cnzymcs, thcy catalyzc thc conjugation of reduced glutathione (GSH, g-glutamylcysteinylglycine) to a wide range of xenobiotics, including carcinogens (eg. benzopyrene) and anti-neoplastic drugs (eg. melphalan and bis { 2-chlroethyl} -1-nitroso urea} (Dulik, Freselau and Hilton, 1986,). The conjugation of glutathione with electrophiles prevents

the interaction of electrophiles with critical target sites such as DNA. This glutathione conjugate maybe excreted as such or, as a result of action by peptidases, can be hydrolyzed to an S-(substituent) cysteine derivative with the loss of glutamic acid and glycine (fig 1.4). The cysteine derivative can then be N-acetylated to form mercapturic acid, a common metabolite of xenobiotics which is excreted in the urine. The S-(substituent) cysteine derivative can also interact with C-S lyases to form mercaptans with the loss of ammonia and pyruvate. The mercaptans are then glucuronidated or methylated by S methyl transferase to form methylsulfinyl compounds which are excreted via bile in the faeces(Bakke and Gustafsson, 1984).

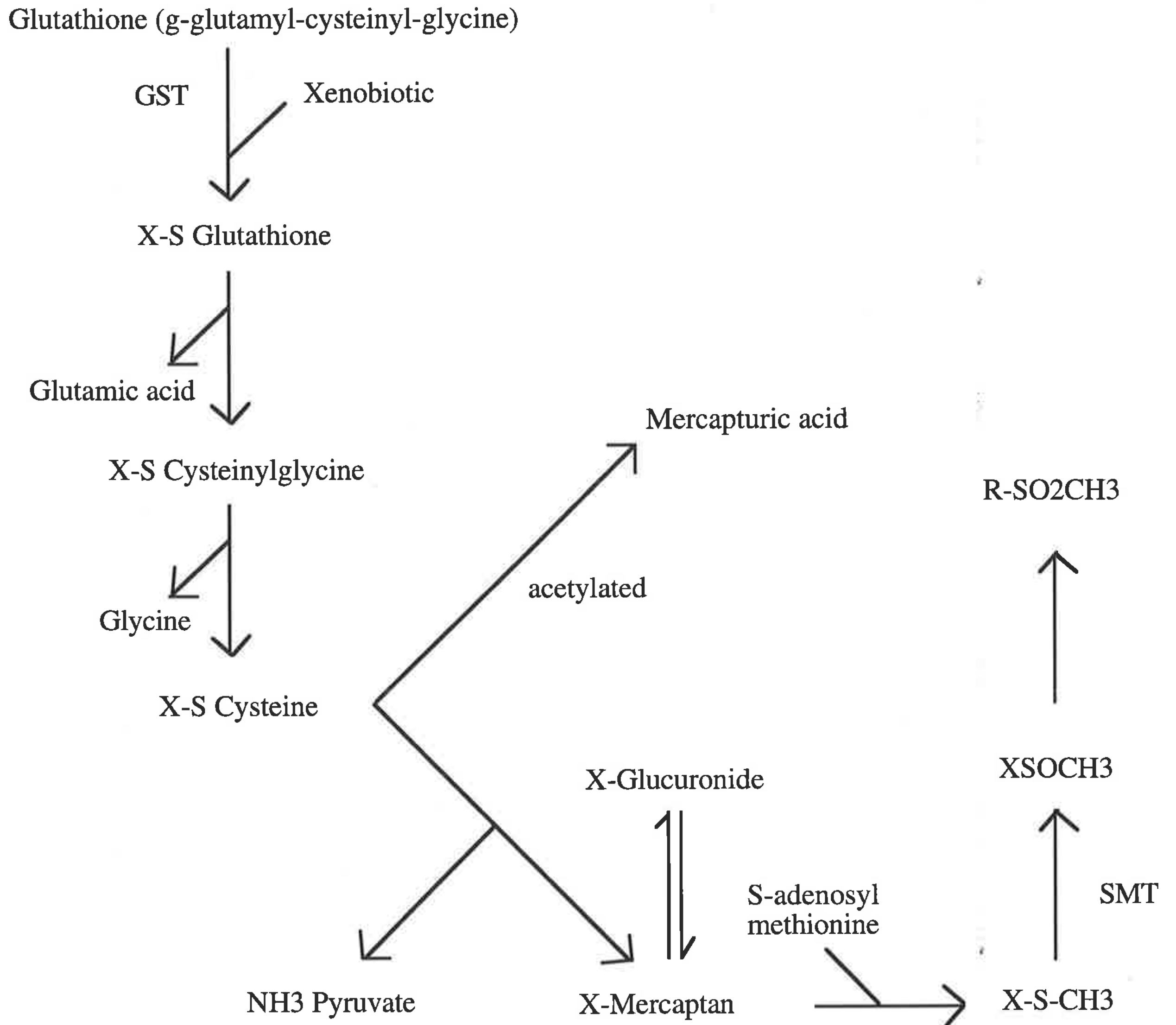


Fig. 1.4 Glutathione metabolic pathways (GST=Glutathione S transferase, SAM=S-adenosylmethionine).

In contrast to their catalytic role as above, the GSTs bind a number of nonsubstrate hydrophobic and amphipathic compounds such as bilirubin, haem, bile acids and steroids (Bhargava, Listowsky and Arias, 1978), (Homma and Listowsky, 1985). One of the nonsubstrate ligands was called Y protein or ligandin until Kaplowitz and colleagues (Kaplowitz, Percy Robb and Javitt, 1973) observed that these proteins also had glutathione S transferase activity. In this

setting, the GSTs may act as an intracellular transport system for organic compounds which have limited solubility in water. Although the binding of some compounds appears to be reversible, the covalent binding of certain reactive electrophilic compounds has beeen observed (Hayes and Wolf, 1988) and may represent an additional protective mechanism. Thus, by both catalytic activity and direct binding, the GSTs protect the cell from a variety of electophilic and free radical intermediates, either exogenous or endogenous. However, the relative contribution of this detoxification system in the protection of the colonic mucosa is not known.

Within a given species, there are a number of isoenzymes of GST which can be separated by isoelectric focussing, chromatofocussing or high performance liquid chromatography (Mannervik, 1985), (Ostlund Farrants *et al*, 1987), (Radulovic and Kulkarni, 1986). These enzymes are ubiqutitous in nature, but vary between species and between tissues within individuals, depending on the stage of development (table 1.2). Reasons for the variable expression in different tissues is unknown but may reflect the differences in stresses on different cell types. As shown in table 1.2, human GSTs can be divided into 3 major classes, GST1, GST2, GST3 (also termed mu (μ), alpha(α) and pi(π) respectively). Historically, this division has been based on different isoelectric points depending on pH on starch gel electrophoresis and more recently, has been shown to be controlled on different genes by in-situ hybridisation (Board and Webb, 1987), (Board, Webb and Coggan, 1989), (De Jong *et al*, 1988).

GST	1(mu)	2(alpha)	3(pi)
Property	Basic	Neutral	Acidic
Isoelectric pt.	7.5-9	6.6	4.8
Chromosome	1	6	11
Tissue	Liver(adult) Kidney Adrenal Stomach	Liver(adult) Kidney	Red blood cells Placenta Liver(foetal) (Respiratory, urological and GI epithelial tracts)

Table 1.2 Showing properties and tissue location of different human GST isoenzymes.

In man, there are three autosomal alleles at the GST1 or µ locus (ie. type 1, type 2 and type 0 or null) with the possible expression of four phenotypes in tissues (1 { genotype 1,0 or 1,1} ; 1-2 { genotype 1,2} ; 2{ genotype 2,0 or 2,2} and 0 { genotype 0,0}). The frequency of these four phenotypes was 18%, 6%, 35% and 41% respectively in an English population (Strange *et al*, 1984b), and 9%, 5% 43% and 43% respectively in a Parisian population (Laisney *et al*, 1984). Both studies were performed on tissues removed at post-mortem. Studies on Indian, Chinese and Caucasian populations have shown that this lack of GST µ phenotype ranged from 31% to 67%, the latter being the only Australian study (Board, 1981). The expression of GSTµ (either present or absent) has been shown to be consistent in various tissues (leucocytes, small and large bowel and liver) in the same individual (Siedegard, De Pierre and Pero, 1985). The GSTµ null phenotype is due to a homozygous deletion of the GSTµ gene (Seidegard *et al*, 1988). High GST activity is inherited in an autosomal dominant manner.

In the liver, the GST 2 or α class consists of 2 major enzymes which have close homology; differing in only 11 amino acids. These 2 enzymes appear to be the products of separate loci that show different levels of expression between individuals (Hayes, Kerr and Cronshaw,

1989), (Rhoads, Zarlergo and Tu, 1987) and between tissues within an individual (Strange *et al*, 1984a), (Suzuki *et al*, 1987).

The isoenzyme known as GST3 or π appears to be the most widely distributed GST isoenzyme and, thus far, a genetic polymorphism has not been shown. GST π levels are increased in neoplastic tissue when compared with non-neoplastic tissue, apparently because of GST π gene activation during malignant transformation. An immunohistochemical method using monoclonal antibodies to GST π demonstrated marked increases in GST π levels in human cancers of the brain, cervix, endometrium, colon, rectum, testis and in fibro- and chondrosarcomas (Kantor *et al*, 1991). These observations suggest that increases in GST expression are a common accompaniment of neoplasia but the biological significance of this association is unclear.

Alterations in the glutathione-based detoxification system have been associated with resistance to alkylating agents, platinum compounds and radiation in many experimental tumour models (Arrick and Nathan, 1984). For example, there is increased expression of GSTs and amplification of GST genes in cell lines selected for resistance to nitrogen mustard and the alkylating agent, chlorambucil (Buller, Clapper and Tew, 1987), (Lewis *et al*, 1988). Puchalski and Fahl (Puchalski and Fahl, 1990) have shown that expression of several recombinant GSTs in cultured cell lines also confers resistance to alkylating agents. These studies suggest that over-expression of GSTs in tumours may be a significant factor in the emergence of resistance to chemotherapeutic drugs, presumably because of drug inactivation through conjugation with glutathione.

The relative activities of GST isoenzymes for different substrates are shown in Table 1.3. Three points are worthy of note. Firstly, GSTμ has the highest activity against 1, chloro, 2,4, dinitrobenzene, a commonly used substrate for the measurement of total GST activity.

Secondly, GST μ has a high activity against trans-stilbene oxide which is used by some to determine the GSTμ phenotype in lymphocytes (Siedegard *et al*, 1986). Thirdly,GST μ has high activity against polycyclic aromatic hydrocarbons, including benzopyrene 4,5 oxide, which are carcinogenic products of incomplete combustion and are found in cigarette smoke, air pollution and coal tar. Other potential carcinogenic substrates for GSTs include nitroaromatic hydrocarbons, aromatic amines, formaldehyde, alkylating agents and nitrosamines.

Substrate	1(Mu)	2(alpha)	3(pi)
1-Chloro-2,4dinitrobenzene	**148**	19	119
Bromsulphalein	**0.58**	0.008	0.007
trans-4Phenyl-3buten-2one	**0.044**	0.009	0.013
trans-Stilbene oxide	**0.049**	0.045	0.013
Benzo(a)pyrene 4,5-oxide	**0.076**	0.009	0.033
1,2-Dichloro-4-nitobenzene	**4.4**	0.062	0.14
4-Hydroxynonenal	**6.0**	1.1	2.6
Leukotreine A4	**0.01**	-	0.002
p-Nitrophenyl acetate	**0.59**	0.011	0.21
Ethacrynic acid	0.12	0.025	**1.4**
1,2Epoxy 3(nitrophenoxy)propane	0.48	0.23	**0.77**
Benzo(a)pyrene 7,8-9,10oxide	0.11	-	**1.1**
Androstene-3,17-dione	0.043	0.035	**0.14**
Cumene hydroperoxide	0.11	**11.6**	0.14
H_2O_2	<0.03	<0.03	<0.03

Table 1.3 Specific activities(umol/min/mg) of mouse glutathione transferases (adapted from Mannervik,table 7(Mannervik and Danielson, 1988).

The high frequency of propagation of the GSTμ null phenotype through many generations raises the possibility that the GSTμ null phenotype has no major disadvantage to survival. However, as most cancers occur in older people, any disadvantage of the GSTμ null phenotype would not be seen until individuals had reproduced and transmitted the GSTμ gene.

Differences in detoxification capacity between individuals eg. whether an individual is of a GSTμ null phenotype or not, may determine susceptibility to injury by certain environmentally derived toxins and carcinogens, particularly epoxides which are metabolized best by GSTμ. Using trans-stilbene oxide (tSBO) as the substrates, Siedegard (Siedegard *et al.*, 1986) reported a significantly reduced frequency of GSTμ in heavy smokers(defined as >30 pack year) with lung cancer(30.4%, n=46) compared to heavy smokers without lung cancer(58.5%, n=65). This difference was not statistically significant in light smokers. This observation suggests that higher activity of GST maybe important in the detoxification of some of the noxious compounds in tobacco smoke which promote lung cancer. However, the frequency of GST-tSBO in patients with squamous cell carcinoma of the lung(usually associated with smoking) was not statistically different to that in control subjects. A further study by Siedegard (Siedegard *et al*, 1990) also showed a significant reduction of GST-tSBO phenotypes amongst all smokers with lung cancer(37.6%, n=125) when compared to controls(57.9%, n=114), although the association with squamous cell carcinoma again failed to reach statistical significance. Surprisingly, however, a highly significant association was found with adenocarcinoma of the lung (p=0.001), a tumour not related to smoking. A subsequent study by another group failed to show a significant association between low activity of GST towards trans-stilbene oxide and the risk of lung cancer, although the results were compatible with a moderate protective effect in heavy smokers with high GST activity (Heckbert *et al*, 1992).

The influence of the GSTμ null phenotype has also been examined in patients with breast cancer and found to have no relationship to prognostic factors such as hormonal receptor and nodal status(Shea *et al*, 1990). Peters, using monoclonal antibodies against GSTμ, found no difference in the distribution of the GSTμ null phenotype in those with breast cancer(59.6%, n=52) when compared to controls(62.5%, n=64)(Peters *et al*, 1990). In another study, the frequency of the GSTμ null phenotype was significantly higher in those with two or more skin tumours with different histological type (SCC, BCC or malignant melanoma) (Heagerty *et al*,

1994). This increased frequency of GSTμ null phenotype was, however, not shown in those with a single type of skin tumour.

Since smokers have a higher incidence not only of lung cancers, but also of oesophageal, laryngeal and bladder cancers, it has been suggested that the carcinogenic substances in cigarette smoke can adversely affect various organs via the systemic circulation. In a similar fashion, we would hypothesise that colonocytes can be exposed to toxic xenobiotics, either from the gut lumen or from the circulation. One possibility is that individuals with the GSTμ null phenotype are more susceptible to colonic neoplasia or to biological changes associated with neoplasia such as enhanced colonocyte proliferation. The latter possibility of a relationship between GST activity and proliferation has been raised by *in vitro* studies using rat liver cells. In these studies, the growth status of cells showed a significant regulatory control on the expression of genes coding for the various isoenzymes of GST (Batist, Woo and Tsoa, 1991). Despite these theoretical considerations, Peters found no difference in the distribution of the GSTμ null phenotype between his controls(62.5%, n=64) and a group of patients with colon cancer(66%, n=50) (Peters *et al.*, 1990).

In colonic tissue, previous studies have shown higher total GST activity in tumours than in the corresponding normal mucosa (Siegers *et al*, 1984), (Peters, Nagengast and Wobbes, 1989)(Mekhail-Ishak *et al*, 1989). Seigers also reported a lower GSH content and higher GST activity in carcinomas from the sigmoid colon than in normal or neoplastic tissues from other regions of the colon, although results for individual regions varied widely and the number of tissues studied was small. The major isoenzyme expressed in colonic tissue is GSTπ with lower levels of GSTμ and GSTα (Peters, Nagengast and Wobbes, 1989). The high level of GSH (exceeding that in the liver) and the presence of GSH-dependent enzymes (GST and GSH peroxidase), in the human intestinal mucosa suggests that these mechanisms have a role in protection of intestinal cells from toxic or carcinogenic damage. Furthermore, colonocyte levels

of glutathione peroxidase, GST and glucuronyl transferase have been shown to decrease with age in Fischer 344 rats, and may be relevant to age-related increases in the incidence of cancer (McMahon, Beierschmitt and Weiner, 1987).

1.4 Cellular Proliferation and Colon Cancer

Abnormal proliferation of cells is an important feature of many diseases, particularly malignancy. A long held hypothesis, substantiated in experimental literature, indicates that accelerated stem cell division in human tissue can increase the rate at which somatic mutations "escape" repair and progress to a neoplasm. This reasoning is supported by the rarity of tumours in slowly dividing tissues (Preston-Martin *et al*, 1990).

Proliferating cells pass through a series of discrete phases in their cell cycle. Howard and Pelc (Howard and Pelc, 1951),who were largely responsible for the modern concept of the cell cycle, described five phases. From the G1(gap 1) phase, cells enter either the G0(resting) or S(DNA synthesis) phase. From the S phase, they enter the G2(gap 2) phase from which they enter the M(mitotic) phase, and then back into G1. Chromosomes are duplicated during the S phase.(Fig. 1.5). Increased cell proliferation is indicated by an increased percentage of cells in the S, G2 and M phases.

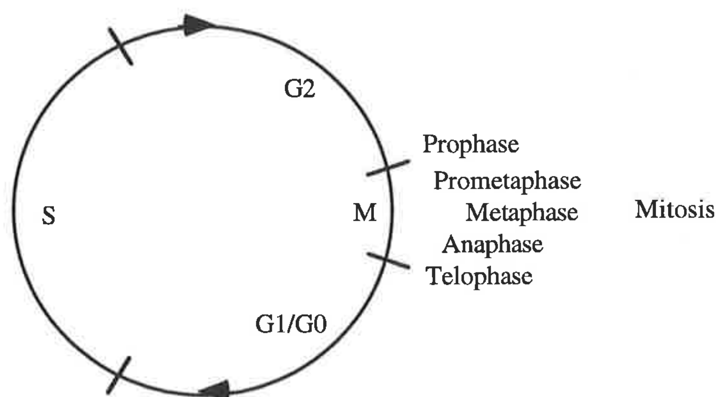


Fig. 1.5 The cell life cycle

The normal human somatic cell, with the 46 chromosomes (23 pairs) is referred to as diploid while a cell with fewer or more than 46 chromosomes is described as aneuploid. Most tumours tend to have a chromosome number near diploid or a high chromosome number either through such mechanisms as non-dysjunction, endoreduplication or cell fusion(Friedlander, Hedley and Taylor, 1984).

Chromosomal analysis, although applicable to leukaemias, has major technical difficulties with solid tumours where only 10-20% of chromosome spreads are interpretable. Also, the identification of individual chromosomes is possible only during metaphase.

Quantitative DNA analysis as in flow cytometry, however, can analyse DNA content on interphase cells. Flow cytometry is characterised by high precision and speed of sample handling. The method of measuring cellular DNA relies on the binding of fluorescent dyes such as propidium iodide to DNA. This binding varies with the amount of DNA in cells in different phases of the cell cycle. In a flow cytometer, laser light illuminates the stream of labelled cells or nuclei to generate a DNA histogram (Fig. 1.6). Two characteristics of the tissue cell population, proliferative activity and ploidy, can be determined from this histogram. A computer then analyses the percentage of cells in the different phases of the cell cycle. The

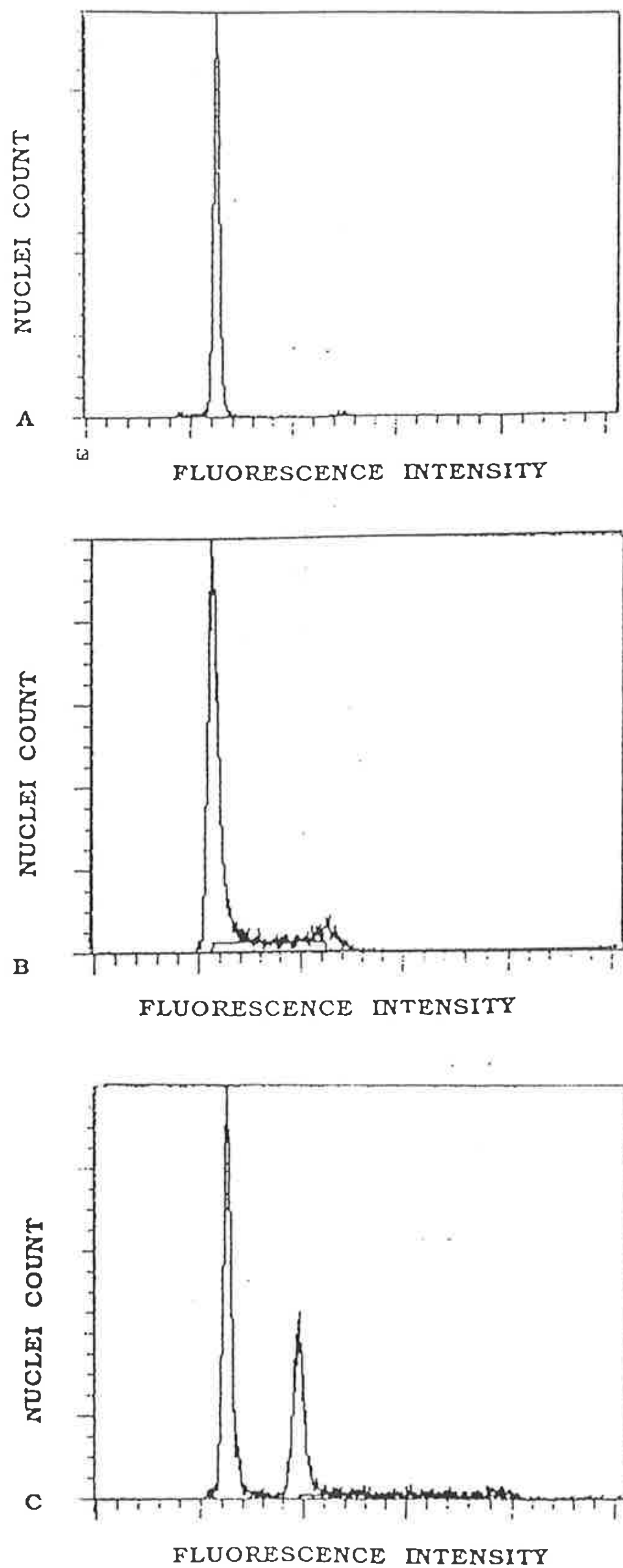


Fig. 1.6 Flow cytometric DNA content in normal and neoplastic colonic tissue.
A: normal colonic mucosa showing a normal diploid nuclear population and a low proliferative activity. B: neoplastic mucosa showing a diploid nuclear population with an increased proliferative activity. C: neoplastic mucosa showing an aneuploid nuclear population near a diploid population.

percentage of cells in S and G_2M obtained from flow cytometry is therefore a quantitative measurement of cell proliferation. The degree of ploidy can be quantitated by the DNA index which represents the ratio of the DNA content of tumour G1 cells to the diploid G1 peak. Thus a DNA index of 0.8-1.2 is synonymous with a diploid DNA content while an aneuploid tumour has a DNA index of <0.8 or >1.2. In most instances, there is good correlation between the chromosome number and cellular DNA content although the resolution of flow cytometry is such that gains or losses of up to 2 chromosomes would not be detected.

Tumour S phase fraction, as determined by flow cytometry, has been closely correlated with tumour labelling index by several investigators (Costa *et al*, 1981), (McDivitt *et al*, 1985). In contrast to labelling studies, flow cytometric studies do not require viable fresh tumour tissue. Both frozen and formalin fixed materials are amenable to study.

The potential for malignant change in the adenoma has been associated with adenoma size, histologic type and the presence of dysplasia (with large adenomas being associated with highest risk). Two groups have reported that the incidence of aneuploidy is related to the size of the adenoma (van den Ingh, Griffioen and Cornelisse, 1985), (Quirke *et al*, 1986). In these reports, aneuploidy was seen in none of 78 adenomas <1 cm diameter, 15 of 88(17%) adenomas 1 to 2 cm in diameter and in 9 of 41 (22%) >2 cm. Conversely, two reports using substantial numbers of cases failed to see a higher frequency of aneuploidy with increasing adenoma size (Banner *et al,* 1987), (Borkje *et al,* 1987). Aneuploidy has also been correlated with adenoma type. Combined results from 3 reports show aneuploidy in 9 of 125 (7.2%) tubular, 17 of 58 (29.3%) tubulovillous and 9 of 41 (22%) villous adenomas (Banner *et al*, 1986), (Quirke *et al.*, 1986), (Sciallero *et al*, 1988). There are also studies that have shown an association of DNA aneuploidy with dysplasia, lending support to the adenoma-carcinoma sequence. Goh and Jass (Goh and Jass, 1986) reported aneuploid adenomas in 5 of 134 (4%)

showing mild dysplasia, 19 of 107 (18%) showing moderate dysplasia and 10 of 28 (36%) showing severe dysplasia. Giaretti and Santi found aneuploid adenomas in 33 of 150 (22%) showing mild-moderate dysplasia, 21 of 45 (47%) showing severe dysplasia and 18 of 25 (72%) with a foci of adenocarcinoma (Giaretti and Santi, 1990). Thus adenomas with foci of carcinoma had a similar incidence of DNA aneuploidy to that of carcinomas. In a smaller study, Banner et al found that 7 out of 17 (41%) adenomas with carcinoma in situ were aneuploid (Banner *et al.*, 1986). A positive association of aneuploidy and dysplasia was also reported in a small study of 35 adenomas by Sciallero et al.(Sciallero *et al.*, 1988). However, Quirke et al in a study of 156 colorectal adenomas found no association of aneuploidy with dysplasia (Quirke *et al.*, 1986).

In colorectal cancer, the combined results of 10 studies (Armitage *et al*, 1985), (Giaretti and Santi, 1990), (Goh and Jass, 1986), (Kokal *et al*, 1986), (Rognum, Thorud and Lund, 1987), (Schutte *et al*, 1987), (Scott *et al*, 1987), (Jones, Moore and Schofield, 1988), (Melamed *et al*, 1985), (Wooley *et al*, 1982) show aneuploidy in 682 of 1020 (67%) tumours. However, there was no consistent association between ploidy and either stage or grade of the primary tumour (Armitage *et al.*, 1985), (Finan *et al*, 1986) apart from one study showing an association between ploidy and tumour stage (Banner *et al*, 1985). There is, however, some evidence that DNA aneuploidy is a poor prognostic marker in colorectal carcinoma. In 1982, Wooley et al reported a striking survival difference between 20 patients with diploid and 13 with aneuploid cancers; their 5 year survival rates were 65% and 7.5% in the two groups, respectively (Wooley *et al.*, 1982). However, an assessment of the relative impact of other prognostic variables was not made, and in fact, 85% of the aneuploid patients but only 50% of the diploid patients had Stage C disease. In another study of 134 patients, Armitage et al found the 5 year survival rate to be 43% for patients with diploid tumours but only 19% for those with aneuploid tumours (Armitage *et al.*, 1985). A significant survival advantage was seen for patients with diploid tumours for each Duke's stage, and ploidy and stage were found to be independent prognostic

factors in a Cox model. In a similar study, Kokal et al found ploidy to be the most important prognostic factor in 72 patients with Stage B and C disease patients; the 5 year survival rate was 100% in the 22 patients with diploid tumours but only 50% in 44 patients with aneuploid tumours (disease-free survival was not ascertained in 8 patients) (Kokal *et al.*, 1986). Scott et al also found that ploidy status and clinical stage were the most important independent prognostic factors affecting 121 patients with rectal carcinoma (Scott *et al.*, 1987). Over the 15 years of follow-up in this report, local recurrence was twice as frequent in resected aneuploid tumours as in diploid tumours. In contrast, Schutte et al found ploidy to be an important prognostic discriminant only for Stage C patients (Schutte *et al.*, 1987). In this subset of 86 patients, 60% of diploid but only 25% of aneuploid patients were alive after 4 years. Overall survival of the whole group of 279 patients with Stage A-D was not significantly associated with ploidy status, although there was a trend towards worse survival for aneuploid patients. In a group of 123 patients, Jones et al reported that aneuploidy had adverse prognostic implications in 59 patients with Dukes B stage but was less important that the surgeon's assessment of operability, pathological classification(grade and stage), and age of the patient in a multivariate analysis of survival (Jones, Moore and Schofield, 1988).

Despite the above reports, Bauer et al did not find a significant difference in the survival of patients with diploid versus aneuploid cancers (Bauer *et al*, 1987). About 50% of his 97 patients had Stage A or D disease, subsets in which others have found prognosis to be relatively unaffected by ploidy. Rognum et al found only a trend towards improved survival when 37 patients with diploid colon cancers were compared with 63 with aneuploidy, with the trend strongest for stage C patients (Rognum, Thorud and Lund, 1987). Finally, Melamed et al in a study of 33 patients found that ploidy had no influence on survival (Melamed *et al.*, 1985).

In stage A or D patients, ploidy status may not define a prognostically different subset, because at early or advanced stages, survival may be more influenced by other factors eg. tumour bulk

than by growth characteristics reflected in aneuploidy.

Another area of interest is the relationship between changes in colonocyte proliferation and the development of adenomas and carcinomas. Patients with neoplasms show expansion of the proliferative zone in crypts and extension of the proliferative zone towards the colonic lumen (Ponz de Leon *et al*, 1988), (Risio *et al*, 1991), (Wilson, Smith and Bird, 1990).

Bauer et al (Bauer *et al.*, 1987) also found that high proliferative activity, defined as > 20% cells in S phase, was significantly correlated with DNA aneuploidy and was a more powerful adverse prognostic indicator than DNA aneuploidy. In 97 patients,the 5 year survival of those with low proliferative activity (60%) was significantly greater than those with high proliferative activity (30%). This difference was even greater in Stage A and B patients together, where the 5 year survival was 82% (S phase <20%) and 43% (S phase > 20%). Schutte et al also showed that a high S phase fraction predicted a significantly worse survival for stage C patients but not for the other stages (Schutte *et al.*, 1987).

Terpstra et al reported increased proliferation, measured by labelling with tritiated thymidine, in normal mucosa of the entire colon in patients with neoplastic disease compared to controls (Terpstra *et al*, 1987). The labelling index { LI} (mean ± SE) was 4.9 ± 0.2 in 16 control patients, 6.2 ± 0.3 in 21 patients with one or more small adenomas (diameter < 1 cm.), 9.1 ± 0.4 in 11 patients with large adenomas and 8.6 ± 0.5 in 13 patients with colon cancer. Ponz de Leon et al also used labelling with tritiated thymidine found similar results: LI (mean ± SE) was 11.3 ± 0.3 in 19 controls, 13.5 ± 0.4 in 21 adenoma patients and 12.5 ± 0.4 in 16 carcinoma patients (Ponz de Leon *et al.*, 1988). It therefore appeared that increased proliferation of the entire colonic mucosa, a "field effect", was a feature of patients with colonic neoplasms and may account for synchronous and metachronous adenomas and carcinomas in other parts of the colon.

Using the slightly different technique of a monoclonal antibody against the thymidine analogue, bromodeoxyuridine (Brdu), several other authors (Risio *et al.*, 1991), (Welberg *et al*, 1990), (Wilson, Smith and Bird, 1990) have again found greater labelling indices in the normal looking colonic mucosa of patients with adenomas and carcinomas than in controls. This further supports the presence of hyperproliferation in those subjects at high risk of developing colon cancer.

In the study to be described, proliferative activity and ploidy of benign and neoplastic colonic mucosa were correlated with metabolic phenotypes and tissue enzyme activities. One drawback, however, is DNA heterogeneity in the samples.

DNA heterogeneity of colorectal carcinoma describes either the presence of diploid or nondiploid(aneuploid) regions within the same tumour. Quirke et al, in 50 colonic tumours, found that 36 tumours(72%) would have been correctly asssigned on the basis of any 1 of 4 (2 superficial, 2 deep) samples taken from a surgically resected tumour whereas 14 (28%) tumours were incorrectly assigned since 1 of the samples fell into a different ploidy group from the others (Quirke *et al*, 1985). There was no difference in results from flow cytometry between randomised superficial or deep samples. Wersto et al analysed the DNA ploidy of 3 separate fresh tissue samples from each of 60 colorectal carcinomas by flow cytometry. He found DNA ploidy was concordant among the 3 samples in 38 cases (63.5%) and that 1 of the 3 was discordant in the remaining 22 cases (36.6%) (Wersto *et al*, 1990). Thus, in about a third of the colonic carcinomas, a single sample showing a diploid DNA histogram would not detect aneuploid DNA patterns which may exist elsewhere in the tumour.

To carry out a case-control study on cellular proliferation in patients with or without colonic neoplasms, it is important to examine the factors that might influence proliferation and hence,

matching. These factors are age, sex, bowel preparation and regional differences in the colon.

Two studies have reported increased labelling with tritiated thymidine of rectal mucosa with increasing age. The LI in rectal mucosa was 9.5± 0.3 (mean ± SE) in 11 subjects aged 30-50, 9.5± 0.3 in 11 subjects aged 51-65, and 12.9± 0.5 in 8 subjects aged 66-90: the last group being 35 % greater than the first (Roncucci *et al*, 1988). Similarly, in a group of 29 subjects with a mean age of 55, the LI of normal rectal mucosa was 7.23, significantly greater than in a group of 59 subjects who had a mean age of 24 and a LI 5.34 (Deschner, Godbold and Lynch, 1988). This represented a 35% increase in the older group. In a smaller study, Fireman et al reported no significant differences, in urban Jews, between 9 men aged under 60 (LI mean ± SD: 6.0± 2.3) and 4 men aged above 60 (LI 6.0± 1.5); but among rural Jews, the LI was significantly greater in 11 men aged above age 60 (LI 8.0± 2.9) than 11 men aged under 60 (LI 5.9± 1.5) (Fireman *et al*, 1989a)

Most reports of cellular proliferation do not separate their control populations by sex. Fireman et al found sex differences only in the rural, but not the urban group (Fireman *et al.*, 1989a). Using tritiated thymidine on normal rectal mucosa, the LI was 6.2 ± 2.2 (mean ± SD) in 13 urban men, no different from a LI of 5.9 ± 1.8 in 17 urban women. In 8 rural men however, the LI of 9.0 ± 2.4 was significantly greater than that of 6.5 ± 1.8 in 14 rural women. No explanation could be given for the above findings.

Bowel preparation with an orally administered nonabsorbable isotonic lavage solution may be important in case-control studies of rectal epithelial proliferation, as these specimens are often taken either during colonoscopies (when there has been bowel preparation) or at rigid sigmoidoscopies (when there is usually no bowel prepartion). Fireman et al showed that rectal epithelial proliferation, using tritiated thymidine, was not significantly affected by orally administered bowel lavage or tap water enemas (Fireman *et al*, 1989b). The LI was 6.9 ± 3.8

(mean ± SD) in 31 subjects who had bowel preparation and 6.0 ± 3.3 in 23 subjects who had no such preparation.

Analysis of any measure of colonic proliferation may need to take into account the site from which the biopsy was taken if there are regional differences in the colon. However, Ponz de leon et al showed no significant differences in the LI(using tritiated thymidine) of biopsies taken from different regions of the colon, classified as caecum(n=7), ascending(n=7), transverse(n=12), descending(n=10), sigmoid(n=10) and rectum(n=19) (Ponz de Leon *et al.*, 1988). Another group reported a nonsignificant trend for reduced thymidine labelling in the distal colon when compared to the proximal colon (LI 9 8.2± 2.2 and 9.4± 3. respectively), thought to be due to an increase in the number of goblet cells(which has a lower LI than columnar cells) in the distal colon (Arai and Kino, 1989).

1.5 Aims and Hypotheses in this Thesis

1. To perform a "case-control" study to compare the frequency of fast acetylators in normal controls to that in patients with colonic adenomas and carcinomas.
   The hypothesis is that patients with neoplasms have a higher than expected frequency of fast acetylators than control subjects.

2. To examine the influence of meat consumption on the relative risk of developing colonic neoplasia in slow and fast acetylators(Fig. 1.7).

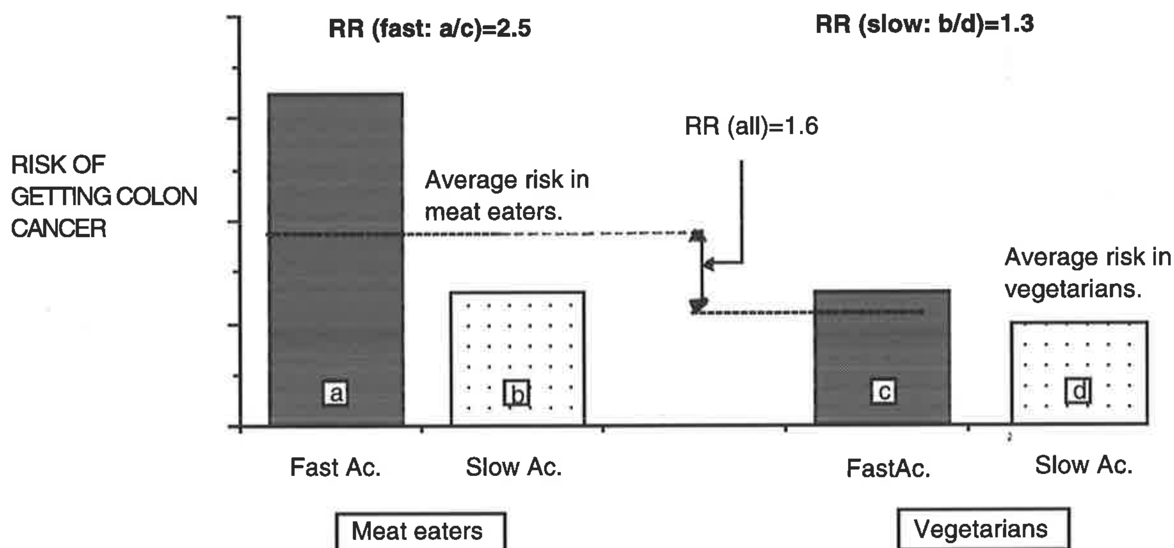


Fig. 1.7 Hypothetical interaction between diet and acetylator phenotype in colon cancer risk. RR=relative risk, Ac=acetylator.

3. To perform a "case-control" study looking at the frequency of the GSTμ null phenotype in controls and patients with colonic carcinoma. The hypothesis is that patients with carcinomas have a higher than expected frequency of the GSTμ null phenotype.

4. To measure the NAT, GST activity and GSH content in normal colonic tissue and in adenoma and carcinoma tissue to obtain a better understanding of changes in activity associated with neoplasia.

5. To determine whether the above factors influence cell proliferation in the colon as assessed by flow cytometry.

The overall aim and hypothesis of this project is illustrated in Diagram 1.2 below.

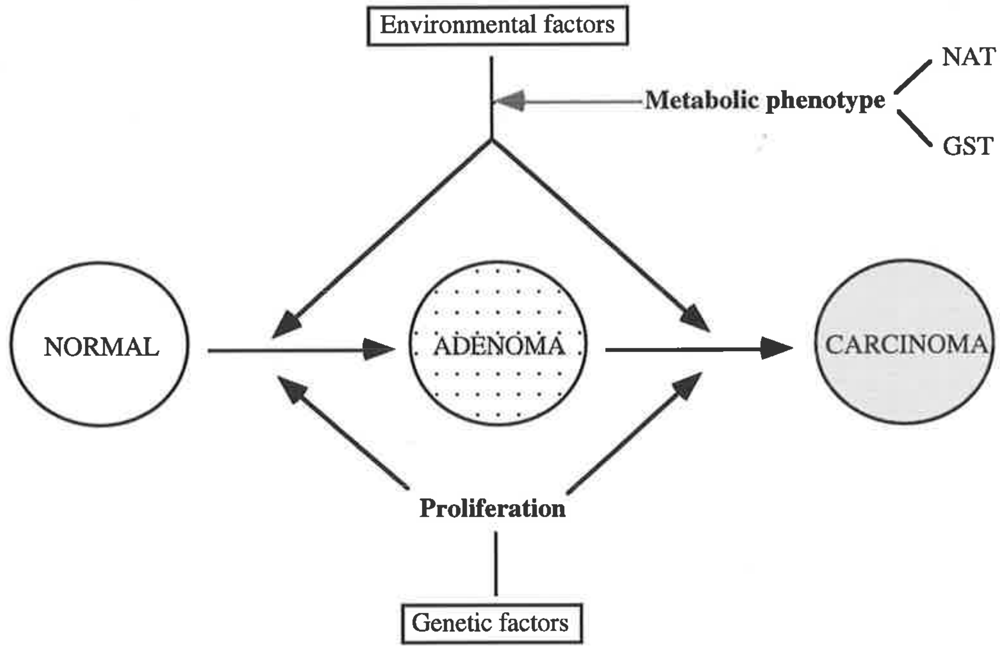


Diagram 1.2 Hypothesis on the aetiology of colorectal neoplasia.

CHAPTER 2 PATIENTS AND METHODS

2.1 Patient criteria and samples

Our study subjects were aged from 40 to 90 years, and were recruited from the Queen Elizabeth Hospital, Woodville, South Australia. They were divided into three groups as follows:

1. Patients who currently have colorectal carcinoma or who have successfully undergone surgical resection for colorectal carcinoma within the past 5 years(1987-1991). Some of these patients are reviewed regularly in the Surgical or Gastroenterological Outpatient Clinics. Patients who have both colorectal carcinoma and a metachronous adenoma, and patients with carcinoma arising from an adenoma (defined as a carcinoma which had breached the muscularis mucosa)were classified in this group.

2. Patients with current adenomas or patients with previous colorectal adenomas who have had adenomas removed at colonoscopy or surgery within the past 5 years (1987-1991). Patients with adenomas containing a carcinoma-in-situ (defined as carcinoma that had not breached the muscularis mucosa) were included this group.

3. The control group consisted of patients who had undergone sigmoidoscopy, barium enema X-ray and/or colonoscopy for gastrointestinal symptoms, but do not have neoplastic lesions. These patients were recruited from the Gastroenterological Outpatient Clinics, and most had functional bowel syndromes including the irritable bowel syndrome, non-ulcer dyspepsia or constipation. These disorders have not been shown to predispose to colonic neoplasia, and there is no evidence for

linkage of these disorders to any of the parameters studied. This control population attended the hospital through similar referral channels to those patients with neoplasia.

The above groups will be referred to as Carcinoma, Adenoma and Normals respectively, in this thesis.

Prior to inclusion in this study, patients underwent routine hematologic and serum biochemical screening to exclude those with serum creatinine, bilirubin, AST, or ALT values greater then 1.5 times the upper limit of the reference range. The groups were matched for age and sex. The histological characteristics of adenomas(dysplasia and type) and carcinomas(grade) reported by several different histopathologists, and the stage of the carcinoma obtained from an examination of the patient records and histopathological reports, were recorded.

Exclusion criteria were as follows:

1. Persons of Oriental background, owing to the disproportionately high prevalence of fast acetylators in this ethnic group.

2. Patients with diseases such as Crohn's disaease, ulcerative colitis, and familial polyposis which are known to predispose to colon cancer.

3. Persons with advanced cancer, for compassionate reasons.

4. Persons with severe, coexisting disease such as cardiac failure, for compassionate reasons.

5. Persons with sulphonamide allergy who are unable to undergo testing for acetylator status.

Patients were recruited either retrospectively or prospectively. Patients recruited retrospectively were obtained either from histology or colonoscopy reports. Histology reports between 1987 and 1989 indicating adenoma or carcinoma of the colon were obtained from the Histopathology Department. Colonoscopy reports bearing the word "polyp" or "carcinoma" between 1990 and 1991 were obtained from the Gastroenterology Department. The names and record numbers on these reports were then used to recall the hospital notes, where further information was obtained to ascertain the appropriateness of these patients for inclusion in the Study. This assessment was based mainly on three criteria; firstly, whether the patient was still alive or not; secondly, whether they lived in the Adelaide metropolitan area (patients living outside Adelaide were avoided due to costs in travel) and thirdly, whether they had any of the exclusion criteria listed above. Letters briefly explaining the project and inviting the patients to participate were then posted. These letters were followed by a telephone call to obtain consent and to arrange an interview.

Prospective patients were recruited from the Gastroenterology Outpatient Clinics or the Surgical Operative Lists. Patients who had colonoscopic polypectomy or bowel resections for colonic adenomas or carcinomas had the research project explained to them prior to their procedure and were studied within three months after the procedure(Diagram 2.1).

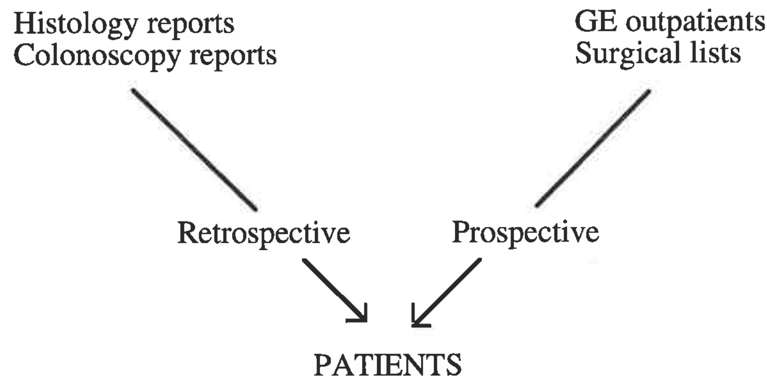


Diagram 2.1. Patient recruitment

There were 110 Normal patients, 89 Adenoma patients and 112 Carcinoma patients that were recruited for acetylator phenotyping. Two-thirds of all the patients in the Adenoma group and half of those in the Carcinoma group were recruited prospectively and the rest, retrospectively. All our Normals were recruited prospectively from the Gastroenterology Outpatient Clinics. Of those where retrospective recruitment was attempted (excluding patients with the exclusion criteria listed above), 50 patients in the Adenoma group were lost for the following reasons: 35 refused(including 2 with incomprehension), 13 were uncontactable and 2 had died. In the Carcinoma group, 38 people were lost with the following reasons: 21 refused(including 2 with incomprehension), 13 were uncontactable and 4 had died. In the Normal group, 19 patients declined to participate.

For the study, most patients attended at the hospital twice. In the first visit, the study was explained to them and they were given a dietary questionnaire for completion at home. No elaboration of any association between food and colonic neoplasia was given. Prior to their second visit, they were given instructions to collect overnight urine after taking a capsule of dextromethorphan (30mg) just before bedtime. This urine collection was for another research project which will not be included in this thesis. Patients fasted after midnight apart from taking essential morning medication.

On their second visit, usually within a week of the first visit, they returned the completed dietary questionnaire and the collected urine. They were then given a glass of orange cordial containing sulphamethazine (at about 0915 hrs) and had blood withdrawn from them one hour later for acetylator phenotyping, serum dehydroepiandosterone and lymphocyte extraction for DNA analysis (GSTu deletion and acetylator genotyping). A further blood sample was collected at 4 hours for acetylator phenotyping. During the time in hospital, an interview was carried out by myself to complete a clinical questionnaire (See Capter 2.2 for details on Questionnaires).

Samples of colonic carcinoma and neighbouring normal tissue were obtained from resected surgical specimens delivered from the operating room. Adenomas were retrieved at colonoscopy. Specimens were sent to the Gastroenterology Laboratory in buffer for biochemical studies and flow cytometry. A schematic representation of the sample collection is shown in Diagram 2.2.below.

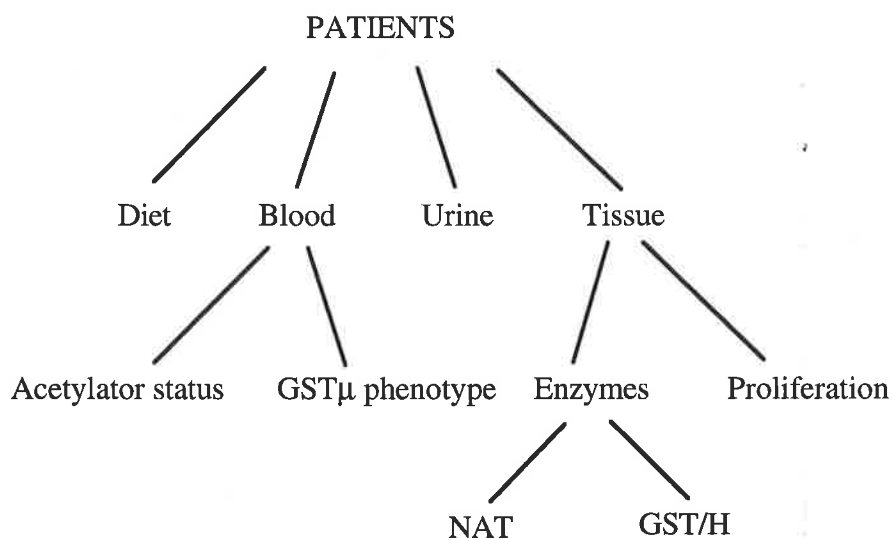


Diagram 2.2. Derivation of and tests done on patient samples

The protocol for this Study was approved by the Queen Elizabeth Hospital Ethics Committee on Human Experimentation and all subjects gave informed consent.

2.2 Questionnaire

2.2.1 Clinical

A record was kept of the patient's address and telephone number. A brief clinical history was obtained, and the blood tests(ie blood count and urea and electrolytes, liver function tests) checked to exclude renal and hepatic failure that might exclude the patient from the study. The weight and height of the patient were recorded and the body mass index(weight{ in kg} /height{ in metre} ^{2}) determined. A drug history and any history of drug allergy was also recorded. The histological characteristics of adenomas(dysplasia and type) and carcinomas(grade) reported by several different histopathologists, and the stage of the carcinoma obtained from an examination of the patient records and histopathological reports, was recorded in the questionnaire.

2.2.2 Dietary

Information concerning usual food and nutrient intake was obtained using a self administered retrospective food frequency quantitative questionnaire, developed by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Division of Human Nutrition, Adelaide. The questionnaire was explained to the patient by myself.

Modified versions of this questionnaire have been used extensively in Australia for national or state population dietary surveys (Baghurst and Record, 1983), (Baghurst *et al*, 1988a), (Baghurst *et al*, 1988b) and for case-control studies of colon, breast and pancreatic cancer (Potter and McMichael, 1986), (Rohan, McMichael and Baghurst, 1988), (Baghurst *et al*, 1991) as well as a prospective study of the determinants of high density lipoprotein

levels(Dwyer *et al*, 1981). The questionnaire has been shown to have good repeatability and to give good correlations with urinary sodium and nitrogen measures, as well as with short-term weight recording techniques, such as four-day records and multiple repeat 24 hour records (Baghurst and Record, 1983), (Rohan and Potter, 1984), (Rohan, Record and Cook, 1987).

The questionnaire includes about 200 food and beverage items. Each food has listed alongside, a standard size of serving which is derived from earlier studies. For each food or beverage, the respondent can change this "standard" serving in an open-ended way to reflect individual usage by writing in a "comment" column. The frequency categories used are also continuous in the sense that four columns are given in which respondents can indicate how often they consume the item "per day", "per week" or "per month" or for "never or very rarely" using symbols (1D, 2D, 1W, 2M or N etc). The open-ended provision for describing the size of serving therefore does not require the respondent to adjust the frequency response to account for changes in size. In addition to these food frequency questions, there were a number of questions on socio-economic class, education level, country of birth and food preparation (e.g. whether meat was cooked-fried, grilled, microwaved or none of above).

Nutrient content per unit weight was calculated using the revised British Food Tables (Paul *et al*, 1978) with additional data relating to Australian foods where data were available, as the nutrient database, and the FREQUAN analysis package (Baghurst and Record, 1984). This analysis of nutrient content reported in the patients' dietary questionnaire was performed by the CSIRO Department of Human Nutrition, Adelaide.

The type of items reported in the patients' diet was as follows:

Water intake(g)	Total CHO(g)	Vit. B12(ug)
Sugars(g)	Sodium(mg)	free folate(ug)
Complex CHO(g)	Potassium(mg)	total folate(ug)
Fibre(g)	Calcium(mg)	Panthothenate(mg)
Nitrogen(g)	Magnesium(mg)	Biotin(ug)

KJoule	Iron(mg)	Alcohol(g)
Protein(g)	Zinc(mg)	Cholesterol(mg)
Total fat(g)	Sulphur(mg)	Added sugar(g)
Sat. fatty acids(g)		
Monounsaturated FA(g)		
Polyunsaturated FA(g)		

Only the results of the protein , fat, carbohydrate and fibre intake would be used in this study (chapter 6).

2.3 N-Acetyl transferase

2.3.1 Systemic phenotype

After an overnight fast, patients were given sodium sulphamethazine (20 mg/kg body weight) dissolved in a glass of water mixed with orange cordial. This drug has a very low incidence of side-effects, and has been used extensively to define acetylator status. After administration, fasting continued for three hours. Blood samples (10 mls each) were withdrawn at 1 and 4 hours after ingestion of the drug. Serum obtained after centrifugation was stored at -70°C. until analysed.

Analysis of acetylator phenotype was performed on the patients' serum using HPLC. A model 440 liquid chromatograph (Waters Associates, Milford, MA) was used with a 20 ul loop injector and a variable wavelength detector set at 254 nm. The HPLC column is a 4.6 x 220 mm Brownlee column with a 3 cm. pre-column, eluted with a mobile phase containing 90% sodium acetate (10 mM, pH 4) and 10% acetonitrile. Under these conditions, sulphadiazine (internal standard), sulphamethazine and acetyl sulphamethazine had retention times of 4, 8, and 10 minutes respectively.

Sulphadiazine in aqueous acetonitrile was used as an internal standard (20 ug/ml). Serum(100 ul) with standard concentrations of sulphamethazine and acetyl sulphamethazine containing 0, 12.5, 25, 50 and 100 ug/ml were mixed with internal standard solutions (100 ul of 20 ug/ml sulphadiazine). 20 ul reaction mixes were then injected into the chromatograph and calibration curves prepared by plotting the peak height ratios from sulphamethazine/ sulphadiazine, and acetyl sulphamethazine/sulphadiazine against the known concentrations of sulphamethazine and acetyl sulphamethazine respectively. The patient's serum samples were mixed with the internal standard, sulphadiazine, and injected into the HPLC. Similar peak height ratios obtained from the patients' plasma were then compared to the previously prepared standard curve of known concentrations, to derive the concentrations of sulphamethazine and acetyl sulphamethazine. (See Appendix Ai for reagents, plasma standards, procedure and HPLC conditions).

The % acetylation was calculated from the ratio of acetyl sulphamethazine to the sum of sulphamethazine and acetyl sulphamethazine multiplied by 100. The acetylator status of a subject was classified as fast if the %acetylation was more than 25% at 1 hour or more than 50% at 4 hours. Conversely, the subject was classified as slow if the % acetylation was less than 25% at 1 hour or 50% at 4 hours. The above method was based on previous studies by R. Whelpton (Whelpton, Watkins and Curry, 1981).

2.3.2 Tissue

Tissue NAT activity was measured in mucosa scraped from adenomas removed at colonoscopy and from normal and carcinoma tissue specimens removed at operation (Diagram 2.3). All specimens were immediately immersed and transported in 0.1 M potassium phosphate (KH_2PO_4) buffer (pH 7.4).

Tissue was homogenized(Ystral homogenizer, model GmbH D-7801, Dottingen, W. Germany) in 4 mls of 0.1M KH_2PO_4 buffer (pH 7.4) and centrifuged at 20,000g (15,000 rpm) at 4°C for 20 minutes (Beckman centrifuge model J2-21M/E with a J21 rotor, California, USA). The supernatants, referred to as cytosols, were removed and placed on ice for NAT assay and for analysis of total protein content.

The NAT assay was carried out in reaction mixes containing 10 ul of 0.4 mM PABA, 10 ul of 0.4 mM SMZ, 9 ul of 150 mM acetyl carnitine, 5 ul of 8mM acetyl coenzyme A and 6.7 ul of carnitine acetyl transferase(26.86 U/ml). All chemicals were dissolved in 0.1M KH_2PO_4 buffer (pH 7.4). Acetyl carnitine is a source of acetyl CoA as shown in the following equation; Acetyl carnitine ----carnitine acetyl transferase---> acetyl CoA + carnitine

The reactions were started by the addition of 50 ul. of cytosol to each of ten reaction mixes and incubated at 37 C. The reactions were stopped by the addition of 50 ul. of acetonitrile (CH_3CN) containing the internal standard, sulphadiazine (20 ug/ml), at 0 (control), 1, 2.5, 5, 15, 30, 45, 60, 75 and 90 minute intervals to each reaction mix respectively. HPLC for the acetylated metabolites were performed on fresh or frozen (-70°C) tissue supernatants. Freezing the tissue supernatants did not affect NAT activity. Reverse phase HPLC was carried out in a model 440 liquid chromatograph (Waters Associates, Milford, MA) with a 20 ul. loop injector and a variable wavelength detector set at 254 nm. The HPLC column is a 4.6 x220 mm Brownlee column with a 3 cm. pre-column, eluted with a mobile phase consisting of 8% acetonitrile and 10 mM sodium acetate (pH 4.0), degassed on the day of use. The column oven was set at a temperature of 35° C and the mobile phase ran at a flow rate of 2 ml/ min by an HPLC pump operating at a pressure of 3000 psi (Waters Chromatography pump).

Peak height ratios of sulphamethazine/sulphadiazine and acetyl sulphamethazine /sulphadiazine

were plotted against known concentrations of sulphamethazine and acetyl sulphamethazine from the standard curve. The stock concentration used for the standard curve was 10 nmol/L, made up of 200 ul. PABA (0.2mM), 200 ul.SMZ (0.2mM), 200 ul.acetyl SMZ (0.2mM) and 200 ul. of water. Serial dilutions of 8, 6, 4, 2 and 1 nmol/L were subsequently made. An aliquot of 50 ul. of these concentrations was mixed with 40 ul. sulphadiazine (internal standard) and 50 ul. water, from which 20 ul. reaction mixes were injected into the chromatograph. A calibration curve was then prepared by plotting similar peak height ratios of PABA/sulphadiazine and acetyl sulphamethazine/sulphadiazine to the known concentrations of PABA and acetyl sulphamethazine. The rate of acetylation was determined by the rate of disappearance of PABA from the reaction mixture and the rate of appearance of acetylated SMZ in the mixture; changes which correspond to the activity of the monomorphic and polymorphic NAT enzymes respectively.(See Appendix A ii for reagents, mobile phases and standard curve.)

Cytosols were also analysed for total protein concentration by the Lowry method (Lowry *et al*, 1951) using bovine serum albumin (BSA; 1mg/ml) as a standard. The protein content of the tissue cytosol (which may be diluted 1 in 5) was obtained by reading off the spectrophotometric absorbance of the cytosol against a calibration curve which had been previously plotted between spectrophotometric absorbance and known concentrations of BSA. The results of the NAT enzyme activity was then expressed in nmols. of substrate acetylated/min/mg. protein.(See Appendix Aiii for Lowry protein assay reagents and procedure.)

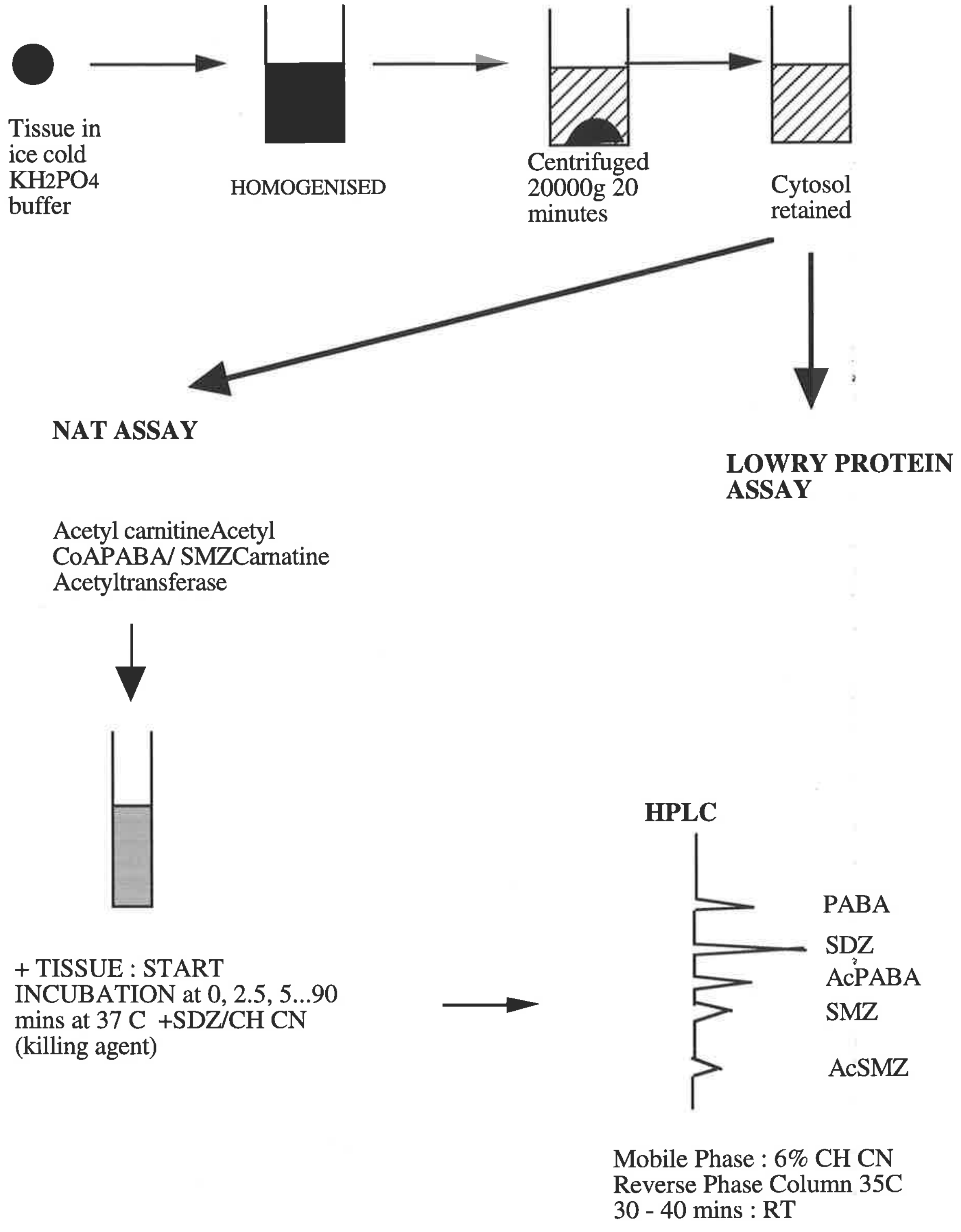


Diagram 2.3. Showing method in measurement of tissue N-acetyltransferase activity.

2.3.3 NAT Genotyping

The genotyping of NAT is classified into 2 steps, discussed under (1) and (2) below:

1. The amplification part of the NAT gene using PCR and suitable primers which anneal to the bases of the NAT gene underlined in fig 2.1. This gives rise to a 704 base pair product containing the diagnostic restriction sites.

ATGGACATTGAAGCATATTTGAAAGAATTGGCTATAAGAACTCTAGGAA
CAAATTGGACTTGGAAACATTAACTGACATTCTTGAGCACCAGATCCGGG
CTGTTCCCTTTGAGAACCTTAACATGCATTGTGGGCAAGCCATGGAGTTG
GGCTTAGAGGCTATTTTTGATCACATTGTAAGAAGAAACCGGGGTGGGT
GGTGTCTCCAGGTCAATCAACTTCTGTACTGGGCTCTGACCACAATCGGT
TTTCAGACCACAATGTTAGGAGGGTATTTTTACATCCCTCCAGTT/**H**AACA
AATACAGCACTGGCATGGTTCACCTTCTCCTGCAGGTGACCATTGACGGC
AGGAATTACATTGT/**T**CGATGCTGGGTCTGGAAGCTCCTCCCAGATGTGG
CAGCCTCTAGAATTAATTTCTGGGAAGGATCAGCCTCAGGTTCCTTGCAT
TTTTTGCTTGACAGAAGAGAGAGGAATCTGGTAC/**K**CTGGACCAAATCAG
GAGAGAGCAGTATATTACAAACAAAGAATTTCTTAATTCTCATCTCCTGCC
AAAGAAGAAACACCAAAAAATATACTTATTTACGCTTGAACCTCGAACAAT
TGAAGATTTTGAGTCTATGAATACATACCTGCAGACGTCTCCAACATCTTC
ATTTATAACCACATCATTTTGTTCCTTGCAGACCCCAGAAGGGGTTTACTG
TTTGGTGGGCTTCATCCTCACCTATAGAAAATTCAATTATAAAGACAATAC
AGATCTGGTCGAGTTTAAAACTCTCACTGAGGAAGAGGTTGAAGAAGTG
CTGAAAAATATATTTAAGATTTCCTTGGGGAGAAATCTCGTGCCAAAACCT
GGTGATGG/**B**ATCCCTTACTATTTAGAATAAGGAACAAAATAAACCCTTGT
GTATGTATCACCCAACTCACTA

Fig. 2.1 DNA sequence of N-Acetyltransferase, showing restriction sites (H for Hinc II, K for Kpn, T for Taq, B for Bam H1) and corresponding bases to primers (underlined).

DNA samples

Lymphocytes were obtained from whole blood by gradient centrifugation on Histopaque 1077 (Sigma, St. Louis, USA){ See Appendix B}. Genomic DNA was extracted from the lymphocytes by proteinase K digestion and phenol/chloroform, and precipitation with ethanol .(See Appendix B for protocol).

Primers

Primers were designed based on the published sequence, with the Tms matched within 1.6^{o}C for each pair.

Sense: GGTCAATCAACTTCTGTACTGGGC Tm=65.1^{o}C

Antisense: TTGGGTGATACATACACAAGGGTT Tm=63.5^{o}C

(Primers were synthesized using the Pharmacia Gene-assembler™).

PCR reaction

PCR amplification of the polymorphic NAT gene was performed on 1 ug. of DNA using 0.5 units Taq polymerase (Boehringer Mannheim, W.Germany) in Boehringer buffer(containing 100 mmol Tris HCL, 15mmol/L $MgCl_2$, 500 mmol/L KCl and 1 mg/ml gelatin), 200 uM dinucleotide triphosphates (dNTPs){ Boehringer Mannheim, W.Germany} , 100 ng each of the primers, in a total volume of 50 ul. An initial denaturation of 94°C for 5 mins. was followed by 45 cycles of 94°C for 1 min., 60°C for 1 min. and 72°C for 1 min. using a FTS-1 Thermal Sequencer(Corbett Research, Australia). This was then concluded by an extension step at 72°C for 7 mins. PCR products were resolved on a 1.5% agarose gel or 8% polyacrylamide gel and stained with ethidium bromide to confirm the presence of the amplified product.

2. The digestion of the PCR product with the restriction enzymes, Kpn 1, Taq 1 and Bam H1, produced different sized fragments that could be distinguished by gel electrophoresis and correlated to the different genotypes. No genotype would have the same pattern as another.

In homozygotes with the Kpn 1 site on both alleles(K+K+), digestion would give rise to 266 and 438 base pair fragments. On agarose or polyacrylamide gel electrophoresis, these would show up as two bands corresponding to these sizes. In a heterozygote(K+Ko), one allele would be cut but not the other, giving rise to three bands. In a homozygote with no Kpn sites on either allele(KoKo), there will be no cutting of the PCR product and therefore one band would be seen on electrophoresis(shown in diagram 2.4i).

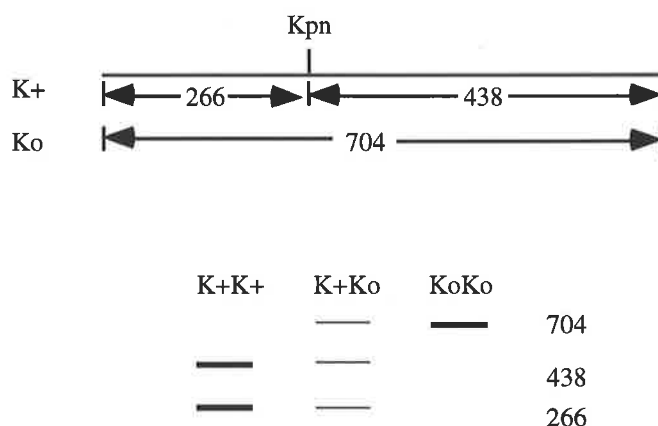


Diagram 2.4i Kpn 1 digestion.

The pattern created by Taq 1 digestion is slightly complicated by the presence of two fixed Taq sites at the 152nd and 548th base pair of the PCR product. In homozygotes with the variable Taq site on both alleles(T+T+), digestion with Taq would give rise to 152, 226, 170 and 156 base pair fragments. The 152 and 156 base pair fragments were seen as one band as their sizes were too close to be resolved separately. In the heterozygote(T+To), digestion of one allele without the variable Taq site, would give rise to 396, 152 and 156 base pair fragments, as well as 152, 226, 170 and 156 base pair fragments from the other allele. In the homozygote without

the variable Taq sites on both alleles(ToTo), digestion would give rise to 396, 152 and 156 base pair fragments only. For simplicity, the three phenotypes could be distinguished by the three different patterns shown in the dotted line box.(Diagram 2.4ii)

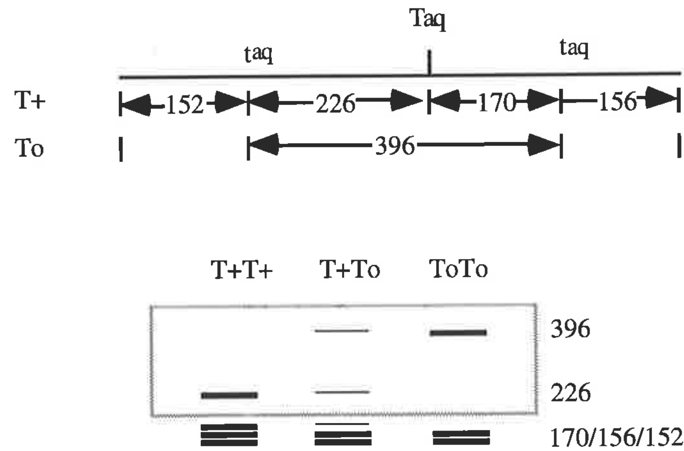


Diagram 2.4ii Taq 1 digestion.

In homozygotes with the Bam H1 site on both alleles, digestion would give rise to 646 and 58 base pair fragments. The 58 base pair fragment is too small to be detected with the above method of electrophoresis. In heterozygotes, Bam H1 digestion would give rise to two bands, the 646 base pair fragment and the uncut 704 base pair product. In homozygotes with no Bam H1 sites on both alleles, there will be no cutting of the PCR product.(Diagram 2.4iii).

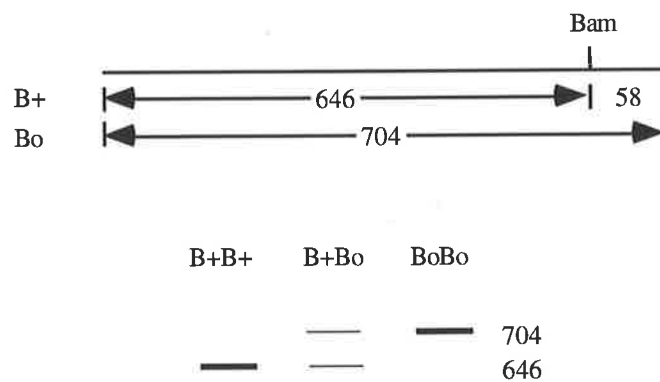


Diagram 2.4iii Bam H1 digestion.

The NAT genotype of an individual could be deduced by the degree of digestion from the three restriction enzymes. Diagrams 2.5i and 2.5ii show digestion with Kpn and Taq, and then with BamH1 respectively; differentiating all the NAT genotypes.

			Kpn 1	
	Degree of digestion	0	50%	100%
	0	-	-	SS2S2
Taq 1	50%	-	S1S2	S2F1/S2S3*i
	100%	S1S1	S1F1/S1S3*ii	F1F1/S3F1/S3S3*iii

Diagram 2.5i Degree of digestion with Kpn 1 and Taq 1 to obtain NAT genotype.

Degree of digestion with Bam H1			
Previous pattern	0	50%	100%
*i	-	S2S3	S2F1
*ii	-	S1S3	S1F1
*iii	S3S3	S3F1	F1F1

Diagram 2.5ii Further digestion with Bam H1 to obtain NAT genotypes.

(Please refer to Appendix Bi for method on lymphocyte extraction and to Appendix Bii for DNA extraction from lymphocytes).

2.4 Glutathione S transferase

2.4.1 Tissue Glutathione S Transferase activity

Tissue GST activity was measured spectrophotometrically using a modification of the method of Habig (Habig, Pabst and Jakoby, 1974). An aryl substrate, 1,chloro - 2,4-dinitrobenzene

(CDNB) and reduced GSH were added to a tissue homogenate with potassium phosphate buffer (diluted 1:10 in most instances unless available tissue was small). The rate of formation of GSH-CBNB under the enzymatic activity of tissue GST was measured by a change in absorbance at 340 nm (Varian DMS 100S UV visible spectrophotometer).

A complete mixture without the enzyme was used as a control. The GST activity was calculated from the following equation utilizing the molar extinction coefficient of 9.6 for the GSH-CDNB conjugate.

$$\text{GST activity} = \Delta\ \text{OD/min} \times M \times D \times V$$

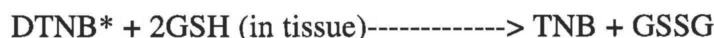
where Δ OD/min = change in absorbance per minute
M = molar extinction coefficient (9.6)
D = GSH dilution factor (10)
V = volume correction factor (5)

The amount of protein in the tissue was estimated by the Lowry method and the results were expressed as nmol/min/mg protein(See Appendix Ci for method and reagents on measuring GST activity).

2.4.2 Tissue glutathione content

Total glutathione content (reduced and oxidized) was analysed by the colorimetric recycling method of Tietze (Tietz, 1969) as modified by Griffith (Griffith, 1980). Equal volumes of tissue homogenate and 5% trichloroacetic acid (TCA, 500ul) were mixed and centrifuged (11000g for 3 minutes) and the supernatant retained for analysis (stored at -70°C). The assay involved the reduction of the aromatic disulphide 5,5'-dithiobis 2 nitrobenzoic acid (DTNB) to TNB by GSH which, in turn, is oxidized to its disulphide linked dimeric form, GSSG. In

order to maintain the reaction, GSSG is reduced back to GSH by the presence of glutathione reductase and NADPH.


DTNB* + 2GSH (in tissue)-------------> TNB + GSSG

GSSG (oxidized) + $NADPH_2$* -----------------------> 2GSH (reduced) + NADP

glutathione reductase (*=added reagent)

The reaction mix of 700 ul NADPH(0.3mM), 100ul DTNB (6.0mM) and 200ul TCA supernatant was incubated for five minutes at room temperature. The reaction was initiated with 20 ul of glutathione reductase (25U/ml). All reagents were made up in 0.125M KH_2PO_4/6.3 mM EDTA buffer, pH 7.4. The absorbance at 412 nm was recorded using the spectrophotometer. The amount of glutathione was estimated from a standard curve generated with GSH concentrations of 40, 20, 10, 5 and 0 nmol.(See Appendix Cii for method and reagents). The amount of protein in the tissue was estimated by the Lowry method so that results was expressed as nmol/mg protein.

2.4.3 GSTμ phenotype

The absence of GSTμ has been ascribed to a homozygous deletion of the gene GST 1 on chromosome 1. The sequence of the coding region was obtained from the Gen Bank nucleotide sequence database and is shown in Fig 2.2. The aim of this method was to amplify up this region using PCR technology and to determine the absence or presence of this region and the subsequent PCR product on agarose gel electrophoresis. If the gene for GSTμ is present, the PCR product (using the primers shown below) , would be 249 bases, including the intron of 95 bases reported by Comstock (Comstock *et al*, 1990).

GCACCAACCA GCACCATGCC CATGATACTG GGGTACTGGG
ACATCCGCGG GCTGGCCCAC GCCATCCGCC TGCTCCTGGA
ATACACAGAC TCAAGCTATG AGGAAAAGAA GTACACGATG
GGGGACGCTC CTGATTATGA CAGAAGCCAG TGGCTGAATG
AAAAATTCAA GCTGGGCCTG GACTTTCCCA ATCTGCCCTA
CTTGATTGAT GGGGCTCACA AGATCACCCA GAGCAACGCC
ATCTTGTGCT ACATTGCCCG CAAGCACAAC CTGTGT*GGGG
AGACAGAAGA GGAGAAGATT CGTGTGGACA TTTTGGAGAA
CCAGACCATG GACAACCATA TGCAGCTGGG CATGATCTGC
TACAATCCAG AATTTGAGAA ACTGAAGCCA AAGTACTTGG
AGGAACTCCC TGAAAAGCTA AAGCTCTACT CAGAGTTTCT
GGGGAAGCGG CCATGGTTTG CAGGAAACAA GATCACTTTT

Fig. 2.2 Nucleotide sequence of cDNAs encoding for human GST mu isoenzyme. Intron of 95 base pairs situated at *. Primers used correspond to those nucleotides which are underlined.

DNA samples

Genomic DNA was extracted from human blood lymphocytes by gradient centrifugation with Histopaque 1077 (Sigma, St. Louis, USA){ See Appendix B} . DNA was obtained using phenol/chloroform extractions and ethanol precipitation.(See Appendix B for protocol).

Primers

Sense	ACAAGATCACCCA GAGCAACGCC	Tm=68.8°C
Antisense	TCTGGATTGTAGCAGATCATGCCC	Tm=66.6°C

(Primers were synthesized using the Pharmacia Gene-assembler™).

PCR reaction

PCR amplification of the GST 1 gene was performed using Boehringer buffer(containing 100 mmol Tris HCL, 15mmol/L $MgCl_2$, 500 mmol/L KCl and 1 mg/ml gelatin), 200 uM dNTPs, 0.5 units Taq polymerase (Boehringer Mannheim, W.Germany), 100 ng each of the primers, 3mM Magnesium in a 25 ul reaction, with 1 ul (approx. 500 ng) of genomic DNA.

The reaction cycle temperatures were 94°C for1 min.,60°C for 1 min. and 72°C for 1min., repeated for 30 cycles in a FTS-1 Thermal Sequencer(Corbett Research, Australia). The above cycles were preceded by denaturation at 94°C for 5 mins. and concluded by an extension step at 72°C for 7 mins. PCR products were electrophoresed on a 1.5% agarose gel.

Procedure for avoiding false positive results

All known precautions (Kwok and Higuchi, 1989) of avoiding PCR product carryover and sample to sample contamination were taken. One "no DNA" reagent control was included within each set of amplifications.

Procedure for avoiding false negative results

This was achieved by amplification of another product (multiplex PCR) to act as a positive control for the reaction. The CD3 gamma gene was amplified using the primers(courtesy of Haematology Department, TQEH) below, to produce a 116 base pair product.

CD3 primers:	Sense	TCTGACTTGTGATGCAGAAGCCA	Tm=68°C
	Antisense	GAGGGTCCTTGGCATTACTTCCC	Tm=72°C

2.5 Cellular Proliferation

Flow cytometry

Rectal biopsies were taken from uninvolved "normal mucosa" from control subjects and from patients with adenomas and carcinomas. Tissue was also obtained from adenomas, mostly removed colonoscopically, and from carcinomas, mostly obtained from surgical resections. These tissues were fixed in 10% formalin and later used for DNA analysis and assessment of cell proliferation.

The proliferative activity was determined by flow cytometry using a modification of the method of Hedley (Hedley *et al*, 1983). The tissues were washed in phosphate buffered saline (PBS) and finely minced with a razor blade. After two further washings, the tissues were incubated in 1.5ml of 0.5% pepsin (Sigma Chem. Co.,St. Louis, Missouri) in 0.9% NaCl and adjusted to pH 1.5 with 2M HCl for 60 mins at 37°C with intermittent vortexing every 20 mins. After enzyme digestion, the suspension of nuclei was washed in PBS and labelled with propidium iodide by incubation in 1 ml PBS solution, pH 7.4, containing 95-98% propidium iodide (0.25 mg/ml, Sigma Chem.Co.) and ribonuclease A type 1-AS (0.5 mg/ml, Sigma Chem. Co.) for 15 mins at 37°C. The tissues were then resuspended in 1 ml PBS and the samples syringed five times using a 23 gauge needle to produce single nuclei suspensions. They were then filtered through a 37 um nylon mesh and stored on ice prior to analysis by flow cytometry.

The DNA content of the tissues was measured with a Facscan flow cytometer (Becton-Dickinson, Sunnydale, California) using an argon laser 15mW. at wavelengh 488 nm. The fluorescence of the stained DNA, which was directly proportional to the cellular DNA, was quantitated after passage through a 610 nm long pass filter to remove any scattered light. Histograms of cell number *versus* linear integrated red fluorescence (proportional to DNA

content) were recorded for 10,000 nuclei at flow rates of about 100 nuclei per second. Data were stored and later analyzed on a Hewlett Packard computer. Electronic gating of the light scatter signal was used to exclude cellular debris. The DNA data analysis was done using Becton-Dickinson CellFIT™ software to analyse cell cycle percentages and DNA Index.

Cell cycle percentages comprising the DNA pre-synthetic phase (G0-G1), DNA synthetic phase (S) and DNA post-synthetic and mitotic phases (G2 + M) were obtained using the RFit model of the Becton-Dickinson CellFIT™ software. The RFIT (Rectangular Fit) analysis model calculates the S phase from a rectangle. The height of the rectangle was the average height of mid-S phase and the width of the rectangle extended from the Go/G1 peak channel to the G2 + M peak channel. The model sums the number of events bounded by this rectangle to determine the number of S phase events. For the other phases of the cell cycle, it first determines the number of events in each half of the histogram and substracts one half of the total number of S phase events. This subtraction yields estimates of the number of cells in G0/G1 and G2 + M. The positions of the peak channels for the G0/G1 and G2+M populations were set manually by the operator.

The percentage coefficent of variation(%CV, defined as s/u x100 where s=standard deviation and u=mean) calculated by the incorporated software for each peak gave a measure of sample variation. Only samples with a %CV $\leq$ 10 were accepted, otherwise samples were repeated and later accepted if the %CV $\leq$ 10.

Most solid tumours, inluding colorectal neoplasms, contain a population with a diploid DNA content and when this was the only cell population present, the tumour was classified as diploid. Tumours which had an additional G1 peak were classified as aneuploid, and the diploid peak was presumed to arise from host cells such as lymphocytes and stroma. The degree of DNA aneuploidy was quantitated as the DNA index (DI) which is the amount of

abnormal DNA relative to normal, calculated as the ratio of the mean channel number of the mucosa of the aneuploid G0-G1 peak to the mean channel number of the mucosa G0-G1 peak. A diploid tumour was defined as having a DNA index of 0.8-1.2 and an aneuploid tumour, <0.8 or >1.2.

2.6 Statistical methods

Descriptive statistics of data were initially derived , giving the mean, SD, SE, range and count frequency. The count frequency was shown as a histogram plot or as points in a regression equation. Data were analyzed using the box and whisker plot. The left and right sides of the box indicate the 25th and 75th percentile of the number of points in the data. The left and right whiskers indicate the 2.5% and 97.5% of all values. Any data points falling outside these whiskers were referred to as "outliers", with the hypothesis that these outliers were unlikely to be from the same sample as points within the box.

Differences between means were compared using the Student's t test and the Mann-Whitney U test for non-parametric values (using Macintosh Statworks™). The significance of proportions was tested using the chi squared(x^2) test. The "odds ratio"(OR) was calculated from a 2x2 table of patient status (eg. cancer or control) *versus* the presence or absence of a trait. The OR is a ratio of the "odds" that the colorectal cancer will occur when the given trait is present to the "odds" that it will occur when the trait is absent. Therefore, an OR of > 1 indicates higher odds in the presence of the trait compared to its absence. The 95% confidence limits of the mean in the odds ratio was calculated by a transformation of approximate limits for ln(OR) (Woolf, 1955). Adjustment for possible confounding variables was performed by the Mantel-Haenszel method(1959).

The degree of association between two variable parameters was measured by the correlation coefficient (r) with a "straight line " association giving a value of +1 or -1. The correlation between two variables was positive if higher values of one variable were associated with higher values of the other, and negative if one variable tended to be lower as the other become higher. A correlation of around zero indicated that there was no linear relation between the two variables(i.e. they are uncorrelated). A part of the variation in one of the variables can be thought of as being due to its relationship with the other variable, and another part as due to undetermined(often "random") causes. The part due to the dependence of one variable on the other was measured by the determination coefficient(r^2).

CHAPTER 3 N-ACETYL TRANSFERASE AND COLORECTAL NEOPLASIA

3.1 Introduction

Two small studies have reported an association of the fast acetylator phenotype with colorectal carcinoma while one larger study has not. The study reported in this thesis is comparable in size to the latter study, and includes a group of patients with colorectal adenoma. The relative importance of an elevated NAT activity in the liver compared to that in the target organ, the colon, is examined in this study by assessing the NAT activity in colonic tissues removed either at colonoscopy or surgery. The actual mechanism of colorectal carcinoma risk in fast acetylators is unknown. Arylamines, derived from cooked meat, have been shown to be pro-carcinogens and to undergo activation by acetylation. A hypothesis was that glucuronidated arylamines were excreted in bile and subsequently underwent different acetylation rates in the colon depending on the acetylator status of the individual. A method of genotyping individuals for their acetylator status had also been developed to enable identification of the NAT heterozygote, previously poorly differentiated by classical biochemical methods, and to provide an alternative and more accurate method of acetylator phenotyping.

3.2 Results

3.2.1 Systemic phenotype

Acetylator status was determined in 311 patients from blood samples obtained at different time intervals after ingestion of sulphamethazine. Blood samples were initially taken at 1 and 4 hours but subsequent results indicated that a 1 hour sample was sufficent to differentiate

between the fast and slow acetylators. In addition, it was more convenient for patients to have only a 1 hour specimen. Therefore, there were 123 patients with a 1 hour sample only, 151 patients with 1 and 4 hour samples, 9 patients with 2.5 and 4 hour samples, 11 with 4 hour samples only, 14 with 1, 2.5 and 4 hour samples and 3 with 1, 2.5, 4 and 6 hour samples. Thus, there were 291 blood samples taken at 1 hour and 188 samples taken at 4 hours.

A frequency histogram of % acetylation at 1 hour showed a bimodal distribution with the cut-off between the two distributions at about 25% (fig.3.1a).

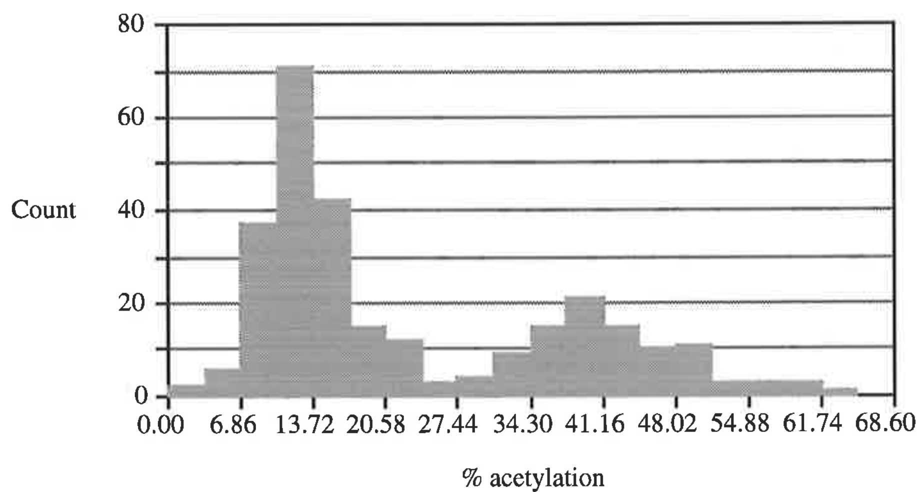


Fig. 3.1a Frequency histogram of % Sulphamethazine acetylation at 1 hour

A plot of % acetytion at 1 and 4 hours against time gave 2 major findings: firstly, a cut-off between slow and fast acetylators of 25% at 1 hour or 50% at 4 hours is appropriate and secondly, the rate of acetylation is not significantly correlated with age(fig. 3.1b and c). The plot therefore shows that the separation between the phenotypes using 25% acetylation at 1 hour, or 50% at 4 hours are suitable for all ages.

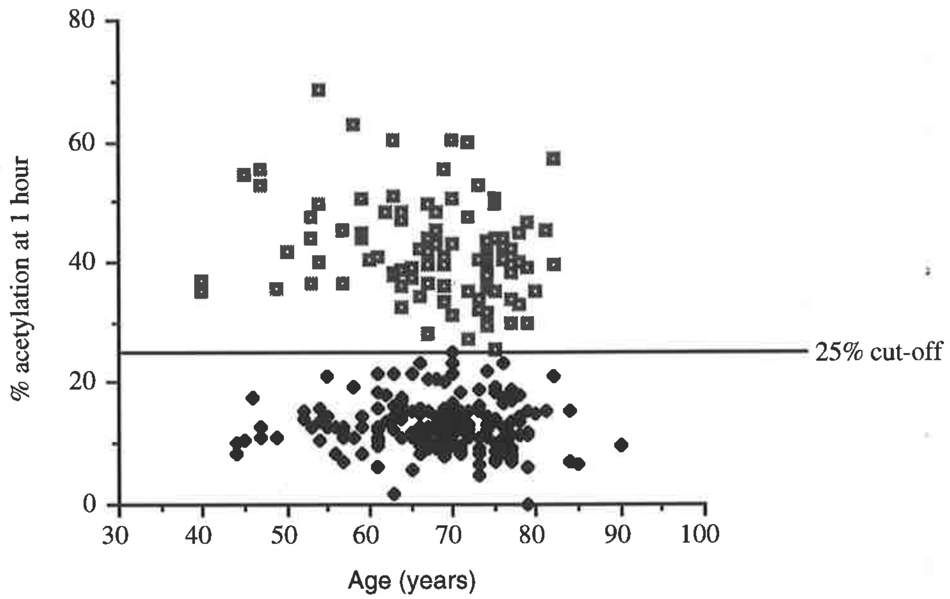


Fig. 3.1b % SMZ acetylation at 1 hour with increasing age showing bimodal distribution, classified as fast if >25% acetylation (■) or slow if <25% acetylation (◆) at 1 hour.

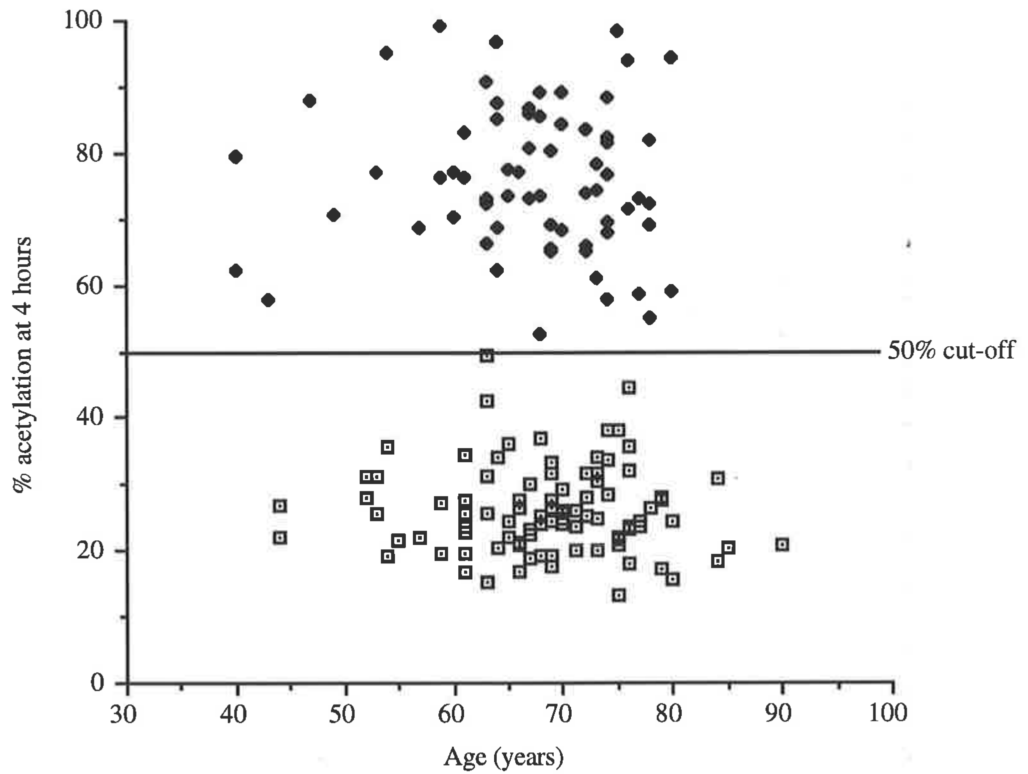


Fig. 3.1c % SMZ acetylation at 4 hours with increasing age, showing bimodal distribution, classified as fast if >50% acetylation(◆) or slow if <50% acetylation(□) at 4 hours.

A plot of % acetylation against time after the ingestion of Sulphamethazine, in fig. 3.2 shows that individuals divide into 2 distinct groups with the dividing line joining 25% and 50% acetylation at 1 and 4 hours respectively (shown as a dashed line).

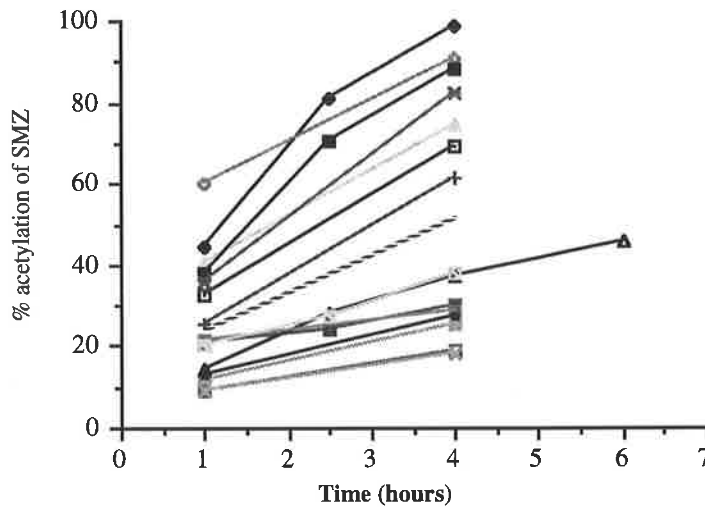


Fig. 3.2 % acetylation of Sulphamethazine with time in 12 individuals, showing separation into 2 groups, classified as fast acetylator above dotted line, and slow acetylator below dotted line.

Acetylator phenotyping was repeated in eight patients to assess repeatability or variability in the results. For all patients, the second test gave identical results to the first test. Four individuals were fast acetylators and four were slow acetylators. At 1 hour, samples taken on both occasions show an average difference of 5% acetylation (SD=3.6).

A total of 311 patients were phenotyped for acetylator status. They consisted of 110 Normal controls, 89 patients with Adenoma and 112 patients with Carcinoma.

The Normal, Adenoma and Carcinoma patient groups were similar in baseline demograpnic and clinical characteristics (table 3.1a) There was a preponderance of males over females in the Normal and Adenoma groups, being more pronounced in the latter, whereas there was a slight preponderance of females over males in the Carcinoma group. Data from a standard textbook

(Shearman and Finlayson, 1989) * is also shown in the last column of table 3.1a for comparison.

Table 3.1a Demographic characteristics in Normal, Adenoma and Carcinoma groups.

		Normal	Adenoma	Carcinoma	Text*
Number		110	89	112	
Sex (male:female)		59:51	57:32	55:57	
Age	mean (SD)	66.8 (8.42)	67.6 (9.87)	68.13 (9.13)	
	median	68.5	69	69	
	range	43-81	40-90	40-84	
Site	Ascending		5(5.6%)	14(12.5%)	15%
	Transverse		4(4.5%)	4(3.6%)	15%
	Descending		8(8.9%)	8(7.1%)	5%
	Sigmoid		38(42.7%)	51(45.5%)	40%
	Rectum		23(25.8%)	30(26.8%)	25%
	Metachronous/Synchronous		11(12.4%)	5((4.5%)	25%

Size of adenoma:		Duke's Stage:		
0-10mm	31(35%)	A	30(26.8%)	5%
11-20 cm	26(30%)	B	46(41.1%)	35%
>20cm	8(9%)	C	30(26.8%)	40%
multiple	17(19%)	D	4(3.6%)	
unknown	7(8%)	unknown	2	
Type of adenoma		**Differentiation:**		
Tubular	22	well	11(11%)	15%
Tub/villous	27	moderate	85(85%)	80%
Villous	8	poor	4(4%)	5%
Unknown	32			

There were significantly more fast acetylators in patients with colorectal carcinoma than in controls. The proportion of fast acetylators in the colorectal adenoma group was between the above 2 groups (See Fig. 3.3, table 3.1b). The "odds ratio" for the increased risk of carcinoma associated with the fast acetylator status was 1.75 (95% C.L.: 1-3). The OR for the statistically

non-significant increased risk for adenoma in fast acetylators was 1.13 (95% C.L.: 0.6-2.1).

Table 3.1b Results of acetylator phenotype in Normal, Adenoma Carcinoma groups.

	Normal		Adenoma		Carcinoma	
Acetylator status	Fast	Slow	Fast	Slow	Fast	Slow
Nunber	33	77	29	60	48	64
Percentage	30	70	32.6	67.4	42.9	57.1
Sex(M/F)	19/14	40/37	18/11	39/21	22/26	33/31

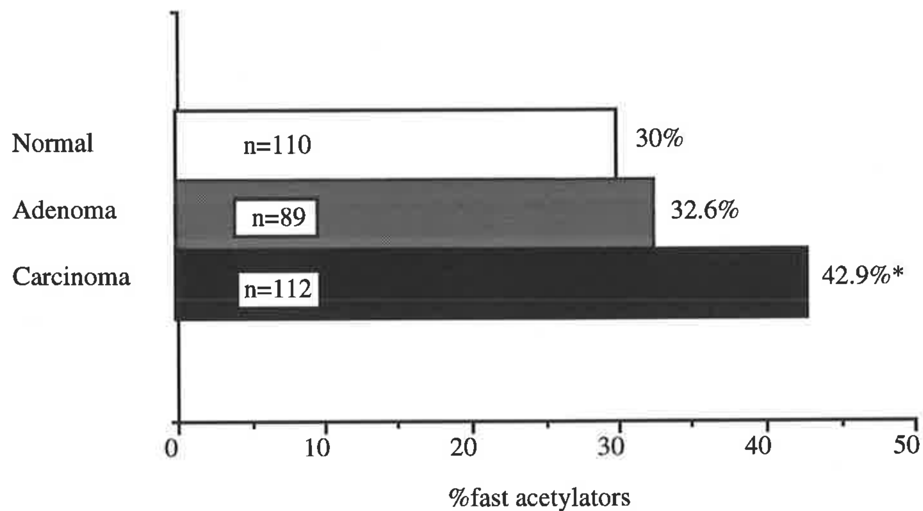


Fig. 3.3 Percentage of Fast acetylators in Normal(□) Adenoma(■) and Carcinoma(■) patient groups. There were significantly more fast acetylators in those with Carcinoma than in Normals($p^* < 0.05$, Chi-squared test).

The difference between the percentage of fast acetylators in Carcinoma patients and Controls is higher in females (18%) than in males (8%) (Fig. 3.3, table 3.1b). The "odds ratio" for the increased risk of carcinoma associated with the fast acetylator status was 1.4 (95% C.L.: 0.7-3) for males and 2.2 (95% C.L.: 1-5) for females. This difference in the OR between the sexes was not significant, perhaps due to low power.

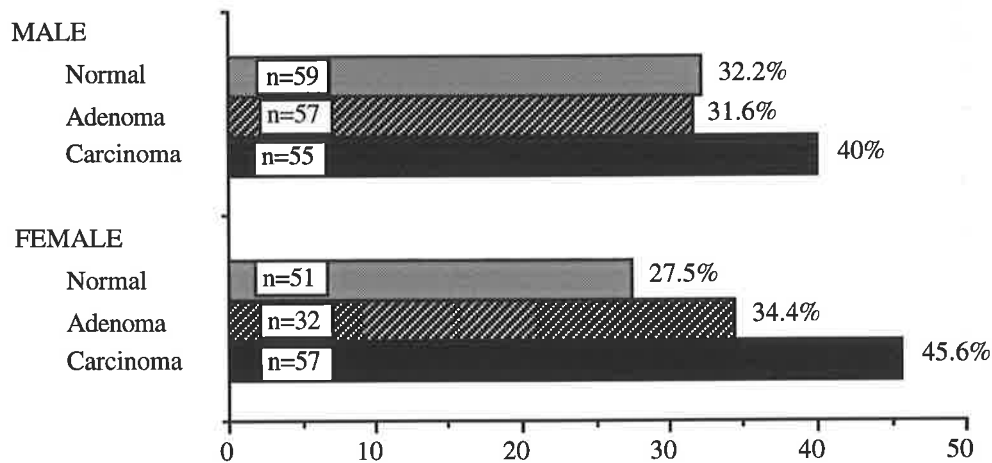


Fig. 3.4 Percentage of fast acetylators in male and female patients in the Normal, Adenoma and Carcinoma groups.

There were no significant differences in the age and sex of fast acetylators compared to slow acetylators(table 3.2a). The distribution of fast acetylators was not correlated with Duke's stage, grade or site of carcinoma (table 3.2a). There was a slight trend, though statistically insignificant, for fast acetylators to develop their carcinomas at a younger age, to have more Dukes stage A or B tumours and to have a preponderance of right sided colonic tumours.

The distribution of fast acetylators was not correlated with the size or type of adenoma(table 3.2b). In this analysis, the acetylator status of 17 patients with multiple adenomas was correlated to the size of the largest adenoma.

Table 3.2a Comparison of fast and slow acetylators who had colonic carcinoma.

		Fast acetylators	Slow acetylators
Age mean (SD)		66.85 (10.42)	69.1(7.98)
Sex (male:female)		22:26	32:31
Duke's stage	A	13 (27.1%)	19 (26.6%)
	B	22 (45.8%)	24 (37.5%)
	C	12 (25%)	18 (28.1%)
	D	1 (2.1%)	3 (4.7%)
Differentiation: well		3 (6.8%)	8 (14.3%)

	mod.	39(81.8%)	46(82.1%)
	poor	2 (4.5%)	2 (3.6%)
Site	Ascending	9 (18.8%)	5 (7.8%)
	Transverse	1 (2.1%)	3 (4.7%)
	Descending	5 (10.4%)	3 (4.7%)
	Sigmoid	17 (35.4%)	34 (53.1%)
	Rectum	13 (27.1%)	17 (26.6%)
	Meta/synchronous	3 (6.25%)	2 (3.1%)

Table 3.2b Comparison of fast and slow acetylators who had colonic adenoma.

		Fast acetylators(%)	Slow acetylators(%)
Size	0-10mm	60.7	48.1
	11-20mm	32.1	40.7
	>20mm	7.1	11.1
Type	Tubular	45	35
	Tub/villous	45	48.6
	Villous	10	16.2

3.2.2 Tissue NAT

Tissue NAT activity was measured in 42 Normal specimens, 22 Adenomas and 40 Carcinomas The Normal tissue was obtained from macroscopically normal colon adjacent to the 40 carcinomas and 2 Adenomas which were removed at operation. The clinical and histological characteristics of the patients and specimens are shown in table 3.3.

There were, therefore, 262 patients who had acetylator status determined alone, 49 who had determination of both acetylator status and tissue NAT activity, and 18 patients who had determination of tissue NAT alone (See diagram 3.1 below).

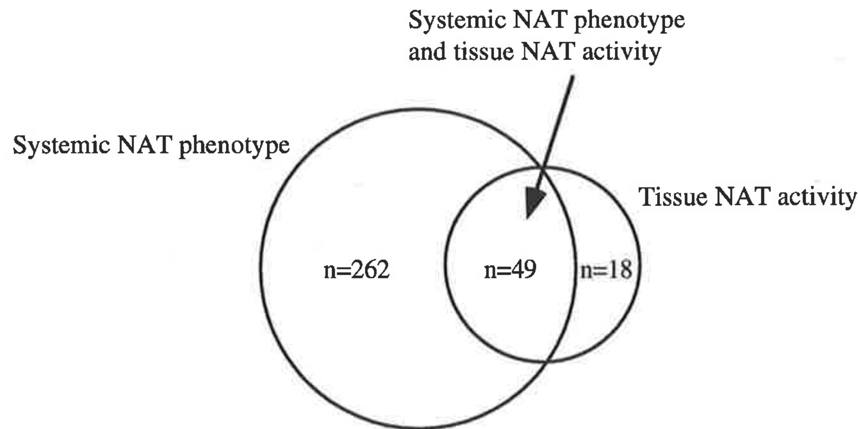


Diagram 3.1 Study of systemic and colonic tissue NAT activity

Table 3.3 Clinical and histological characteristics of patients and specimens.

		Normal	Adenoma		Carcinoma	
Number		42	22		40	
Age	Mean (SD)	67.12 (11.98)	72.78 (8.33)		66.90 (12.12)	
		overall mean (SD) = 68.23 (11.51)				
	Range	40-89	52-89		40-89	
Sex	M:F	26:16	17:5		26:14	
Size of Adenoma			0-10mm	9(41%)	Duke's stage	
			11-20mm	6(27%)	A 7(17.5%)	
			>20	5(23%)	B 19(47.5%)	
			Unknown	2(9%)	C 12(30%)	
Type of Adenoma			Tubular	5(23%)	D 2(5%)	
			Tub-villous	11(50%)	Grade	
			Villous	3(14%)	Well	3
			Unknown	3(14%)	Moderate	32
					Poor	1
					Unknown	4

Tissue NAT distribution

There appeared to be a bimodal distribution in the tissues for polymorphic NAT activity, with the cut-off between the two populations at approximately 0.312 nmols/l/min (table 3.4, fig.3.5a). There would be 2 NAT activity values between 0.604 and 0.75 nmol/l/min if the three "outliers" were not included in the analysis. Investigation revealed no mistake with the "outliers" and they were not excluded from analysis.

Histogram plot for the monomorphic tissue NAT activity showed no evidence of a bimodal distribution (table 3.4, fig.3.5b).

Table 3.4. Descriptive statistics of NAT tissue activities in all tissues

Polymorphic

mean=0.219	Standard deviation=0.167	number=92
median=0.189	Standard error=0.017	range=0.022-0.750

Whiskers and Box plot showed three "outliers" with values, 0.655 (Normal), 0.638(Adenoma) and 0.750(Carcinoma).

Monomorphic

mean=23.71	Standard deviation=16.67	number=107
median=21.43	Standard error=1.61	range=0.75-97.49

Whiskers and Box plot showed one "outlier" with a value of 97.49 (Adenoma).

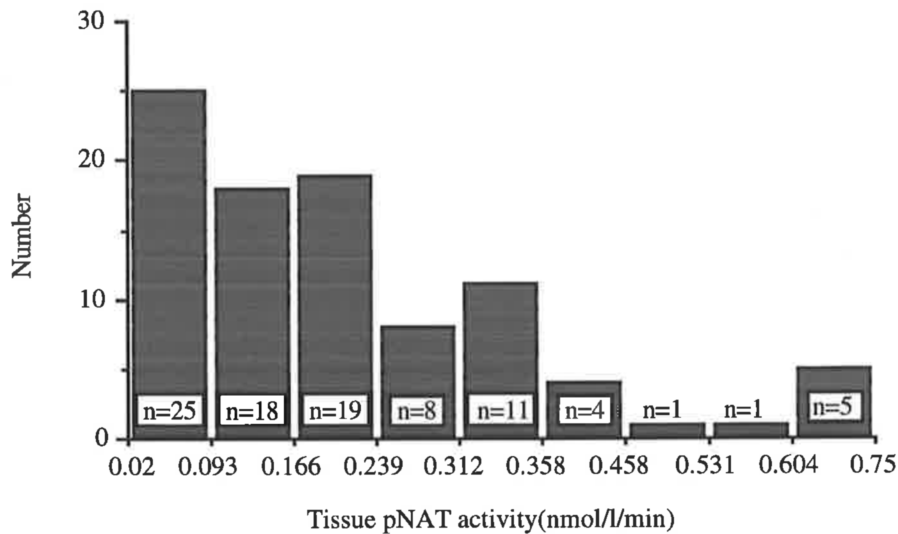


Fig. 3.5a Histogram plot of polymorphic NAT activity(expressed as mean) in all tissues.

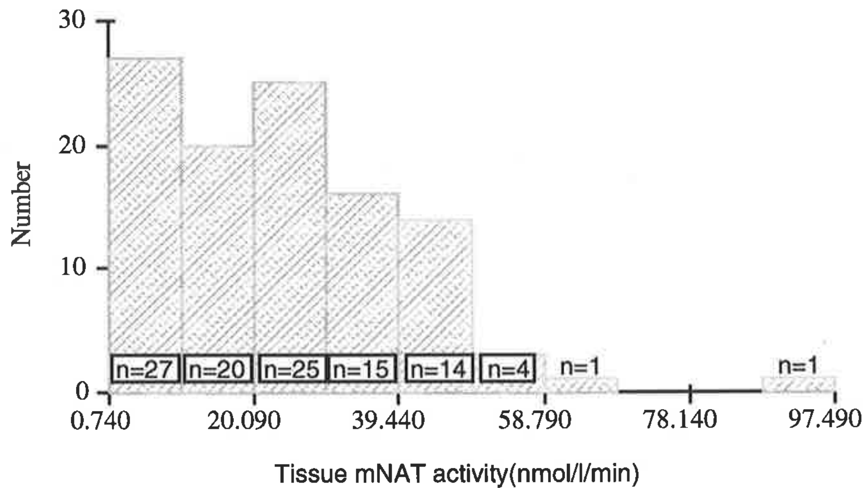


Fig. 3.5b Histogram plot of monomorphic NAT activity(expressed as mean) in all tissues.

NAT activity in uninvolved mucosa and neoplastic colon

There were no significant differences in the monomorphic or polymorphic NAT activity between the three tissue types (See table 3.5, Fig. 3.6). The monomorphic NAT activity is 100-fold greater than the polymorphic NAT activity using sulphamethazine as the substrate.

Table 3.5 Monomorphic and polymorphic NAT activity in Normal, Adenoma and Carcinoma tissues

		Normal	Adenoma	Carcinoma
NAT	Monomorphic			
	Number	40	22	40
	Mean (SE)	22.92 (2.15)	27.42 (5.01)	22.13 (2.43)
	Range	2.6-50.35	0.75-97.49	0.77-57.83
	Polymorphic			
	Number	38	18	36
	Mean (SE)	0.22 (0.02)	0.21 (0.03)	0.22 (0.03)
	Range	0.05-0.66	0.05-0.64	0.02-0.75

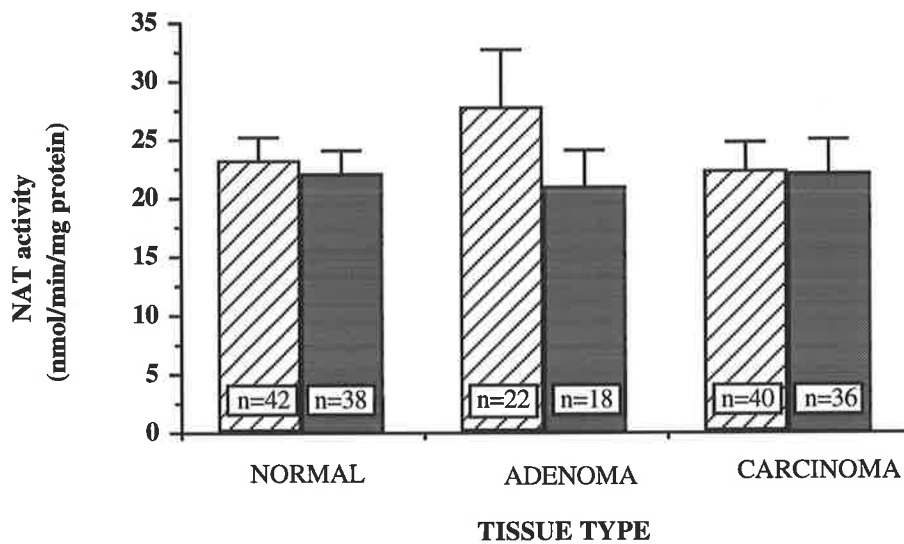


Fig. 3.6 Monomorphic(▨) and polymorphic(■) NAT activity in Normal, Adenoma and Carcinoma colorectal tissue. Monomorphic NAT activity is significantly greater (100 fold) than polymorphic NAT activity in all tissue types. Results expressed as Mean (SE) with the polymorphic NAT activity multiplied 100 fold.

NAT activity between Slow and Fast acetylators in the tissues

There were no significant differences in monomorphic NAT or total NAT activity between the Fast and Slow acetylators in the Normal, Adenoma and Carcinoma tissues (table 3.5, fig. 3.7)

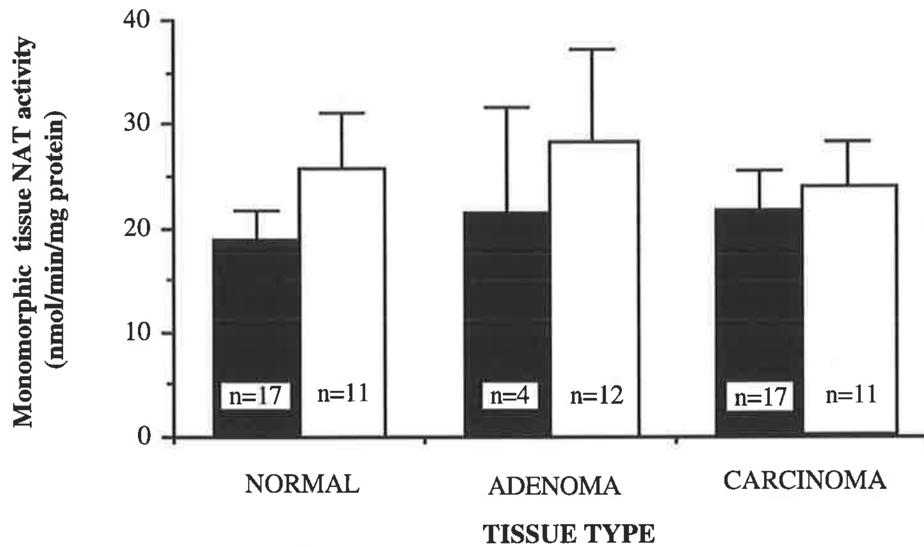


Fig. 3.7 Monomorphic tissue NAT activity of fast(■) and slow(□) acetylators in Normal, Adenoma and Carcinoma tissue.

However, the polymorphic NAT activity was significantly greater in fast acetylators than in slow acetylators in all tissue types (table 3.5, fig. 3.8).

Table 3.5 Monomorphic and polymorphic NAT activity of Fast and Slow acetylators in Normal, Adenoma and Carcinoma tissues.

	Normal		Adenoma		Carcinoma	
Monomorphic						
	Fast	Slow	Fast	Slow	Fast	Slow
Number	17	12	4	14	17	11
Mean	18.77	25.48	21.4	29.34	21.58	24.2
SE	2.85	4.98	10.13	7.26	3.83	4.58
Range	2.6-45.63/	3.35-50.35	3.74-49.55/	0.75-97.49	0.86-46.33/	0.77-43.88

Polymorphic						
	Fast	Slow	Fast	Slow	Fast	Slow
Number	15	13	4	11	16	11
Mean	0.29	0.13	0.31	0.19	0.3	0.12
SE	0.04	0.02	0.02	0.05	0.06	0.03
Range	0.07-0.66/	0.06-0.24	0.09-0.31/	0.05-0.64	0.02-0.75/	0.03-0.31

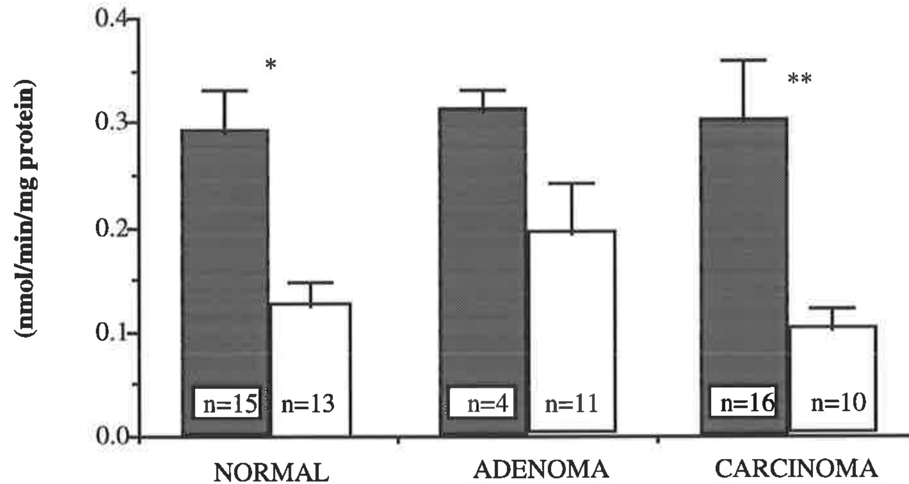


Fig. 3.8 Polymorphic tissue NAT activity of Fast (■) and Slow (□) acetylators in Normal, Adenoma and Carcinoma colorectal tissue. The NAT activity of normal and carcinoma tissues in Fast acetylators was significantly greater (p*=0.004, p**=0.02, Student's t test) than in Slow acetylators.

NAT activity in different regions of the colon

There was significantly higher monomorphic NAT activity from carcinoma tissue from the transverse colon than from the ascending colon.

There were no significant differences in tissue polymorphic NAT activity in normal and carcinoma tissues from different regions of the colon.(Table 3.7, fig. 3.9, fig. 3.10)

Table 3.7 Monomorphic and polymorphic tissue NAT of Normal and Carcinoma tissues in the different regions of the colon.

Monomorphic NAT

	Ascending	Transverse	Descending	Sigmoid	Rectum
Normal tissues					
Number	10	4	6	19	3
Mean	19.26	21.73	17.4	26.7	23.85
SE	3.36	2.54	5.66	3.52	13.25
Range	3.95-40.87	15.95-27.74	3.58-36.97	2.6-50.35	3.35-48.65
Carcinoma tissues					
Number	10	4	5	18	3
Mean	12.79	38.70	24.76	23.08	21.12
SE	3.98	8.22	6.23	3.29	11.75
Range	0.93-36.46	21.59-57.83	2.45-38.96	0.86-46.26	0.77-41.47

Polymorphic NAT

	Ascending	Transverse	Descending	Sigmoid	Rectum
Normal tissues					
Number	9	4	5	17	3
Mean	0.19	0.34	0.27	0.21	0.17
SE	0.04	0.15	0.1	0.02	0.08
Range	0.05-0.42	0.06-0.66	0.06-0.61	0.09-0.39	0.08-0.33
Carcinoma tissues					
Number	7	4	5	18	2
Mean	0.2	0.19	0.37	0.19	0.23
SE	0.1	0.09	0.12	0.04	0
Range	0.02-0.75	0.05-0.44	0.06-0.66	0.03-0.51	-

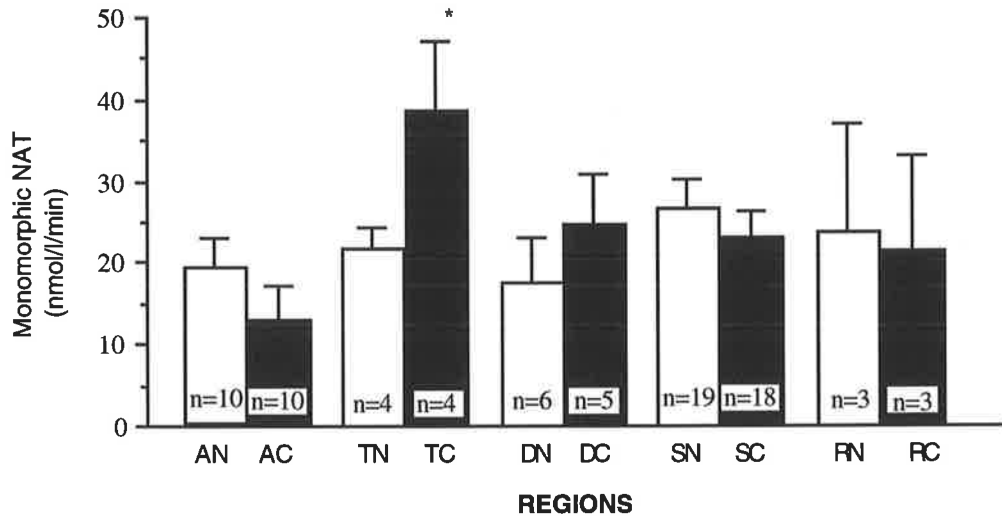


Fig. 3.9 Monomorphic NAT activity in the different regions (A:ascending, T: transverse, D: descending, S: sigmoid, R: rectum, in Normal (N) and Carcinoma (C) tissues, expressed as mean(SE). The transverse colon (TC) is significantly greater than the ascending colon (AC) in carcinoma tissue(p*<0.05, Mann-Whitney U test).

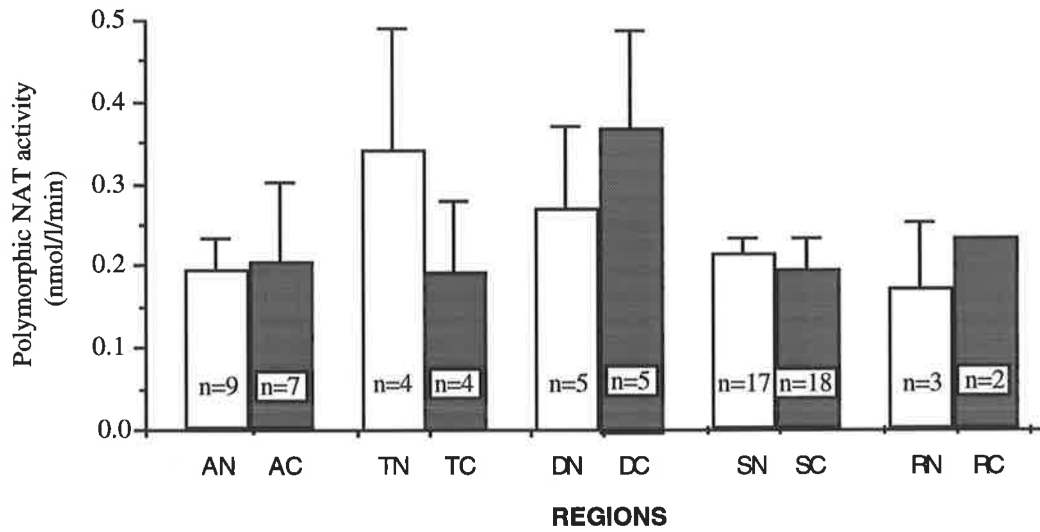


Fig. 3.10 Polymorphic NAT activity in the different regions (A:ascending, T: transverse, D: descending, S: sigmoid, R: rectum, in Normal (N) and Carcinoma (C) tissue, expressed as mean(SE).

NAT activity and gender

There was higher tissue polymorphic NAT activity in Normal and Carcinoma tissue from females than males, although this was statistically significant only in the Normal tissue(table 3.8, fig. 3.11). In the 22 pieces of colonic tissue from male patients, 7 were from fast acetylators, 8 from slow acetylators and 7 of unknown acetylator status, whereas from the 16 pieces of colonic tissue from female patients, 8 were from fast acetylators, 3 from slow acetylators and 5 of unknown acetylator status. This suggests that the higher polymorphic NAT activity seen in tissues of female patients than in tissues of male patients were because there were more fast acetylators in the female group(50%) than there are in the male group(32%). There were no significant differences in the monomorphic NAT activity in Normal and Carcinoma tissue between males and females(Fig. 3.12).

Table 3.8 Sex differences in polymorphic and monomorphic NAT activity in Normal and Cancer tissues.

Polymorphic NAT

	Male/Normal	Female/Normal	Male/Cancer	Female/Cancer
no.	22	16	22	14
mean (SE)	0.16 (0.02)	0.31 (0.04)	0.18 (0.04)	0.27(0.66)

Monomorphic NAT

	Male/Normal	Female/Normal	Male/Cancer	Female/Cancer
no.	26	16	26	14
mean (SE)	25.18 (2.95)	20.18 (3.12)	23.63 (3.15)	19.36 (3.75)

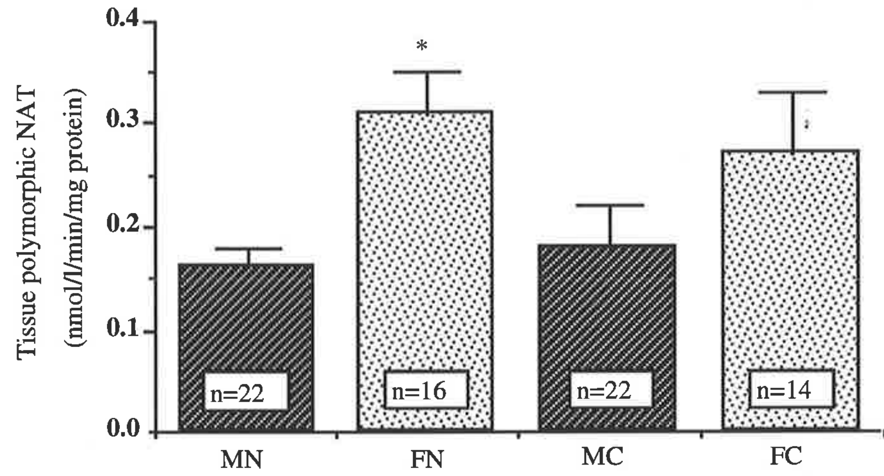


Fig. 3.11 Polymorphic NAT activity in male(M) and female(F) in Normal(N) or Carcinoma(C) tissue, expresmed as mean(SE). Tissue NAT activity in the Normal tissue was greater in female than in Male(p*=0.002, Mann-Whitney U test).

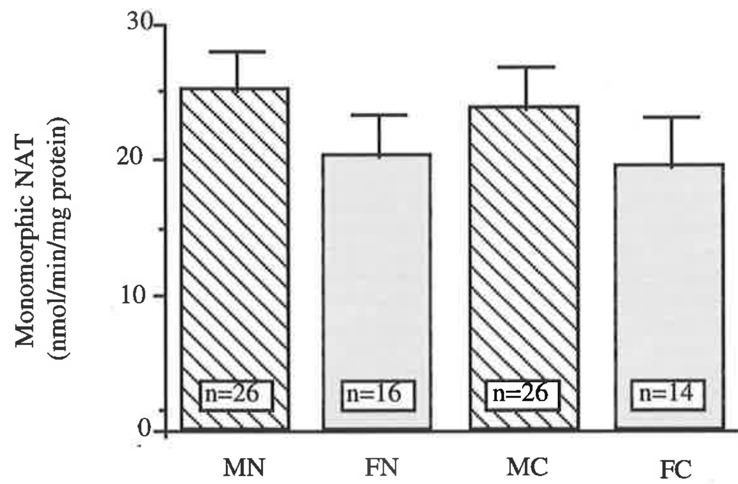


Fig 3.12 Monomorphic NAT activity of males(M) and females(F) in Normal(N) and Carcinoma(C) tissues, expressed as mean(SE).

NAT activity and age

There was no significant correlation of age with tissue mNAT activity in the Normal(r=-0.2) or Carcinoma group(r=-0.03) or with tissue pNAT activity in the Normal(r=-0.18) or Carcinoma group(r=-0.23){ data not shown} .

NAT activity and size/type of Adenoma

The NAT activity in intermediate sized adenomas(11-20mm) was significantly less than in small adenomas(<10mm) or large adenomas(>20mm)(table 3.9, fig. 3.13). Even if the "outlier" in the small adenoma group with a value of 0.638 was removed, the intermediate group is still significantly less than the small adenoma group.

Table 3.9. Relationship of polymorphic NAT activity and adenoma size

Size(max. diameter)	number	NAT activity(mean { sd})
0-10 mm	7	0.326 (0.155)
11-20mm	6	0.107 (0.05)
>20 mm	4	0.216 (0.014)

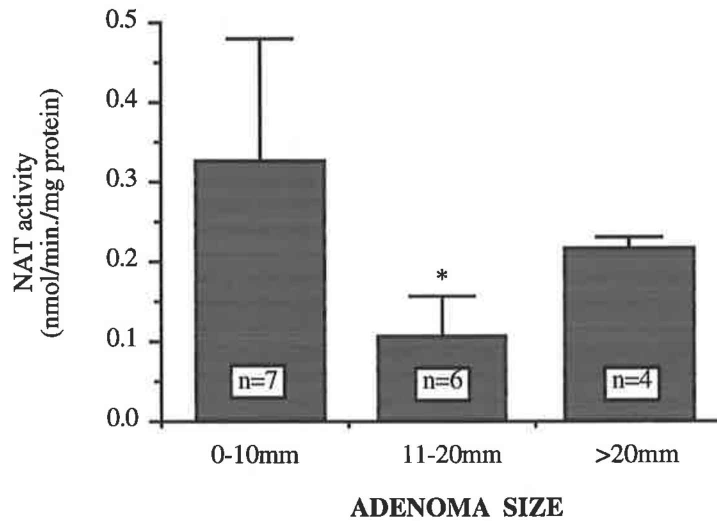


Fig. 3.13 Polymorphic NAT activity in different sized adenomas,expressed as mean(SD). The NAT activity in the intermediate sized adenomas was significantly less than the small adenoma(p*=0.007) and the large adenoma (p*=0.003, Mann-Whitney U test).

There were no significant differences in monomorphic NAT activity in adenomas of different sizes(data not shown). Similarly, there were no significant differences in monomorphic or polymorphic NAT activity in the three different histological types of adenomas(data not shown).

NAT activity and Duke's stage

There were no significant differences in the monomorphic or polymorphic NAT activity either in the Normal or Carcinoma tissue between the different Duke's stages of Carcinoma (table 3.10, fig. 3.14a and b)

Table 3.10 Relationship of NAT activity with the Duke's stage of the Carcinoma.

Monomorphic NAT

Dukes	A	B	C	D
Normal tissue				
Number	7	19	12	2
Mean (SE)	25.11(5.34)	22.85(3.11)	20.68(3.97)	26.76(15.16)
Carcinoma tissue				
Mean (SE)	19.93(3.71)	24.66(3.82)	20.57(4.91)	15.22(8.41)

Polymorphic NAT

Dukes	A	B	C	D
Normal tissue				
Number	7	17	12	1(not analysed)
Mean (SE)	0.2(0.03)	0.21(0.04)	0.24(0.05)	
Carcinoma tissue				
Number	6	18	11	1(not analysed)
Mean (SE)	0.21(0.06)	0.2(0.04)	0.26(0.07)	

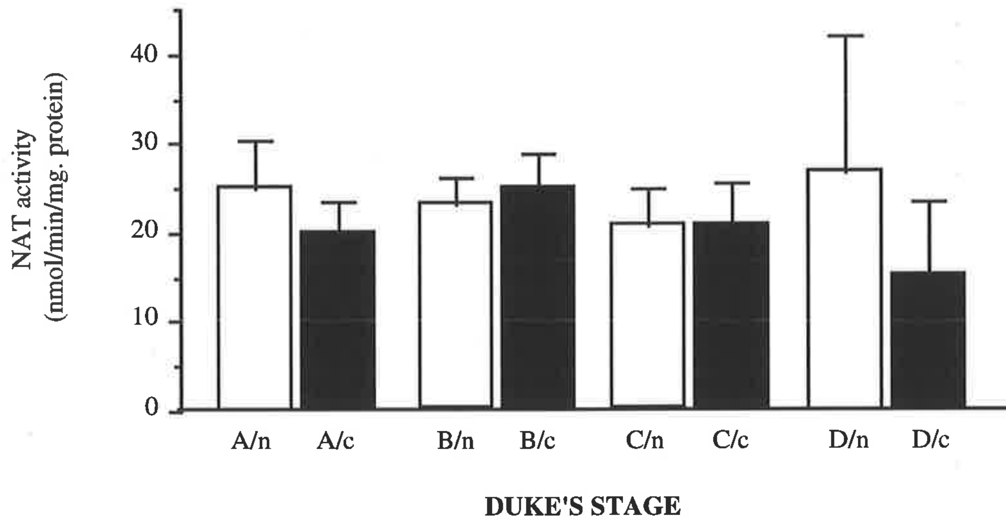


Fig. 3.14a Monomorphic NAT activity in the different Duke's stages, in Normal(n) and Carcinoma(c) tissues, expressed as mean(SD).

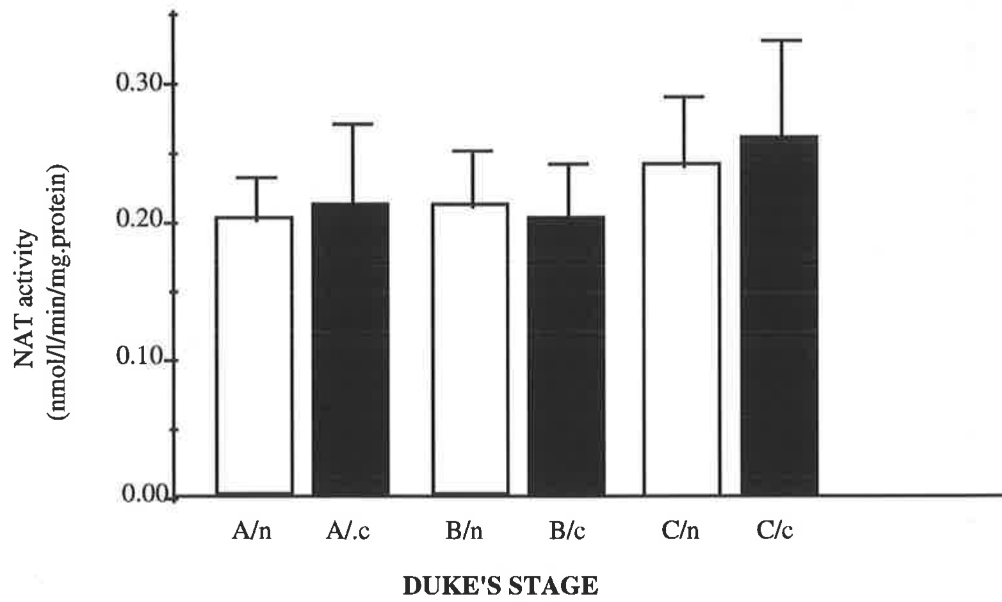


Fig. 3.14b Polymorphic NAT activity in the different Duke's stages, in Normal(n) and Carcinoma(c) tissues, expressed as mean(SD).

NAT activity and carcinoma grade

There was no significant difference in NAT activity between the well and moderately differentiated carcinomas(table 3.11)

Table 3.11 Table showing NAT activity and differentiation in carcinoma tissue

	Well	Moderate
Number	3	32
mean	0.1	0.24
SD	0.03	0.2

Correlation between tissue and systemic acetylator status

The correlation between tissue NAT and the acetylator status could be examined in 36 individuals. If we regard tissue values above 0.312 as "fast"(F), and values below 0.312 as "slow"(S), then correlation with systemic acetylator status would be shown as follows(table 3.12):

Table 3.12 showing correlation between acetylator phenotype in uninvolved normal mucosa from Cancer patients(N/C){ 1} , and in cancer tissues(C){ 2} .

{ 1} Phenotype	Tissue N/C	No.	{ 2} Phenotype	Tissue C	No.
F	F	6	F	F	8
F	S	9	F	S	8
S	S	13	S	S	11
S	F	0	S	F	1

Thus, if an individual was a fast acetylator, the probablity of his normal tissues being "fast" is 6/15 or 40%, and being "slow" is 60%. Similarly, the probability of his cancer tissue being "fast" is 8/16 or 50%, and being "slow" is 50%. If an individual was a slow acetylator, the probability his normal tissues being "slow" is 100%, and his cancer tissue being "slow" is 11/12 or 92%.

The above results show a high likelihood(40-50%) of a falsely low tissue NAT activity if you are a fast acetylator. This would be best explained by enzyme decay caused by the transportation time and time taken to assay the activities of the enzymes. This is estimated to vary between 30 to 90 minutes.

Correlation between the different types of tissues is also poor with discrepant results (that is, "fast" or "slow" in either of the two tissues) in 10/34 or 29% of the tissues(table 3.13)

Table 3.13 showing correlation between Normal tissues from Cancer patients(N/C) with Cancer tissues(C).

N/C	C	No.
F	F	4
F	S	4
S	S	20
S	F	6

3.2.3 NAT Genotype

Distinctive PCR products containing the NAT gene could be seen on electrophoresis consistent with the expected size of 704 base pairs. Digestion with Kpn 1, Taq 1 and Bam H1 restriction enzymes produced the expected fragment sizes for the various genotypes which could be identified on electrophoresis (shown in figs. 3.15a and 3.15b).

Fig. 3.15a. PCR product of NAT gene and digestion with Kpn 1 restriction endonuclease. *Lane 1*, pBr322/Hins Eco marker (bands of 998, 634, 517, 396, 344, 298, 220/221, 154 and 75); *Lanes 2-9*, PCR product; Lanes 10-18, Kpn 1 digests of PCR product (*Lanes 10-12*, homozygotes with both Kpn 1 alleles; *Lanes 13-16*, heterozygotes with Kpn allele; *Lane 17*, homozygote with no Kpn alleles); *Lane 18*, negative (no DNA) control; *Lane 19*, pUC/Hpa 2 marker (bands of 501/489, 331, 242, 190, 147 and 111/110).

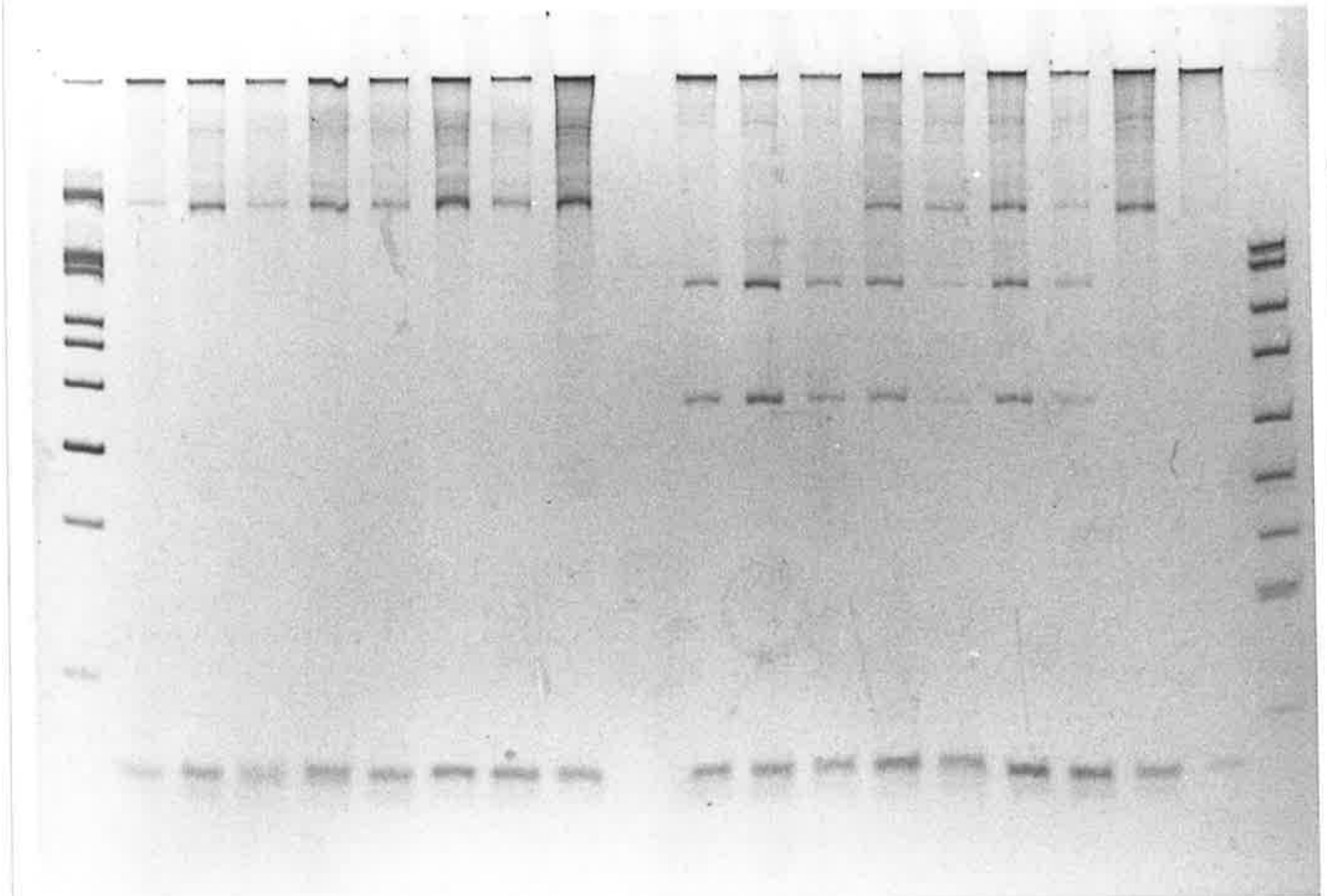
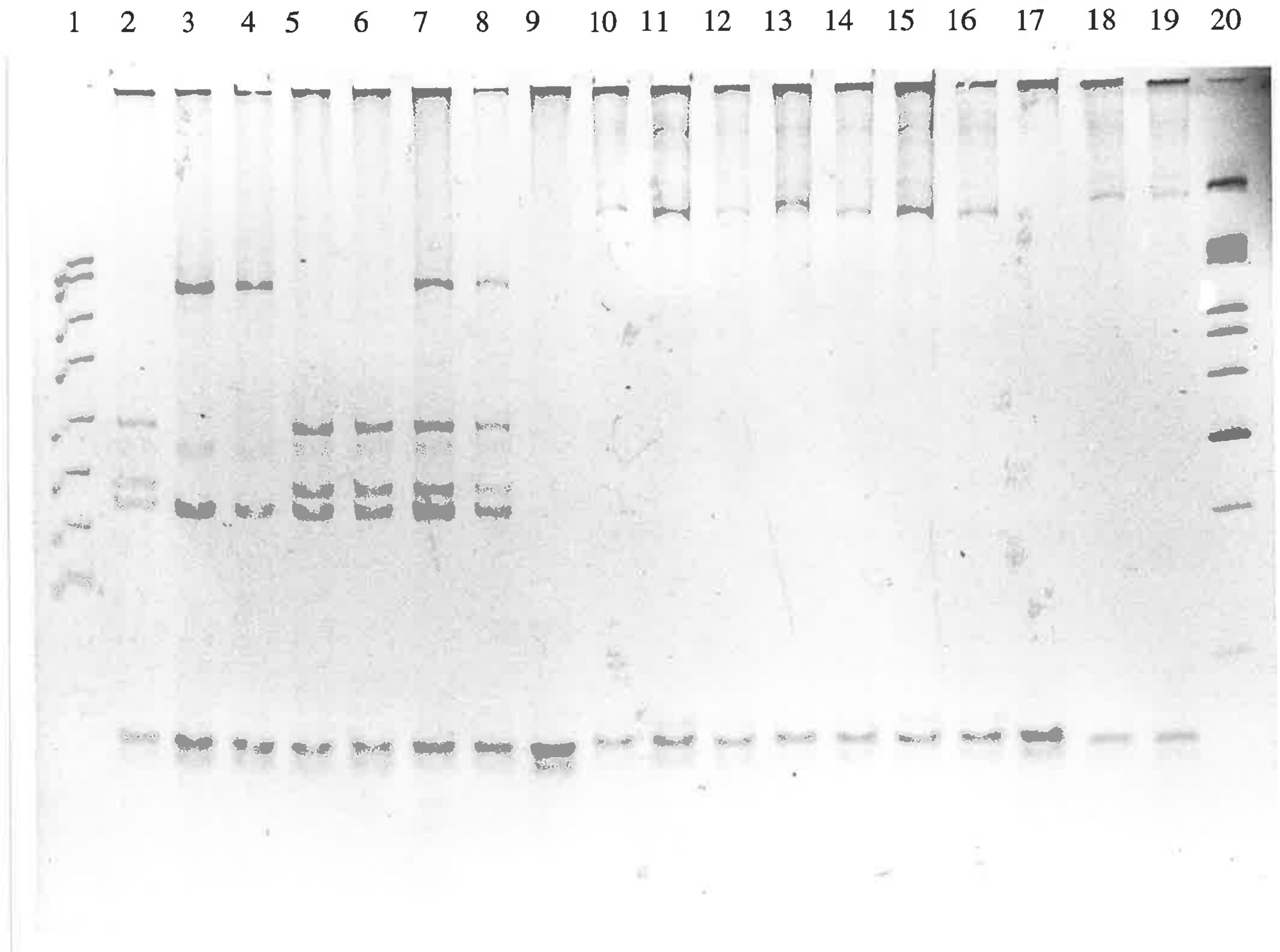


Fig. 3.15b. Taq 1 and Bam H1 digests of NAT product. *Lane 1*, pUC/Hpa 2 marker (bands of 501/489, 331, 242, 190, 147 and 111/110); *Lanes 2-9*, Taq 1 digests of PCR product (*Lanes 2, 5 and 6*, homozygote with both Taq alleles; *Lanes 7 and 8*, heterozygote with 1 Taq 1 allele; *Lanes 3 and 4,* homozygote with no Taq 1 alleles); *Lanes 10-12, 14-17*, homozygotes with both BamH 1 alleles; *Lane 13*, heterozygote with 1 BamH 1 allele; *Lanes 18 and 19*, NAT gene PCR product; *Lane 20*, pBR322/Hins Eco marker (bands of 998, 634, 517, 396, 344, 298, 220/221, 154 and 75).



Concordance between acetylator phenotyping and genotyping

A comparison between acetylator biochemical phenotyping and genotyping was made in 53 individuals. There was concordance in 52 out of 53(98.1%) individuals between acetylator biochemical phenotyping and genotyping.

Biochemical phenotyping was based on % acetylation at 1 hour in 35 individuals, on 1 and 4 hours in 14 individuals and on 4 hours in 4 individuals .

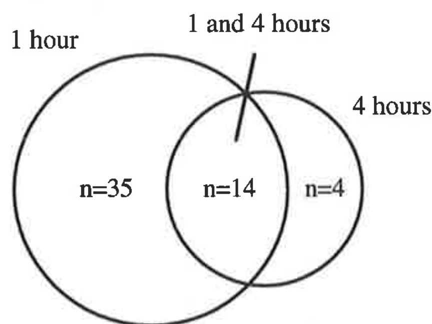


Diagram 3.2 Acetylator phenotyping and genotyping.

There was good concordance between acetylator biochemical phenotyping at 1 hour and genotyping, as shown in fig. 3.16. There were 41 homozygote slow acetylators identified by genotyping, who were also classified as slow acetylators biochemically with % sulphamethazine acetylation below 25% at 1 hour. Seventeen of 18 heterozygote and homozygote fast acetylators were classified as fast acetylators biochemically with acetylation above this 25% threshold. There was no obvious explanation for the one heterozygote who had acetylation below 25% at 1 hour.

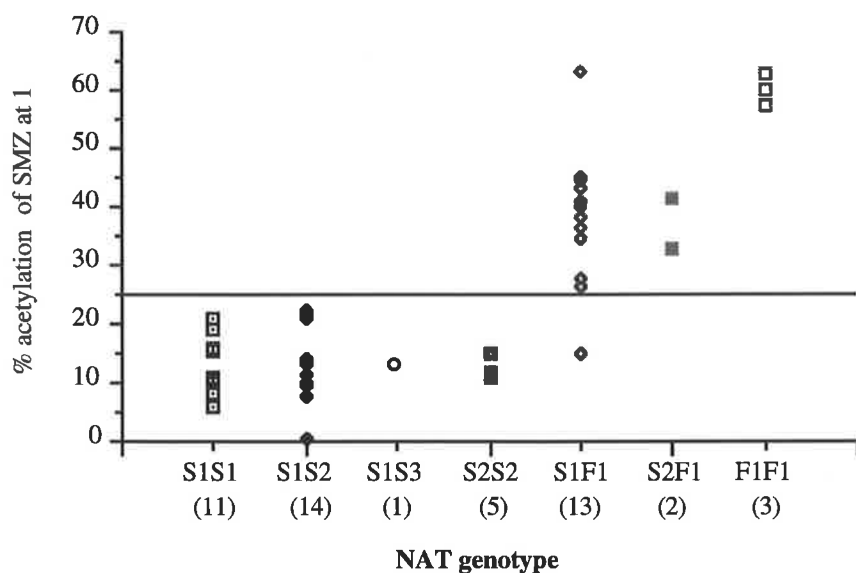


Fig. 3.16 Concordance between acetylator phenotyping at 1 hour and NAT genotyping. The horizontal line at 25% acetylation divides the fast(above line) and slow(below line) acetylators biochemically. The numbers of patients are shown in parenthesis.

Similarly, there was good concordance between biochemical phenotyping at 4 hours and genotyping. All homozygote slow acetylators identified by genotyping were slow biochemically and, all heterozygotes and homozygote fast acetylators were fast biochemically (fig. 3.17).

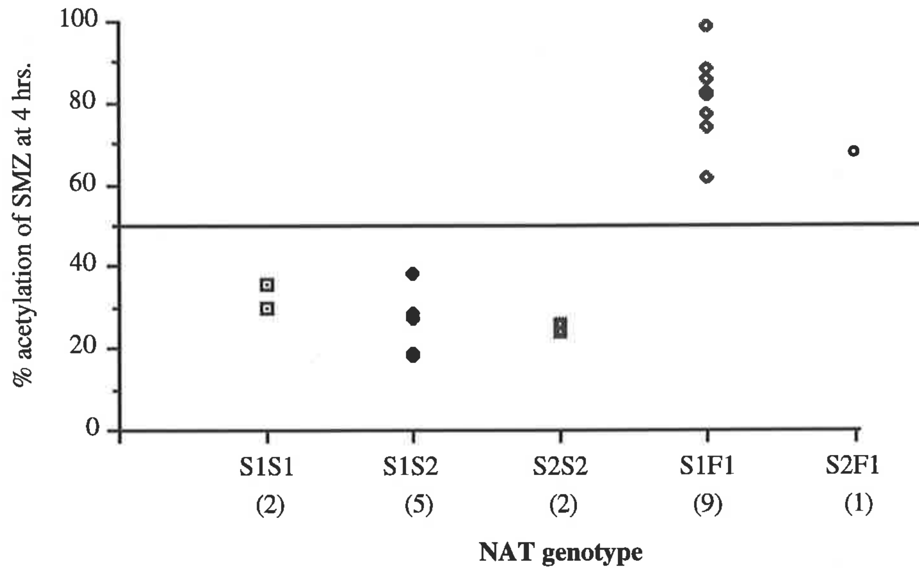


Fig. 3.17 Concordance of biochemical phenotyping at 4 hours and NAT genotyping. The horizontal line at 50% acetylation divides between fast (above line) and slow (below line) acetylators biochemically. The numbers of patients are shown in parenthesis.

Comparison of allelic frequencies in controls and colorectal cancer patients

Clinical characteristics were similar in controls and those with colorectal cancer. The Duke's stage of those with cancer indicate there were more early Duke's stage cancer than that reported in standard textbooks. The allelic frequency results indicate that there were more fast alleles (25%) and less slow alleles (75%) than in controls (fast,16% and slow, 84%)(table 3.14).

Table 3.14 Clinical characteristics and allelic frequencies of controls and colorectal cancer patients.

	Controls	Colorectal cancer
Number (male:female) :	25 (12:13)	28 (13:15)
Mean age (SD) :	67 (9)	70 (8)
Dukes stage (A/B/C/D) :		18% / 54% / 25% / 3%

Allelic frequencies

F1	16	25
S1	52	52
S2	30	23
S3	2	0

3.3 Summary

There were significantly more fast acetylators in patients with colorectal carcinoma than in controls, compatible with the findings from two other centres (Ilett *et al.*, 1987), (Lang, Chu and Hunter, 1986). The OR was 1.75. This would suggest that fast acetylation is a risk factor for the development of colorectal carcinoma. There may also be a greater risk in females than males with the OR for females at 2.22 and for males, 1.4. Adjusting the association of fast acetylators and colorectal carcinoma for sex again gave an OR of 1.75. The demographic and clinical characteristics are similar in controls and in those with colonic adenomas and carcinomas indicating the absence of any confounding variables in patient recruitment. However, the percentage of fast acetylators in controls in this study (30%) was lower than the average of 42% fast acetylators from 20 selected prevalence studies (listed on page 10), and also falls outside their 95% confidence limits. The method of phenotyping is similar in those studies when compared to this study. However, half of the studies listed on page 10,.were done more than 20 years age. The more recent studies of Ilett and Lang looking at the frequency of fast acetylators in controls and colorectal cancer are very similar to this study(fig. 3.18). One obvious difference between the studies on acetylator status and colonic neoplasia (including this study) and with the prevalence studies in page 10, is that hospital-based controls were used in the studies on acetylator status and colonic neoplasia while community-based controls were used in the prevalence studies. There were, however, no apparent uncontrolled confounding

variables for the recruitment of increased numbers of slow acetylators or lesser numbers of fast acetylators in the control group. Ladero's study, which showed no difference in the frequency of fast acetylators in controls and carcinoma, probably used hospital-based controls(fig.3.18) (Ladero, Gonzalez and Benitez, 1991). There was no difference in the distribution of the slow-fast acetylator phenotype with adenoma grade or type, nor with Duke's stage, histologic grade or site of carcinoma.

To support the hypothesis that fast acetylators produce increased amounts of glucuronidated arylamine metabolites which are then deglucuronidated in the colon and undergo increased O-acetylation, it was desirable to demonstrate increased activity of NAT in colonic tissue. The results showed that , although total NAT activity (which is composed mainly of monomorphic NAT) was no different in the normal, adenoma or carcinoma tissues, the polymorphic NAT activity was significantly higher in fast acetylators than in slow acetylators. This finding persists despite correlational studies between tissue NAT activities and the acetylator status of patients which suggests that there may be falsely low tissue NAT levels in fast acetylators due to enzyme decay.

The findings in this study are consistent with the possibility that putative carcinogenic substrates could be activated by polymorphic NAT, even though most NAT activity is derived from the monomorphic isoenzyme. This study was able to discern a slow-fast acetylator phenotype in the colonic tissues, using sulphamethazine as a substrate This could not be shown in another study although only a small number of cytosols were used (Turesky *et al.*, 1991). Kirlin et al., although able to segregate individuals into the slow-fast acetylator phenotype from the colonic tissues, based it on a polymorphic distribution of NAT activity towards PABA (Kirlin *et al.*, 1991), a substrate that was found in this study to have a monomorphic distribution in the colon.

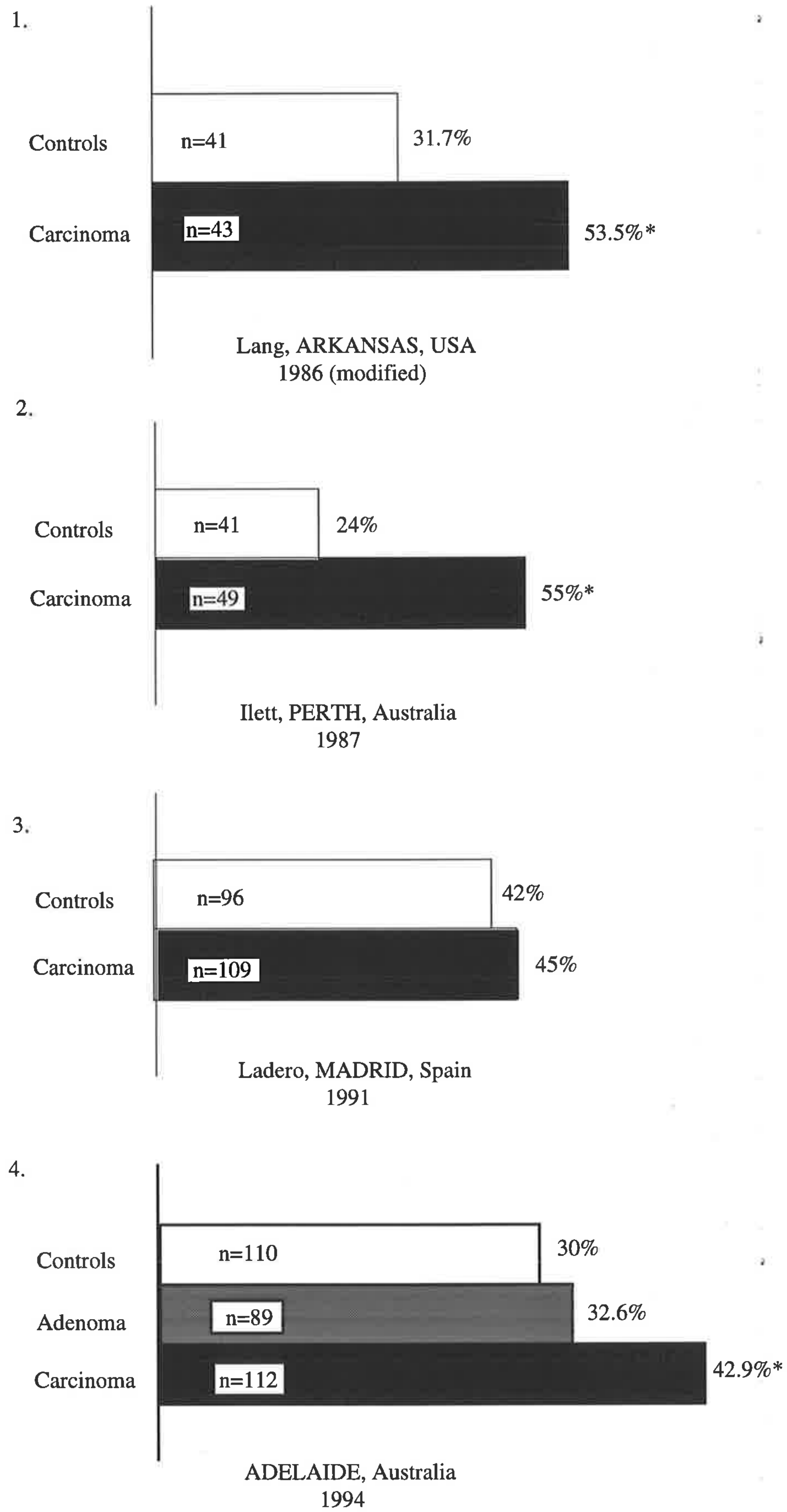


Fig. 3.18 World data showing % fast acetylators in colorectal neoplasia (this study's results is no. 4). Significant differences are shown with an asterisk.

There were no differences in the monomorphic or polymorphic NAT activity with increasing age or in the different parts of the colon. The polymorphic NAT activity was higher in females than males, but only in the normal tissue. This could explain the apparent greater risk of female fast acetylators to develop colorectal carcinoma than male fast acetylators.

The polymorphic NAT activity was significantly higher in the intermediate (11-20mm) sized adenomas than in the small or large adenomas. The significance of this is not known. There were no differences in the monomorphic or polymorphic NAT activity with increasing adenoma size, with the different types of adenoma, or between the different Duke's stages or histologic grade of carcinoma.

A method of genotyping individuals for their acetylator status was also developed for identification of the NAT heterozygote. Good concordance was obtained between biochemical phenotyping and genotyping for acetylator status. Allelic frequencies of the F1, S1, S2 and S3 alleles were 16%, 52%, 30% and 2% respectively. Comparison of these results with 2 other studies by Hickman et al. and Deguchi et al.(Hickman and Sim, 1991), (Deguchi, Mashimo and Suzuki, 1990) confirm that the majority of Caucasians are slow acetylators, with a predominant S1 allele, whereas the Japanese are mostly fast acetylators (and when slow acetylators, they are mostly of the S2 allele). A study comparing the NAT allelic frequencies between 25 controls and 28 colorectal cancer patients showed a trend towards a higher frequency of the F1 allele in the latter group, consistent with the results from the larger biochemical phenotyping study. In addition, genotyping offers the potential for examining archival tissue specimens for the determination of acetylator status.

CHAPTER 4 GLUTATHIONE S TRANSFERASE AND COLORECTAL NEOPLASIA

4.1 Introduction

Glutathione S transferases (GST) are a family of enzymes that mediate glutathione conjugation to a variety of xenobiotics including carcinogens and anti-neoplastic drugs. The level of GST and GSH in the colonic tissues may therefore be important in protection against the development of neoplasia. GST consists of 3 isoenzymes; α, μ, and π. As there is a genetic polymorphism affecting GSTμ, it is possible that the GSTμ null phenotype (individuals who lack the isoenzyme GSTμ) might have impared detoxification of carcinogens and a greater predisposition to the development of carcinoma. This study determined the total GST activity and GSH content, using standard biochemical methods, in colonic specimens of normal and neoplastic tissue. It also compared the frequency of the GSTμ null phenotype in controls and in patients with colorectal carcinoma. A method for determining the GSTμ null phenotype was developed using PCR. This method involved a positive control to distinguish between the inherited absence of the gene and a false negative DNA amplification in the PCR reaction.

4.2 Results

4.2.2 Tissue Glutathione S Transferase

Total GST activity against the aryl substrate, 1, chloro, 2, 4 dinitrobenzene(CDNB) was measured in cytosols from 59 normal specimens, 25 adenomas and 57 carcinomas. All normal tissue was macroscopically free of neoplasia and was taken from approximately 10 cm away

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from any visible neoplasm in the surgical specimens.Two of 18 adenomas had foci of in-situ carcinoma, 4 of 18 had metachronous colorectal carcinoma and 1 had a synchronous carcinoma and adenoma of the colon. The 57 carcinoma specimens were from 56 patients; one patient provided tissue from 2 carcinomas operated on different times from different parts of the colon. Patient characteristics, type and size of the adenomas, and the stage of carcinomas are shown in table 4.1 below. There were no significant differences in the patient characteristics.

Table 4.1 Clinical and histological characteristics of the three patient groups

		Normal	Adenoma		Carcinoma
Number		59	25		57
Age	Mean(SD)	68.71 (12.26)	71.04 (10.91)		68.61 (12.38)
	Range	40-90	40-85		40-90
Sex	M:F	36:23	18:7		35:22
Type of Adenoma			Tubular	5(20%)	Dukes stage
			Tub-villous	11(44%)	A 14(24.5%)
			Villous	6(24%)	B 26(45.6%)
			Unknown	3(12%)	C 14(24.5%)
Size of Adenoma			0-<1 cm.	10(40%)	D 3(5.3%)
			1-2 cm.	8(32%)	Grade
			>2 cm.	5(20%)	Well 7
			Unknown	2(8%)	Moderate 48
					Poor 2
Site	Ascending		0		16(28%)
	Transverse		1		6(11%)
	Descending		1		6(11%)
	Sigmoid		0		25(44%)
	Rectum		23		4(7%)

GST activity in uninvolved mucosa and neoplastic colon

By using the Box and Whiskers plot(Macintosh Statworks™), four and two values in the

Normal and Carcinoma groups respectively, were outside the "whiskers" and are referred to as "outliers". Investigation revealed no mistakes with these values and they were included in the statistical analysis. If excluded, the mean(SE) of the Normal and Carcinoma groups would be 44.8 (3.48) and 61.02 (4.83).

In our sample of specimens, GST activity in the carcinoma group was significantly greater than in the Normal tissue, irrespective of whether or not the "outliers" were included in the analyses. Adenoma GST activity was intermediate between Normal and Carcinoma tissue(See table 4.2, fig. 4.1).

Table 4.2 GST activity(nmol/l/min) in Normal, Adenoma and Carcinoma tissues

		Normal	Adenoma	Carcinoma
GST	Mean(SE)	53.31 (5.36)	59.25 (6.76)	67.78 (6.86)
	Range	10.03-221.41	9.47-119.26	2.18-322.47
GST Ca/N ratio (n=57)		Mean(SE) = 1.49(0.11)		
		Range = 0.01-5.01		

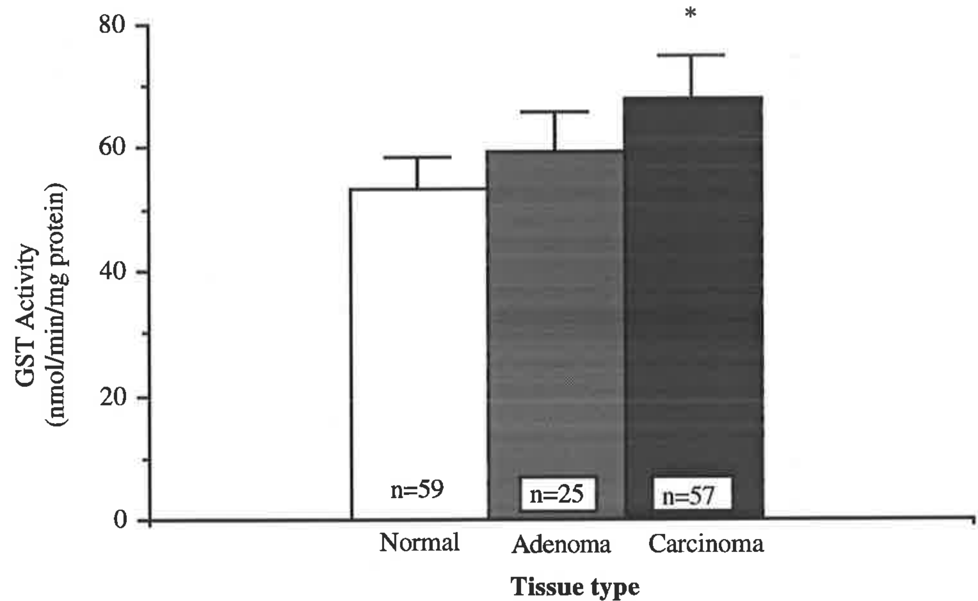


Figure 4.1 Glutathione S Transferase (GST) activity in normal and neoplastic colorectal tissue. Values expressed as nmol CDNB conjugated/min/mg protein. GST activity in Carcinoma tissue was significantly greater than in Normal tissue (p*<0.05, Mann- Whitney U test).

The great variability among individuals makes it important that comparisons be made between carcinoma tissue and normal adjacent mucosa from the same individual. In individual specimens, the GST activity in Carcinoma is, on average, 1.5 times greater than its neighbouring Normal tissue (Table 4.2).

The GST activity in neighbouring Normal tissue was weakly positively correlated with the GST activity in Carcinomas ($r=0.454$, $r^2=0.2$, $p<0.05$).

The frequency distribution of GST activity in Normal tissue showed a normal distribution slightly skewed to the left. The frequency distribution in the Carcinoma tissue was suggestive of a bimodal distribution. The number in the Adenoma group were too few to draw conclusions (Fig. 4.2a-c).

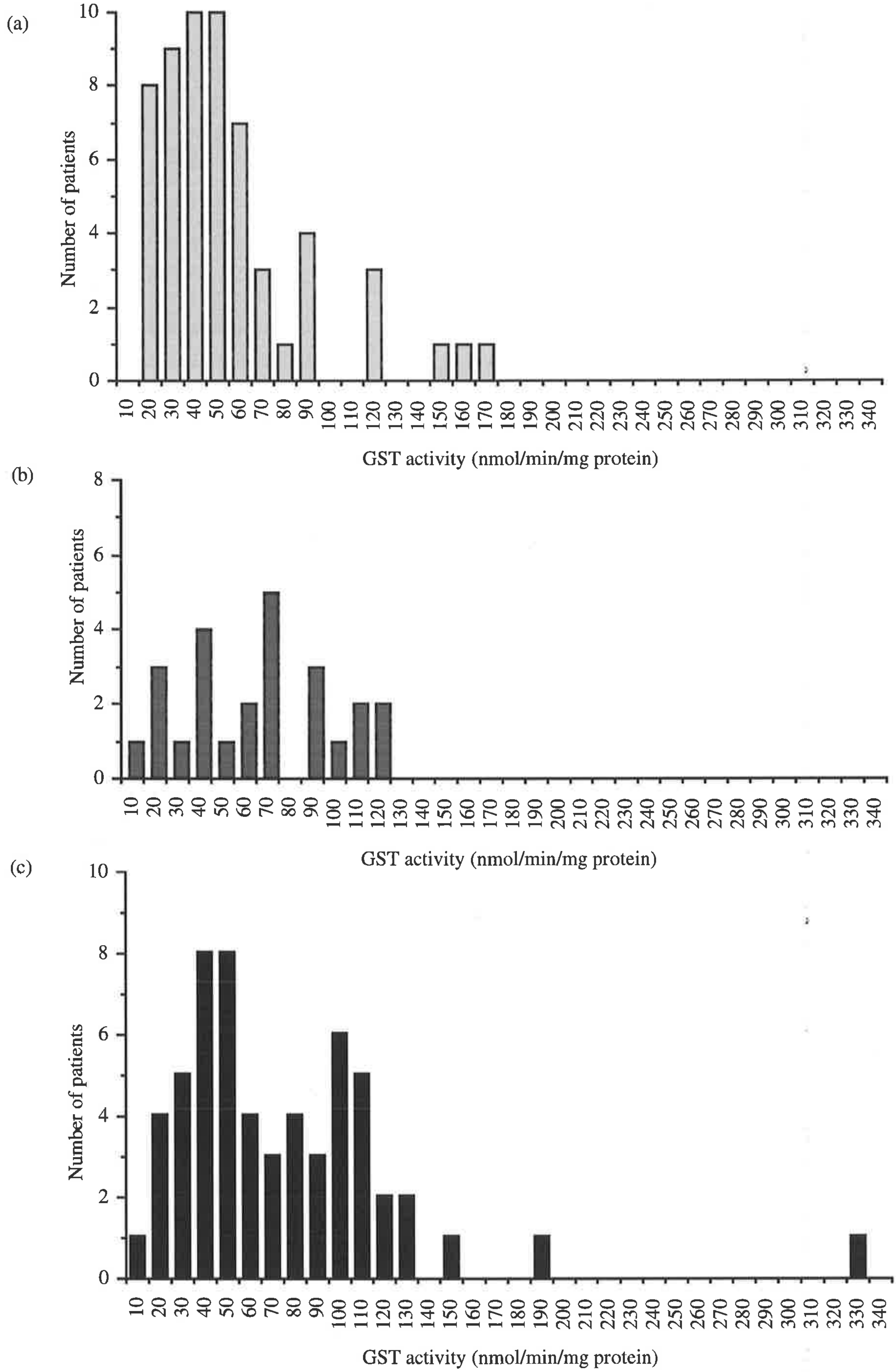


Figure 4..2 Frequency distribution of GST activity in Normal, Adenoma and Carcinoma groups (a,b,c respectively.)

GST activity in different regions of the colon

The regions of the colon were divided into the ascending, transverse, descending, sigmoid and rectum (See Table 4.3). The GST activity in Normal and Carcinoma tissue were not statistically different in the different regions of the colon.

Table 4.3 GST activity (nmol/l/min, mean { SE}) in different regions of the colon in Normal, Adenoma and Carcinoma tissues

	Normal	Adenoma	Carcinoma
Ascending	49.46 (8.75)		62.9 (9.54)
Transverse	50.48 (20.66)		98.86 (33.27)
Descending	50.30 (16.27)		101.95 (46.3)
Sigmoid	52.45 (8.51)		55.04 (6.53)
Rectum	61.30 (13.70)	56.37 (7.44)	103.41 (8.70)

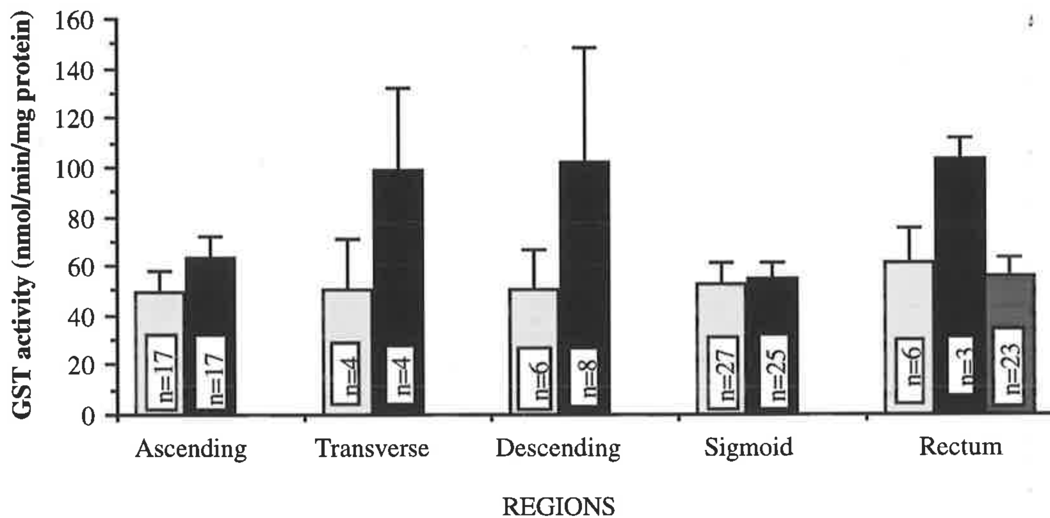


Fig. 4.3 GST activity (expressed as mean{SE}) in different regions of the colon in normal (□) adenoma(■) and carcinoma (■). Twenty-three of the 25 adenomas were in the rectum.

GST activity and gender

As shown in Fig. 4.4, gender had no effect on tissue GST activity.

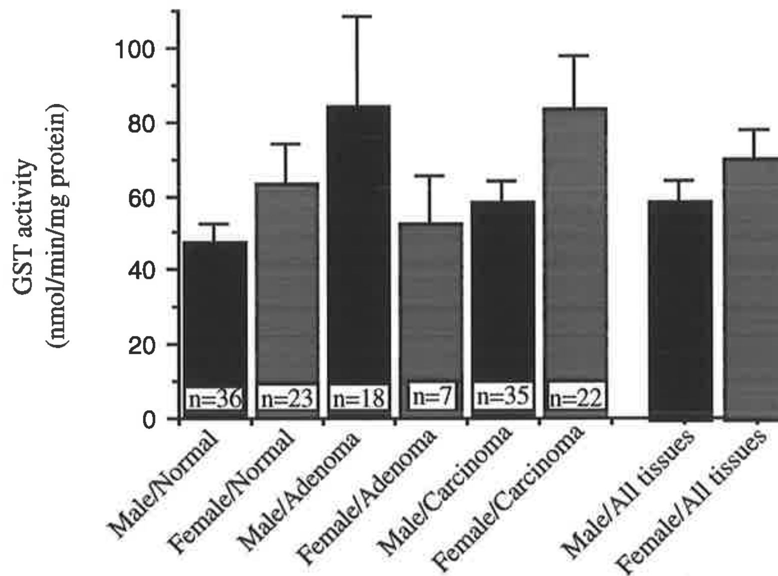


Fig. 4.4. GST activity (expressed as mean {SE} between Male(■) and Female (■) patients in Normal, Adenoma, Carcinoma groups and in all tissues.

GST activity and age

Linear regression analysis showed no correlation between age and GST activity in Normal (r=0.1), Adenoma (r=0.2) or Carcinoma (r=0.1) groups.

GST activity and size/type of Adenoma

GST activity was significantly greater in large adenomas (>20 mm.) than in small adenomas (0-10 mm.). The GST activity of adenomas 11-20 mm in size was intermediate between the above

groups. GST activity was positively correlated with adenoma size ($r=0.43$, $r^2=0.19$, $p<0.05$, using multiple regression analysis). (See fig. 4.5)

Table 4.4 Relationship between GST activity and size of adenomas.

Size(max. diameter)	GST activity (mean{ SE})
0-10 mm.	45.91 (8)
11-20 mm.	68.68 (12.13)
>20 mm.	81.87 (11.34)

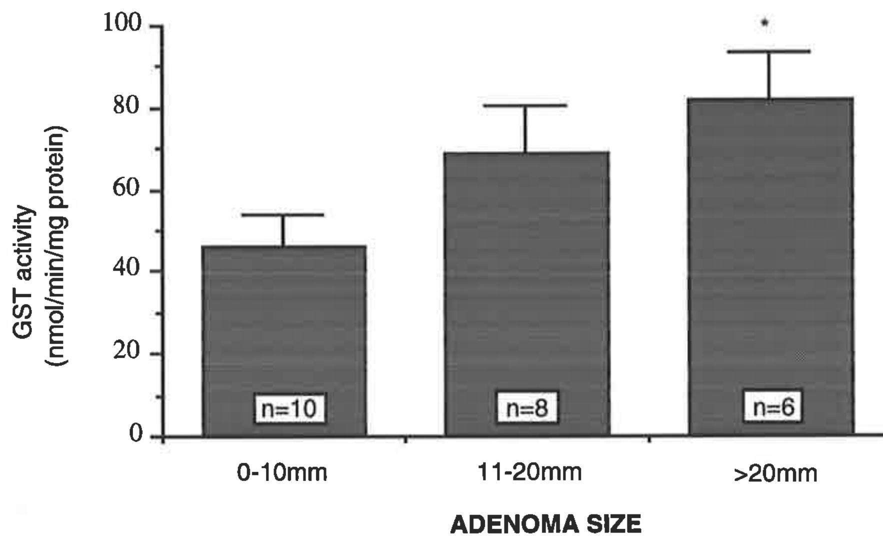


Fig. 4.5 GST activity in the 3 different sized adenomas, expressed as mean(SE). Adenomas >20mm diameter has significantly greater GST activity than adenomas 0-10mm diameter(p=0.007*, Students t test{unp aired}).

There was also a positive trend for increasing GST activity with histological grade, progressing from tubular to tubulovillous to villous adenomas, but differences were not statistically significant, perhaps due to the small numbers in each group(Fig.4.6)

Table 4.5. Relationship between GST activity and type of Adenoma

Type	GST activity (mean { SE})
Tubular	44.26 (19.68)
Tub-villous	53.45 (9.02)
Villous	73.11 (13.45)

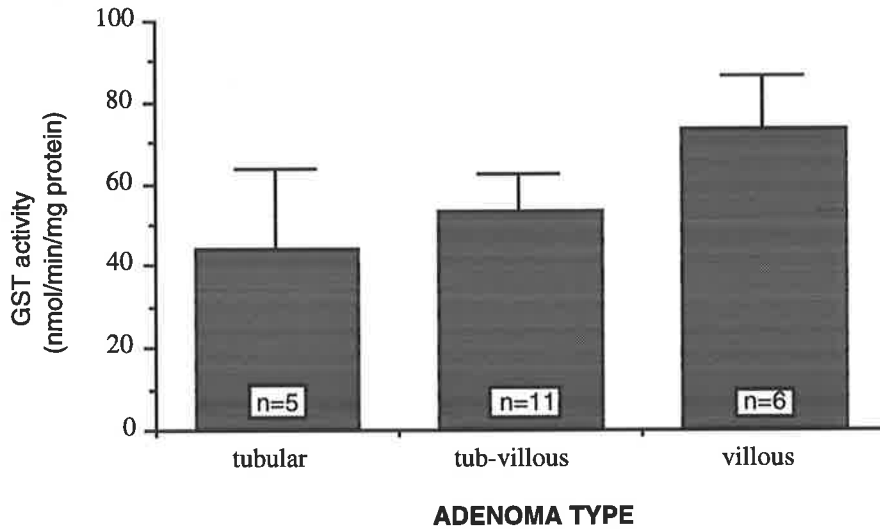


Fig. 4.6 GST activity(expressed as mean{SE}) in the 3 histological types of Adenomas.

GST activity and Duke's stage

GST activity was not influenced by the Duke's stage, either in carcinoma tissue or in adjacent normal mucosa (Fig.4.7).

Table 4.6 Mean (SE) of GST activity in Normal and Carcinoma tissues in the different Duke's stages.

Duke's/Tissues	Normal	Carcinoma
A	58.95 (9.29)	69.84 (10.07)
B	55.98 (9.77)	60.61 (7.28)
C	49.35 (10.66)	85.3 (22.23)
D	40.43 (11.01)	34.24 (2.41)

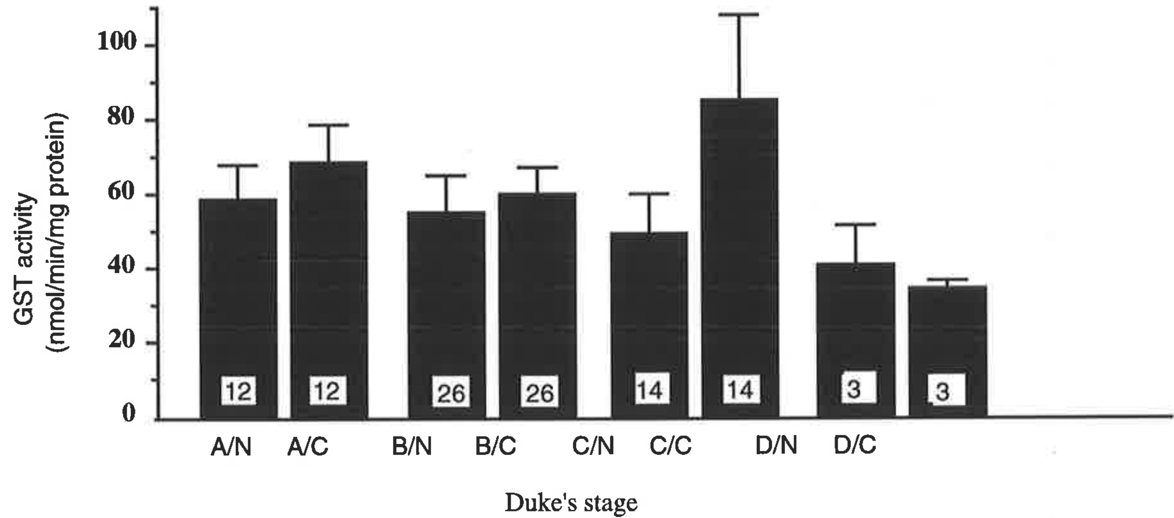


Fig. 4.7 GST activity in the Normal(N) and Carcinoma(C) tissues of the different Duke's stages, expressed as mean(SE).

GST activity and carcinoma grade

There were no significant differences in GST activity between well, moderately and poorly differentiated carcinomas (see table 4.7).

Table 4.7 GST activity in the three grades of carcinoma

	Well	Moderate	Poor
number	7	48	2
mean	54.92	70.28	28.89
SD	31.66	53.66	2.45

Relationship between tissue GST and GSH

The GST activity appeared to be unrelated to GSH content in the same tissues; the GST activity remaining fairly constant relative to varying concentrations of tissue GSH.

4.2.3 Tissue Glutathione content

Total GSH content (oxidized and reduced) was measured in 58 Normal, 25 Adenoma and 56 Carcinoma specimens as described previously. The patient and histological characteristics of the three groups are shown in table 4.8 below.

Table 4.8 Clinical and histological characteristics of the three patient groups

		Normal	Adenoma		Carcinoma
Number		58	25		56
Age	Mean (SD)	68.4 (12.05)	71.04 (10.91)		68.29 (12.16)
Sex	M:F	35:23	18:7		35:21
Type of Adenoma			Tubular	5(20%)	Dukes stage
			Tub-villous	11(44%)	A 13(23.2%)
			Villous	6(24%)	B 26(46.4%)
			Unknown	3(12%)	C 14(25%)
Size of Adenoma			0-<1 cm.	10(40%)	D 3(5.4%)
			1-2 cm.	8(32%)	
			>2 cm.	5(20%)	
			Unknown	2(8%)	
Site	Ascending		0		15(27%)
	Transverse		1		6(11%)
	Descending		1		6(11%)
	Sigmoid		0		25(45%)
	Rectum		23		4(7%)

GSH content in uninvolved mucosa and neoplastic colon

Using the Box and Whiskers plot(Macintosh Statworks™), there were two "outliers" in the Normal and Adenoma groups and three "outliers" in the Carcinoma group. Investigation revealed no mistakes with these values and they were included in the statistical analysis. If excluded, the mean (SE) in the Normal, Adenoma and Carcinoma groups would be 19.35 (1.09), 25.11 (2.7), 25.7 (1.57)

GSH content was significantly greater in neoplastic (adenoma and carcinoma) tissue than in normal colorectal tissue, irrespective of whether the analyses were done with or without the "outliers". There was no difference in GSH content between Carcinoma and Adenoma groups (See table 4.9 fig. 4.8).

Table 4.9 GSH content in the Normal, Adenoma and Carcinoma tissues

		Normal	Adenoma	Carcinoma
Number		58	25	56
GSH	Mean (SE)	22.49 (2.52)	33.28 (7.39)	30.30 (3.37)
	Range	7.58-155.26	1.77-197.60	8.91-180.77

GSH Ca/N ratio (n=57) Mean (SE) = 1.59 (0.18)

Range = 0.47-9.5

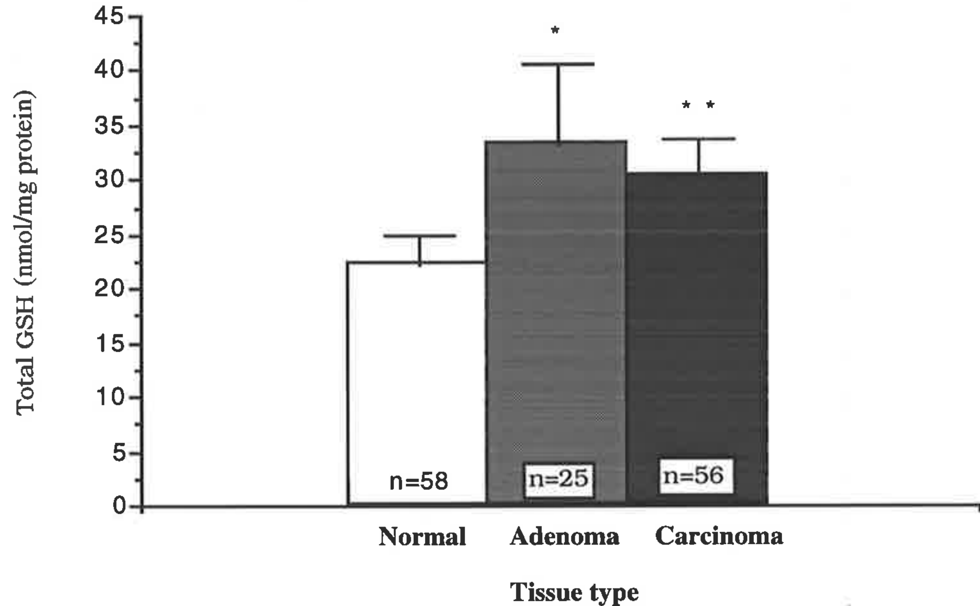


Figure 4.8 Glutathione concentrations (GSH) in normal and neoplastic colorectal tissue, expressed as mean (SE). GSH concentration in Adenoma tissue significantly greater than in Normal tissue (p**<0.005) and in Carcinoma tissue significantly greater than in Normal tissue (p*<0.05, Mann-hitney U test)).

The GSH content in neighbouring Normal tissue was weakly positively correlated with GSH content in the Carcinomas ($r=0.367$, $r^2=0.135$, NS)

The frequency distribution of GSH values in the three tissue groups suggested a normal dsitribution(Fig.4.9).

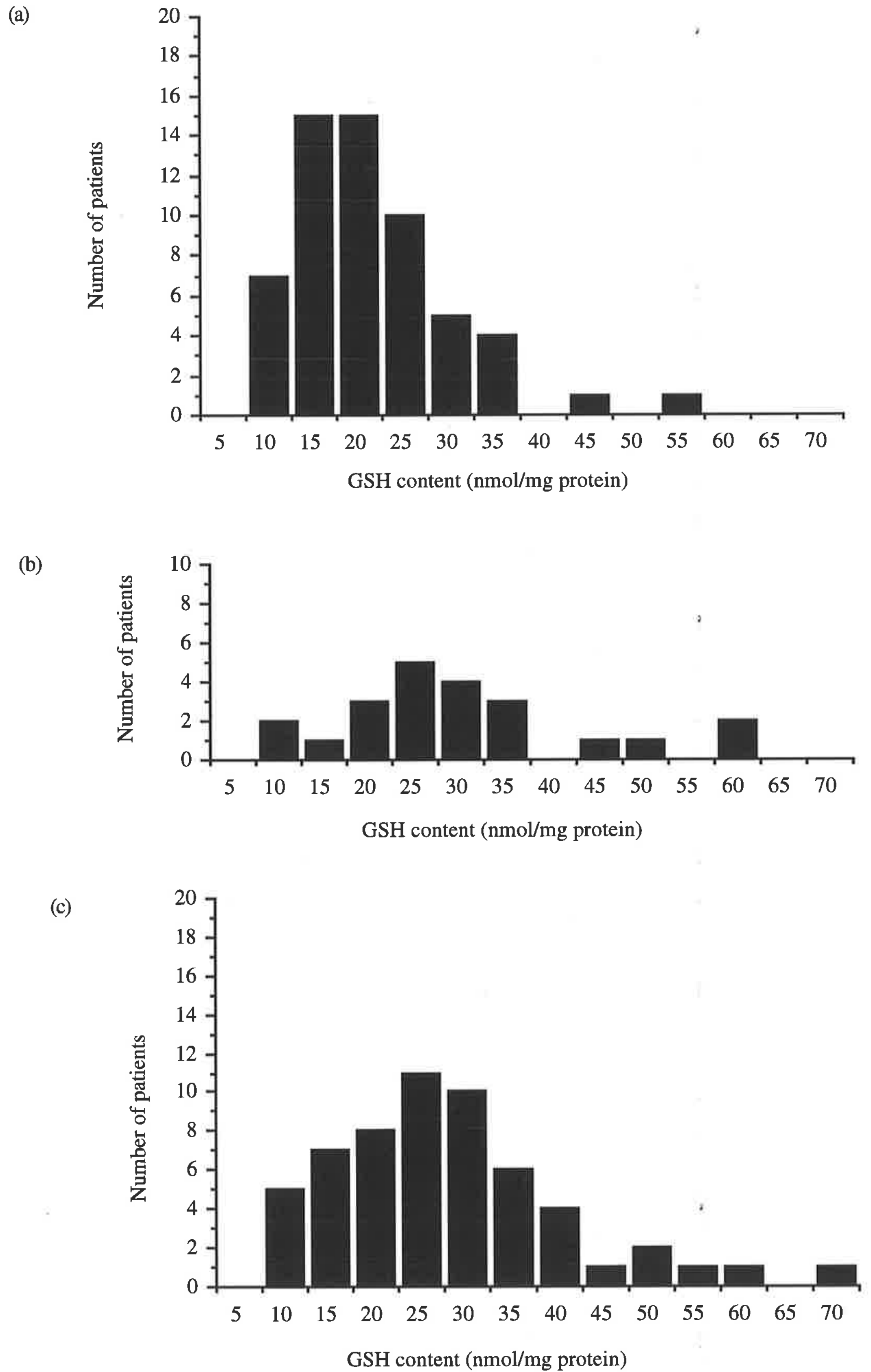


Figure 4.9. Frequency distribution of GSH content in Normal, Adenoma and Carcinoma groups (a,b and c resp.).

GSH concentrations in different regions of the colon

The regions of the colon were divided into ascending, transverse, descending, sigmoid and rectum. There were significantly higher concentrations of GSH in carcinomas from the sigmoid colon than in carcinomas from the ascending colon. No other significant differences were found (See table 4.10, Fig 4.10).

Table 4.10 GSH concentrations (nmol/mg protein, mean { SE}) in different regions of the colon in Normal, Adenoma and Carcinoma tissues

	Normal	Adenoma	Carcinoma
Ascending	20.10 (2.55)		21.77 (1.85)
Transverse	16.08 (2.21)		66.94 (37.9)
Descending	22.00 (5.68)		19.89 (3.49)
Sigmoid	24.92 (5.27)		31.71 (3.52)
Rectum	24.31 (4.54)	32.7 (7.70)	23.55 (7.04)

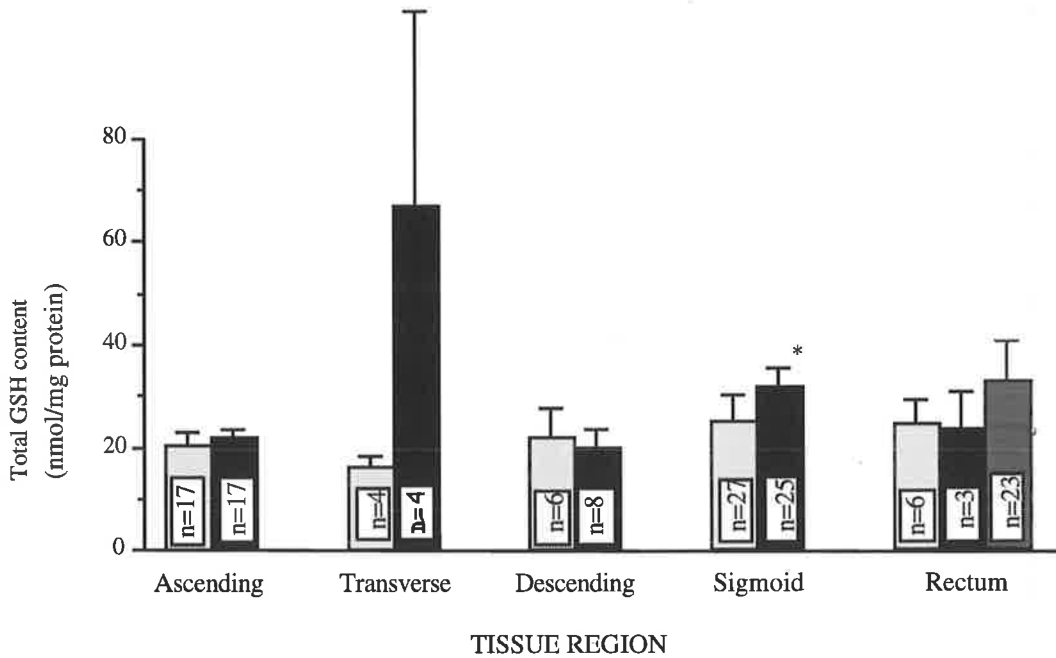


Fig. 4.10 Total GSH content(expressed as mean ± SE) in different regions of the colon in normal(□), adenoma(■) and carcinoma(■) groups. GSH content in the sigmoid colon was significantly greater than in the ascending colon (p*<0.05, Mann-Whitney U test).

GSH content and gender

There was no difference in tissue GSH concentrations between males and females.

GSH content and age

Linear regression analysis showed no correlation between age and GSH concentrations in Normal (r=0.1), Adenoma (r=0.1) or Carcinoma (r=0) groups.

GSH content and size/type of Adenoma

There was no significant relationship between GSH content and size of colonic adenomas. Multiple regression analysis showed a weak negative correlation($r=-0.2$, $r^2=0.17$, NS)(See table 4.11)

Table 4.11. Relationship between the GSH content and size of adenomas.

Size	GSH concentration (mean, { SE})
0-10 mm.	40.92 (17.79)
11-20 mm.	29.88 (5.8)
>20 mm.	30.57 (6.9)

There was no significant relationship between GSH content and the type of adenoma. The mean in the tubular and villous groups appeared to be inappropriately raised by one "outlier" in each group(whose individual values were 197.6 and 56.78nmol/mg protein, respectively)(see table 4.12).

Table 4.12. Relationship between GSH content and type of adenoma.

Type	GSH concentration (mean, { SE})
Tubular	61.36 (35.15)
Tub-villous	24.1 (3.18)
Villous	34.58 (6.28)

GSH content and Duke's stage

There was a significantly greater GSH content in Duke's A carcinomas compared to the normal tissue from patients with Duke's A and B. There were, otherwise, no significant differences in GSH concentrations between Duke's stages in either tissue groups(Table 4.13, fig.4.11).

Table 4.13 Mean (SE) of GSH content in Normal and Carcinoma tissues between the different Duke's stages.

Duke's/Tissue	Normal	Carcinoma
A	21.33 (2.17)	34.34 (3.62)
B	20.32 (2.29)	25.78 (2.64)
C	26.43 (9.39)	36.47 (11.57)
D	21.89 (2.16)	23.47 (4.15)

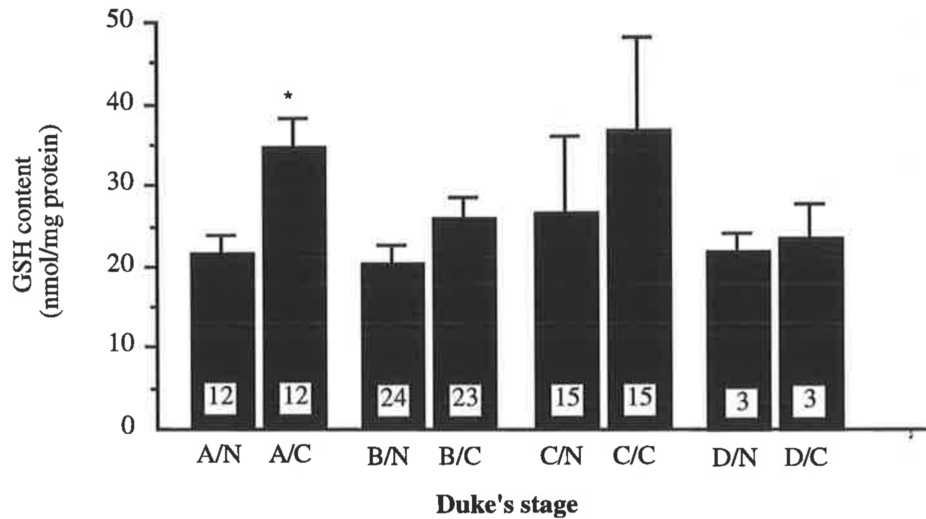


Fig. 4.11 GSH content in Normal(N) and Carcinoma(C) tissues, in the different Duke's stages, expressed as mean(SE). Carcinoma tissues of Dukes A(A/C) was significantly greater than Normal tissues of Dukes A and B(A/N & B/N),{p<0.05*, unpaired t test}

GSH concentrations and carcinoma grade

There were no significant differences in GSH concentrations between well, moderately or poorly differentiated carcinomas.

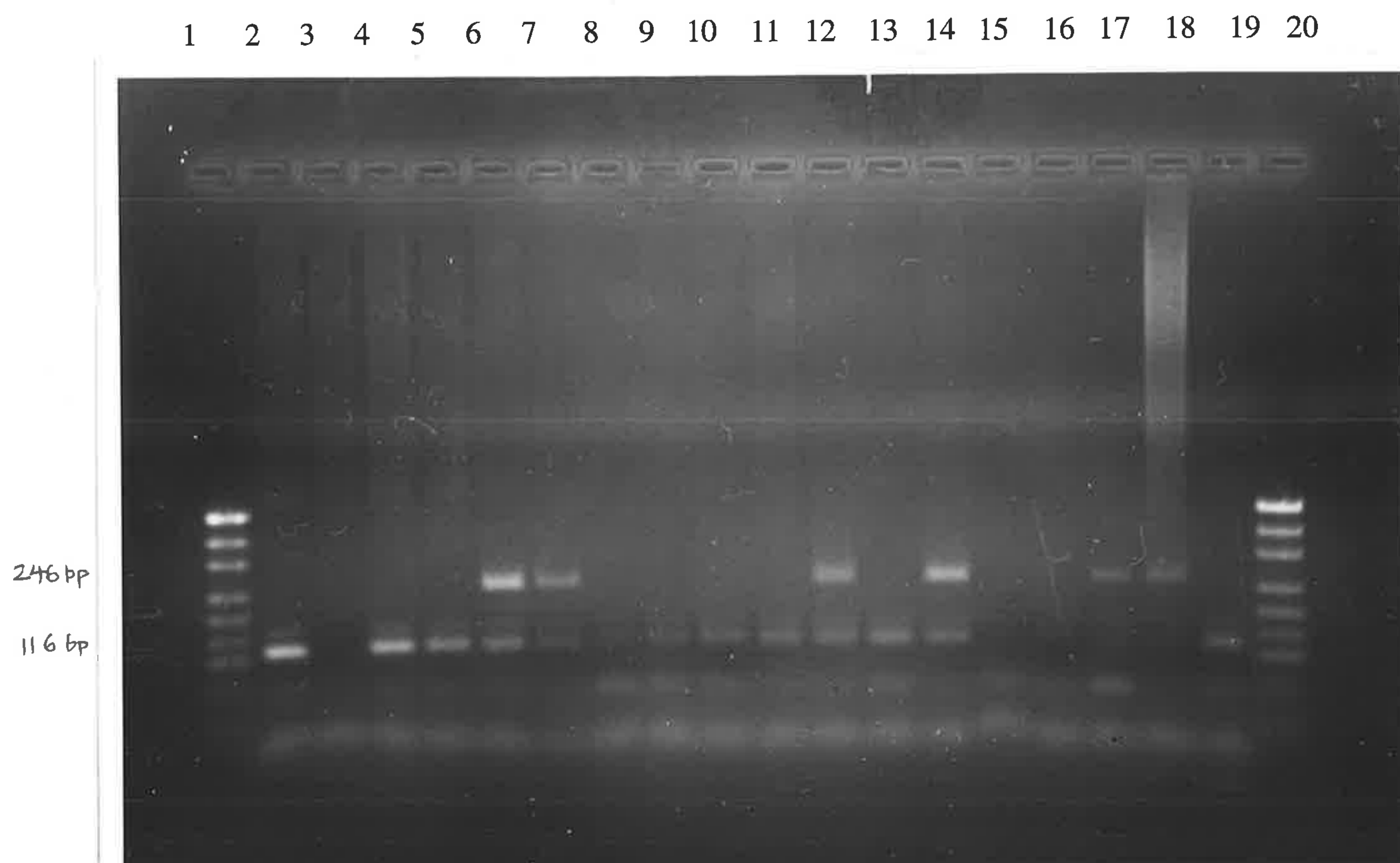
Table 4.14 GSH concentrations in the different grades of carcinoma.

	Well	Moderate	Poor
No.	5	50	2
mean	23.24	31.22	24.49
SD	17.41	26.17	0.79

4.2.3 GST mu(μ) phenotype

The presence of GSTμ was detected by PCR, with subsequent agarose gel electrophoresis of the product and staining with ethidium bromide. The absence of the gene is shown by the absence of the PCR product, but in the presence of the CD_3 gene product acting as a positive control for the reaction. An example of a reaction is shown in fig. 4.12.

Fig. 4.12. Agarose gel electophoresis showing the presence (*lanes 6, 7, 12, 14, 17 and 18*) and absence (*lanes 2, 4, 5, 8-11, 13 and 19*) of PCR product of GSTμ gene (246 base pairs). Positive controls with CD3 gene (116 base pairs) show failed PCR reaction in *lanes 3, 15 and 16*. *Lanes 1 and 20*, pUC/Hpa 2 marker (bands of 501/489, 331, 242, 190, 147 and 111/110).



Multiplex PCR with β globin, ABO blood group, HLA and NAT gene were tried before the CD3 gene and gave unsatisfactory results.

Preliminary experiments showed that magnesium concentrations in the range 1.5 to 3 mM in the reaction gave equally bright bands on gel electrophoresis.

There were 9 false negative results (ie. CD_3 gamma gene PCR amplification negative) with 6 in the Normal group and 3 in the Carcinoma group. This constituted 10.7% of the total number of samples examined. A possible cause for these false PCR results was faulty DNA preparation. They were excluded from further analysis.

The clinical characteristics of the patients recruited are shown below (table 4.15).

Table 4.15 Clinical characteristics of patients phenotyped for GSTμ.in the Normal and Carcinoma groups

	Normal	Carcinoma
Number	34	41
Age: Mean (SD)	66.2 (9.7)	70.1 (8.6)
Range	43-81	47-84
Male:Female	16:18	19:22
		Duke's stage
		A 12 (29%)
		B 19 (46%)
		C 9 (22%)
		D 1 (3%)

Frequency of GSTμ null phenotype in normal and carcinoma patients

There was no significant difference in the frequency of the GSTμ null phenotype in those with colorectal carcinoma compared to normal controls(See table 4.16).

Table 4.16 Frequency of GSTμ null phenotype in the Normal and Carcinoma groups.

		Normal(n=34)	Carcinoma(n=41)
GSTμ	+	13(38.2%)	16(39%)
	-	21(61.8%)	25(61%)

GSTμ phenotype and GST activity

There were 25 colonic specimens where GSTμ phenotype were ascertained and GST activity measured. GST activity in carcinoma tissues from GSTμ null phenotype individuals was significantly greater than in normal or carcinoma tissues from GSTμ positive individuals. (See table 4.17, fig. 4.13).

Table 4.17 GST activity in GSTμ positive or null phenotyped carcinoma patients

	GSTμ +		GSTμ -	
Number	10		15	
Tissue	Normal	Carcinoma	Normal	Carcinoma
Number	10	10	15	15
Mean (SD)	36.5 (19.3)	48.2 (32.0)	61.7 (46.9)	83.25(48.9)

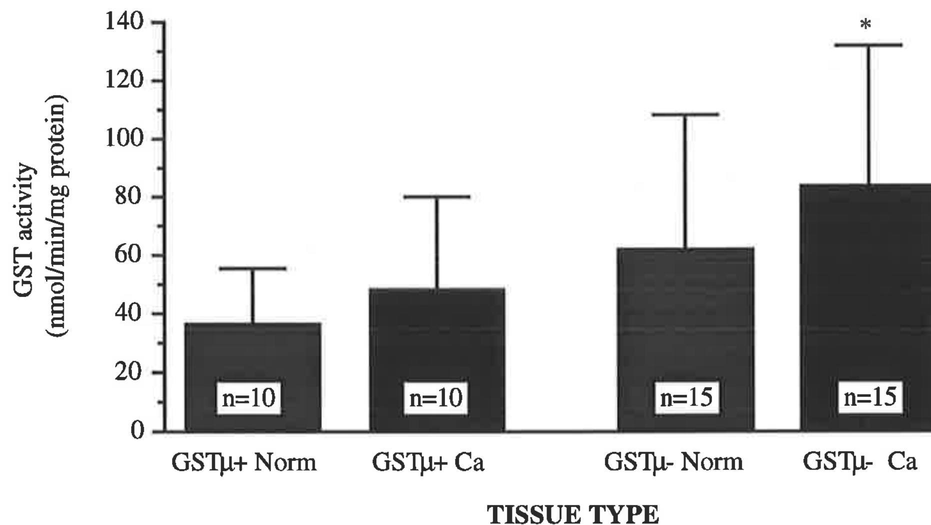


Fig. 4.13 GST activity in Normal and Carcinoma tissues of GSTμ positive or negative phenotype patients. GST activity was significantly greater in GSTμ negative carcinoma tissues than in GSTμ+ normal (p=0.007*) or carcinoma tissues (p=0.027*, Mann-Whitney U test).

4.2.4 Tissue protein

The total protein concentration was measured using the Lowry assay in 63 normal specimens, 27 adenomas and 57 carcinomas . Normal tissue was obtained from 58 operative specimens containing carcinomas, 4 operative specimens without neoplastic conditions and 1 operative specimen containing an adenoma. The descriptive statistics of the samples are shown in table 4.18.

Table 4.18 Descriptive statistics of the Normal, Adenoma and Carcinoma groups.

	Normal	Adenoma	Carcinoma
Number	63	27	57
Mean(mg/ml)	4.294	4.788	4.849
SD	2.39	5.73	2.69
Median	3.769	3.497	4.299
Range	0.19-12.84	0.63-24	1.41-13.17

There were no significant differences in the mean protein concentrations in the three tissue types studied (See fig. 4.14). Values of protein concentrations were skewed to the left in all tissue types.

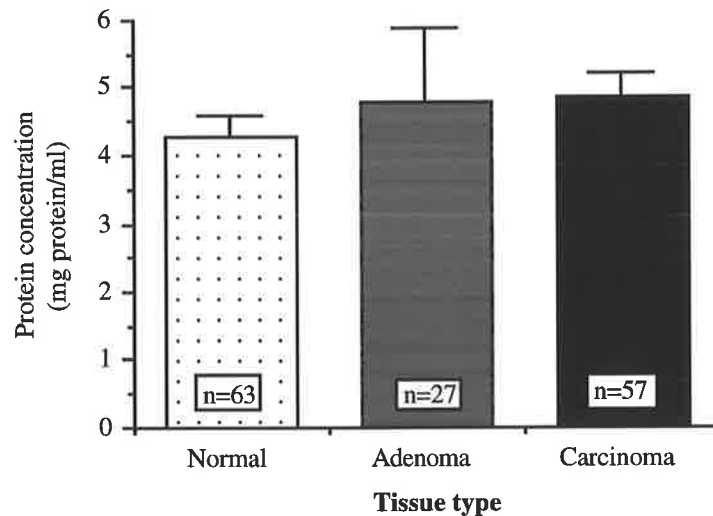


Fig. 4.14 Protein concentrations in normal and neoplastic tissue, expressed as mean(SE).

There was no correlation in protein concentration with sex, age or site of removal of normal tissue (data not shown) or between protein concentrations in the normal tissue adjacent to carcinomas and the carcinomas ($r=0.265$, $r^2=0.07$, NS).

The protein concentration showed a significant positive correlation with wet weight in a random sample of 6 normal and 5 carcinoma specimens ($r=0.8$, $r^2=0.63$, $p=0.004$)(Table 4.18, Fig 4.15).

Table 4.18 Protein content and weight weights in Normal and Carcinoma tissues.

	Normal	Carcinoma
Protein (mean, SD)	4.66 (2.0)	3.74 (1.5)
	418.82 (114.3)	338.96 (82.8)

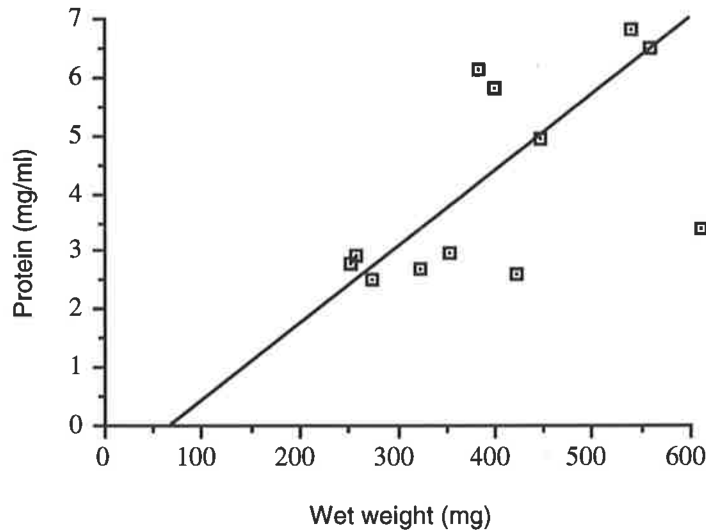


Fig. 4.15 Correlation between protein concentration and wet tissue weight.

4.3 Summary

This study showed that total GST activity was higher in neoplastic tissue than in normal tissue; a finding which corrobarates results from other centres(Siegers *et al.*, 1984),(Peters, Nagengast and Wobbes, 1989)(Mekhail-Ishak *et al.*, 1989). The activity of GST was not influenced by gender, age or site of carcinoma. However, there was increased GST activity with increased adenoma size, achieving statistical significance between adenomas of >20 mm and adenomas of 0-10 mm. However, no association of GST with adenoma type or with carcinoma stage or histologic grade was demonstrable.

The reasons for elevated levels of GST in neoplasia remain unclear; possibilities include enzyme induction in response to the neoplastic process and enzyme induction as an integral component of metabolic changes in neoplastic cells.

GSH, the substrate for GST, was increased in neoplastic tissue, perhaps in response to increased GST activity. There was no difference in colonic GSH content with gender or age. There was a significantly higher GSH content in cancers of the sigmoid colon than in cancers of the ascending colon. The numbers of carcinoma in the other sites were small and this difference between the sigmoid and ascending colon may represent a higher GSH content in the distal than in the proximal colon. The result was different to that of Seigers who found a lower GSH content in carcinomas from the sigmoid colon than from other regions. This discrepancy may be due to the wide variation in the results obtained both in this and in Seiger's studies, and in the small numbers in the latter study. There was no association between GSH content in the tissues and the size or type of adenoma. There was higher GSH content in carcinoma tissue compared to normal tissue at all Duke's stages, but differences only achieved statistical significance in stage A carcinoma. There was no relationship of GSH content with the histologic grade of carcinoma.

The reasons for an elevated GSH level in colonic neoplasia remains unclear but may include enhanced activity of synthetic enzymes, suppressed activity of degrading enzymes or impaired release of GSH from cells.

The percentage of GSTμ null phenotype individuals was similar in controls to those with carcinoma. Thus, the GSTμ null phenotype is unlikely to predispose to colonic carcinoma. However, GSTμ null phenotype individuals have a higher GST activity in their carcinoma tissue than in the carcinoma tissue from GSTμ positive individuals. This suggests that the GSTμ null phenotype individuals, although lacking in GSTμ, may respond to neoplasia by

over-production of the other GST isoenzymes. GSTπ has been shown in other studies to predominate in the colon and to increase with malignant transformation(Kantor *et al.*, 1991). This finding is supported by Batist et al. who showed increased total GST activity in colon cancer and, by using rat mRNA probes, showed increased mRNA expression for GSTπ but reduced expression for GSTμ. A number of studies have shown higher total GST activity in various cancers including colon, stomach, oesophagus and lung but not in breast, kidney and liver. Most of these studies have corrobarated observations in this study showing only a modest increase in GST activity(30%-100%) and considerable variation between individual tumours(Cook, Pass and Iype, 1991)(Howie, Forrester and Glancey, 1990)(Peters, Wormskamp and Thies, 1990). The low levels of GSTμ in colonic tissue and the ability of other isoenzymes to maintain total GST activity may explain why GSTμ null phenotype individuals are not at higher risk for developing colorectal carcinoma.

An alternative explanation for higher GST activity and GSH levels in neoplastic tissue than in normal tissue could be lower concentrations of protein in neoplastic tissue resulting in a spurious increase in GST and GSH levels. This explanation seems unlikely as there were no significant differences in the protein concentrations of normal tissue, adenomas and carcinomas.

CHAPTER 5 CELLULLAR PROLIFERATION AND COLON CANCER

5.1 Introduction

Altered cellular proliferation has been shown to be an intermediate risk marker in the progression of tissues to neoplasia, as shown by an increase in proliferative index and aneuploidy with increasing degrees of neoplasia in the colonic adenoma-carcinoma sequence. Several studies have also suggested that the uninvolved rectal mucosa of patients with colonic adenomas and carcinomas shows increased proliferative activity, raising the possibility that this might be used as a biomarker for carcinoma risk (Risio *et al.*, 1991), (Welberg *et al.*, 1990), (Wilson, Smith and Bird, 1990) .

The major aims of this study, were firstly, to use cellular proliferation, (as measured by flow cytometry) to determine the potential involvement of metabolic phenotypes (acetylator status and GSTμ null phenotype) and tissue enzyme levels (NAT, GST, GSH) in the development of colonic neoplastia. Secondly, to determine the proliferative index by flow cytometry in the uninvolved rectal mucosa and to assess its use as a biomarker of risk for developing colorectal neoplasia.

As the discriminatory value of proliferative index by flow cytometry may be influenced by confounding variables such as age, sex, site of sampling and colonic lavage,these factors were also investigated in this study. The presence of any biological dose variation in proliferation is further investigated by correlation of the proliferative index with colonic adenoma size and type, and with carcinoma stage and grade.

5.2 Results

Proliferative index, ploidy and tissue type

The proliferative indices of the normal rectal mucosa of Normal, Adenoma and Carcinoma groups and in the diploid and aneuploid tissues of adenomas and carcinomas are shown in Table 5.1. The mean(SD) DNA index of the aneuploid adenomas was 1.44 (0.2) and that of aneuploid carcinomas, 1.6(0.3).

Table 5.1 Proliferative indices in the normal mucosa of Normal, Adenoma and Carcinoma patient groups and in the diploid and aneuploid tissues of adenomas and carcinomas.

	n/n	n/ad	n/ca	ad(dip)	ad(aneu)	ca(dip)	ca(aneu)
No.	45	43	89	23	5	45	17
Mean	8.67	8.23	8.72	13.54	28.08	16.32	30.57
SD	4.36	3.97	4.87	8.55	6.48	9.15	9.15
SE	0.65	0.61	0.51	1.78	2.90	1.36	2.22

There were no significant differences in the proliferative indices of the normal rectal mucosa between the Normal, Adenoma and Carcinoma groups. The PI was significantly higher in adenomas and carcinomas than in normal tissues, and significantly higher in aneuploid than in diploid neoplastic tissue(fig. 5.1). A high proliferative index was correlated with DNA aneuploidy.

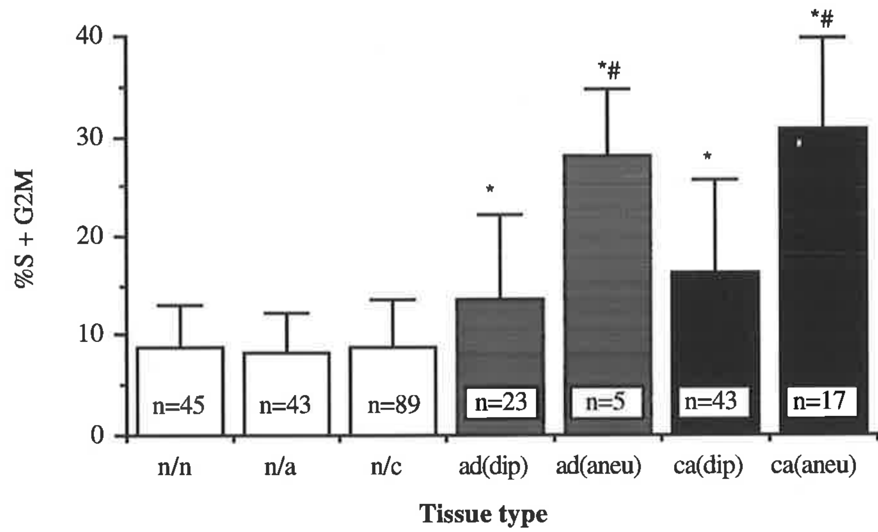


Fig. 5.1 Proliferative index(PI, expressed as mean{SD}) in normal mucosa from Normal, Adenoma and Carcinoma groups, and in diploid and aneuploid adenomas and carcinomas. PI was significantly greater in adenomas and carcinomas than in normal mucosa(p*<0.05), and greater in aneuploid than diploid neoplasms(p#<0.05, Mann-Whitney U test)

Proliferative index and age

There was no significant correlation between age and proliferative index (fig. 5.2).

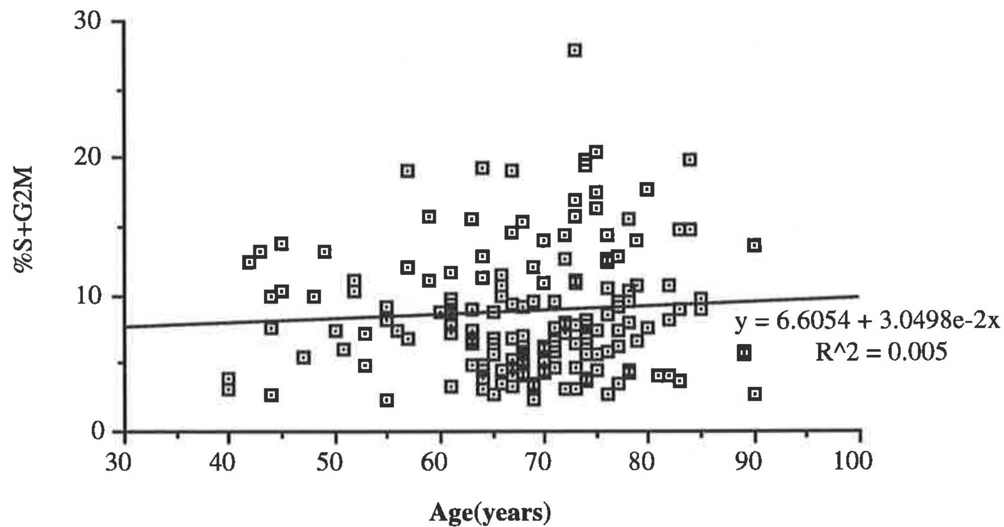


Fig. 5.2 Proliferative index of normal tissue in Normal, Adenoma and Carcinoma groups(n=170) with increasing age.

Proliferative index and sex

There were no significant differences in the proliferative index between male and female individuals in the normal, adenoma or carcinoma tissues(Table 5.2, fig. 5.3).

Table 5.2 Proliferative index in the three tissue types between males and females.

	Normal		Adenoma		Carcinoma	
	Male	Female	Male	Female	Male	Female
No.	94	84	15	13	37	25
Mean	8.84	8.29	13.55	19.12	21.74	18
SD	4.79	4.17	8.3	11.05	11.49	10.34

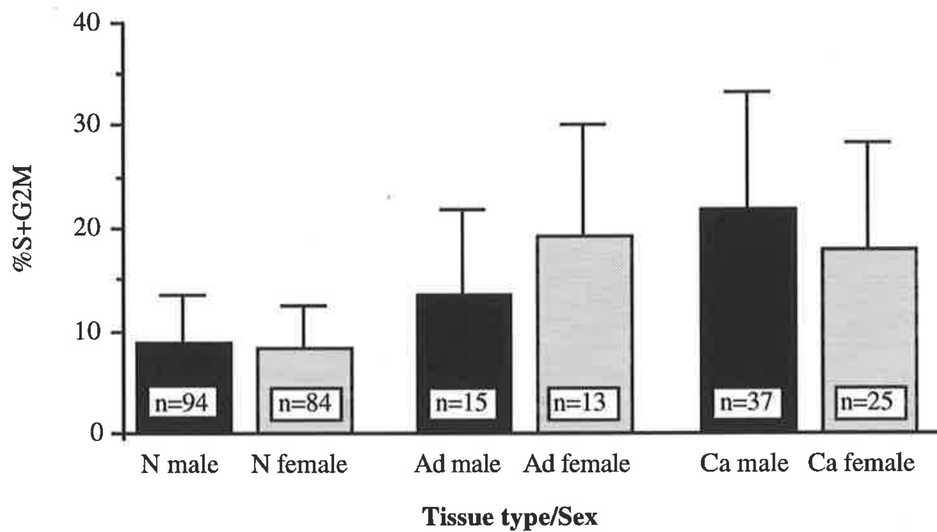


Fig. 5.3 Proliferative index (expressed as mean{SD}) in normal, adenoma and carcinoma tissue in male and female patients.

Proliferative index and size of adenoma

There was a positive non-significant trend for increased proliferative index with increasing size of adenoma(fig. 5.4).

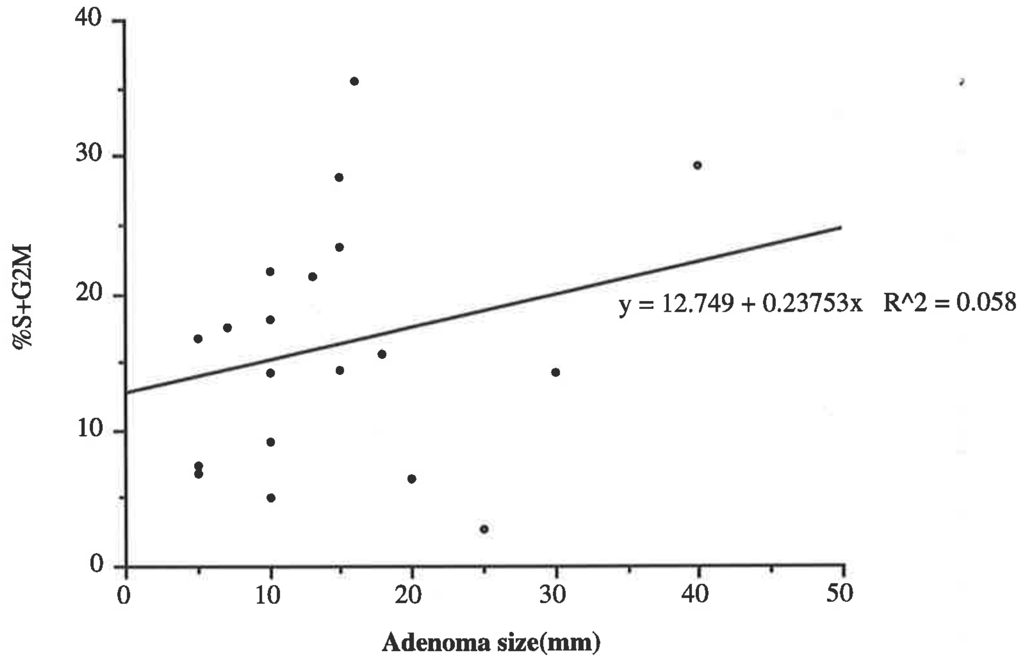


Fig. 5.4 Proliferative index and adenoma size.

Proliferative index and type of adenoma

Proliferative index was significantly higher in tubulovillous and villous adenomas than in tubular adenomas(Table 5.3, fig. 5.5).

Table 5.3 Relationship between PI and adenoma type

	Tubular	Tubulovillous	Villous
No.	7	10	6
Mean	9.43	19.03	18.12
SD	4.68	11.71	6.85

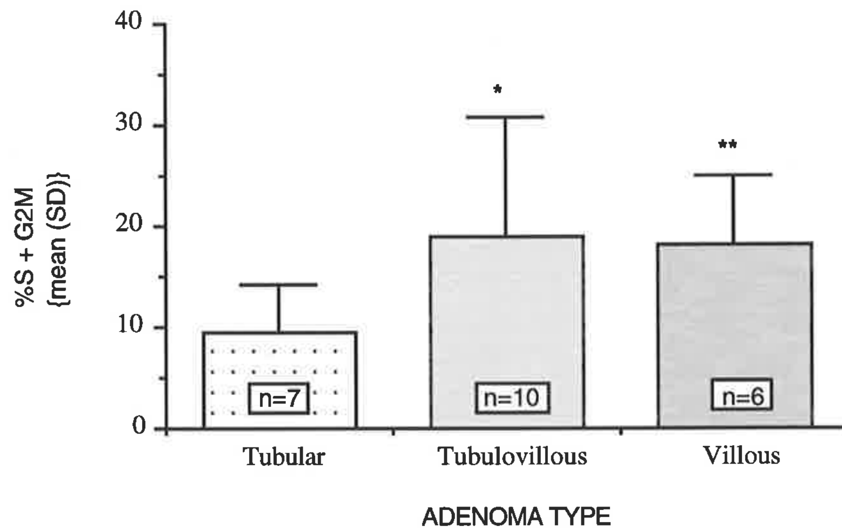


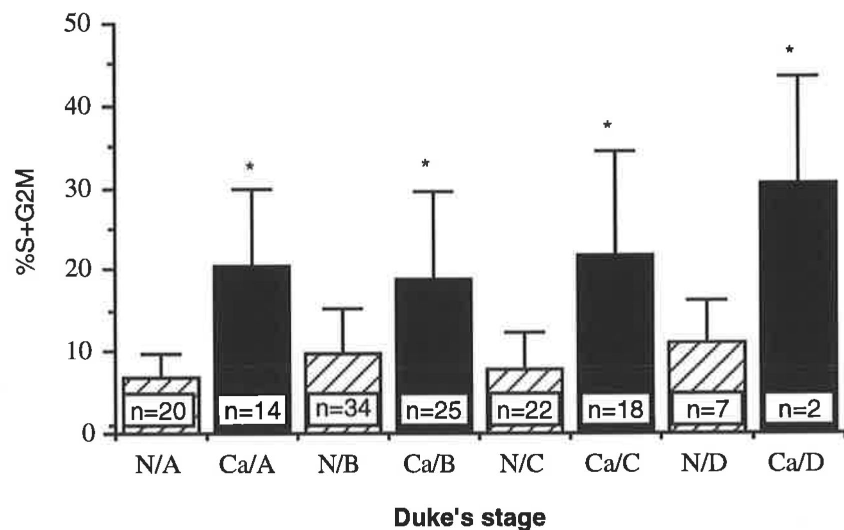
Fig. 5.5 Proliferative indices in the three adenoma types. %S+G2M was significantly higher in tubulovillous and villous adenomas than in tubular adenomas (p=0.049*, p=0.011** Mann-Whitney test)

Proliferative index and Duke's stage of carcinoma

There was no differences in the PI between the various Duke's stages in normal or carcinoma tissues. The carcinoma tissue had a significantly higher PI than normal tissue at all stages(table 5.4, fig.5.6). There was no difference in the frequency of aneuploidy between the different Duke's stages.

Table 5.4 PI in normal and carcinoma tissue of different Duke's stages.

	N/A	Ca/A	N/B	Ca/B	N/C	Ca/C	N/D	Ca/D
no.	20	14	34	25	22	18	7	2
mean	6.71	20.31	9.86	18.89	7.64	21.70	10.91	30.65
SD	3.13	9.71	5.49	10.56	4.69	12.73	5.3	12.94
%A		28.6%		24%		27.78%		50%



Aneuploid	4(29%)	6(24%)	6(33%)	1(50%)

Fig. 5.6 Proliferative index(PI, expressed as mean{SD}) in normal mucosa of patients, and carcinoma of different Duke's stages. The carcinoma has a PI significantly greater than in normal tissue at all stages ($p^* < 0.05$, Mann-Whitney U test).

Of the 17 patients with aneuploid carcinomas, their Duke's stages were Duke's A in 4 (23.5%), B in 6 (35.3%), C in 6 (35.3%) and D in 1 (5.9%). These were not significantly different in

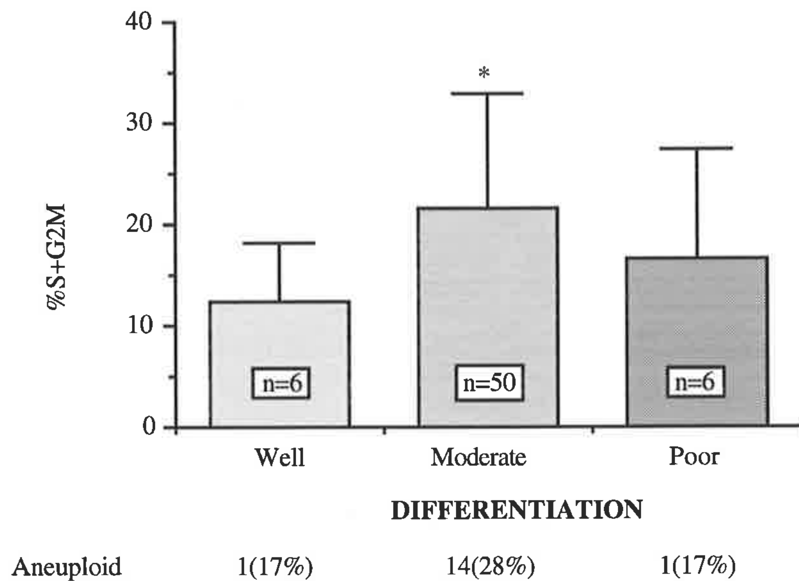
frequency to diploid carcinomas where Duke's stage A was found in 10 (23.3%) B in 19(44.2%), C in 13 (30.2%) and D in 1 (2.3%).

Proliferative index and grade of carcinoma

The proliferative index was significantly greater in moderate than in well-differentiated carcinomas. The PI of poorly differentiated carcinomas were between the other two grades(table 5.5, fig. 5.7). There was no difference in the frequency of aneuploidy between the different carcinoma grades.

Table 5.5 PI in carcinomas of different grades(differentiation)

	Well	Moderate	Poor
No.	6	50	6
Mean	12.37	21.6	16.65
SD	5.74	11.28	10.82
Aneu.(%)	1 (17%)	14 (28%)	1 (17%)



Aneuploid	1(17%)	14(28%)	1(17%)

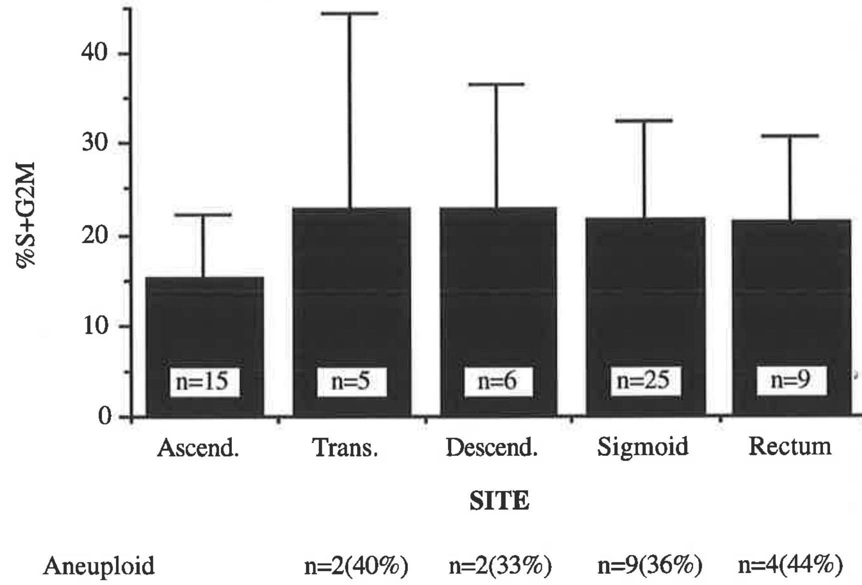
Fig. 5.7 Proliferative index (PI, expressed as mean{SD}) in carcinomas of well, moderate and poor differentiation. PI was significantly greater in carcinomas of moderate than well differentiation(p=0.03*, Mann-Whitney U test).

Proliferative index and site of carcinoma

The PI of sigmoid carcinomas was significantly higher than in those from the ascending colon. There were, otherwise, no significant differences between carcinomas from different regions(table 5.6, fig.5.8). There was no difference in the frequency of aneuploidy in carcinomas from the different regions of the colon.

Table 5.6 PI in carcinomas from the different regions of the colon.

	Asc.	Trans.	Desc.	Sigmoid	Rectum
number	15	5	6	25	9
mean	15.31	22.84	22.85	21.71	21.18
SD	7.02	21.51	13.58	10.59	9.22
% aneuploid	0	2(40%)	2(33.3%)	9(36%)	4(44%)



Aneuploid		n=2(40%)	n=2(33%)	n=9(36%)	n=4(44%)

Fig. 5.8 Proliferative index(expressed as mean{SD}) in the different regions of the colon

Proliferative index and systemic acetylator phenotype

There were no significant differences in the PI between fast and slow acetylators, in the normal specimens, adenomas or carcinomas(table 5.7, fig. 5.9). There were 29 patients (8 fast, 21 slow acetylators) in the Normal group, 32 (12 fast, 20 slow) in the Adenoma group and 65 (28 fast, 37 slow) in the Carcinoma group. PI was significantly higher in neoplastic tissue than in normal tissue irrespective of acetylator status

Table 5.7 PI in the tissues between fast and slow acetylators.

	Normal		Adenoma		Carcinoma	
	Fast	Slow	Fast	Slow	Fast	Slow
number	48	78	6	10	21	18
mean	8.85	8.55	13.84	15.34	21.78	22.19
SD	4.65	4.66	6.66	9.98	10.78	12.17

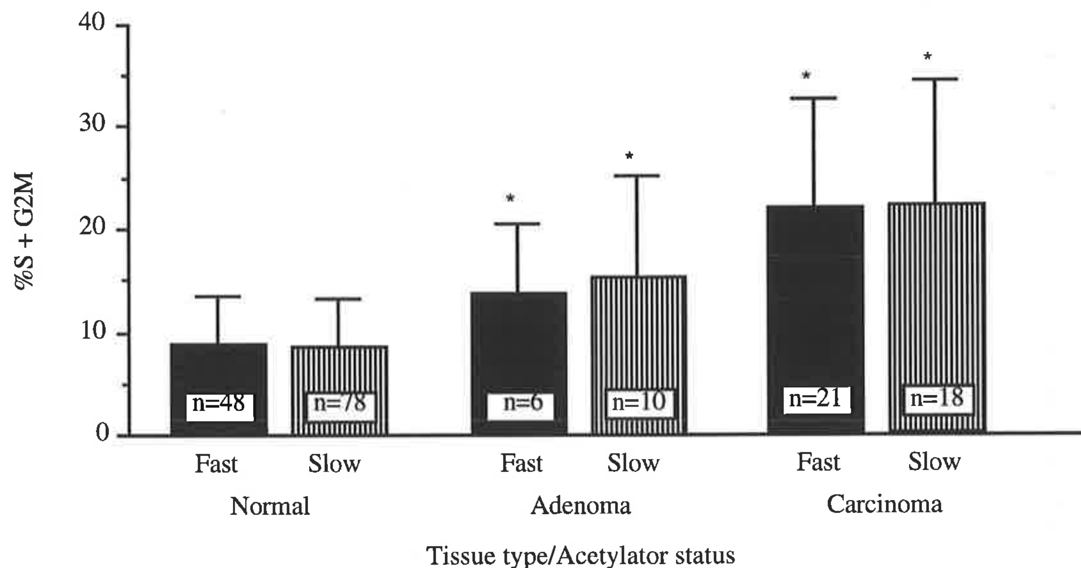


Fig. 5.9 Proliferative index(PI, expressed as mean{SD}) in normal, adenoma and carcinoma tissue between fast and slow acetylators. PI was significantly greater in neoplastic tissue than in normal tissue(p*<0.05, Mann-Whitney U test)..

Proliferative index and tissue NAT activity

There was no significant correlation between NAT activity and the PI of normal mucosa (n=21, r=0.05, p=0.84 for monomorphic NAT; n=19, r=0.21, p=0.39 for polymorphic NAT) or with the PI of carcinoma tissue(n=17, r=0.04, p=0.87 for monomorphic NAT; n=34, r=0.02, p=0.9 for polymorphic NAT). There were insufficient numbers to examine the correlation between adenoma PI and NAT activity.

Proliferative index and GST activity

There was no significant correlation between GST activity and the PI of normal mucosa (n=43, r=-0.24, r^2=0.05) or with the PI of carcinoma tissue (n=45, r=0.23, r^2=0.05).

Proliferative index and GSH content

There was no significant correlation between GSH content and the PI of normal mucosa (n=44, r=0.18, r^2=0.03) or with the PI of carcinoma tissue (n=46, r=0.43, r^2=0.18).

Proliferative index and GST μ phenotype

The proliferative index was significantly higher in carcinomas than in normal tissue or normal tissue obtained from patients with colonic carcinoma, irrespective of GSTμ phenotype. However in carcinoma tissue, GSTμ null phenotype individuals had a higher PI than GSTμ positive individuals(table 5.8, fig. 5.10).

Table 5.8 PI in the tissues between GSTμ null and GSTμ positive phenotype individuals.

	Normal		Normal/Ca. patient		Carcinoma	
	GSTμ-	GSTμ+	GSTμ-	GSTμ+	GSTμ-	GSTμ+
No.	4	3	16	11	13	10
mean	10.49	10.67	8.85	10.85	30.91	19.17
SD	4.1	6.4	8.6	10.9	10.7	8.0

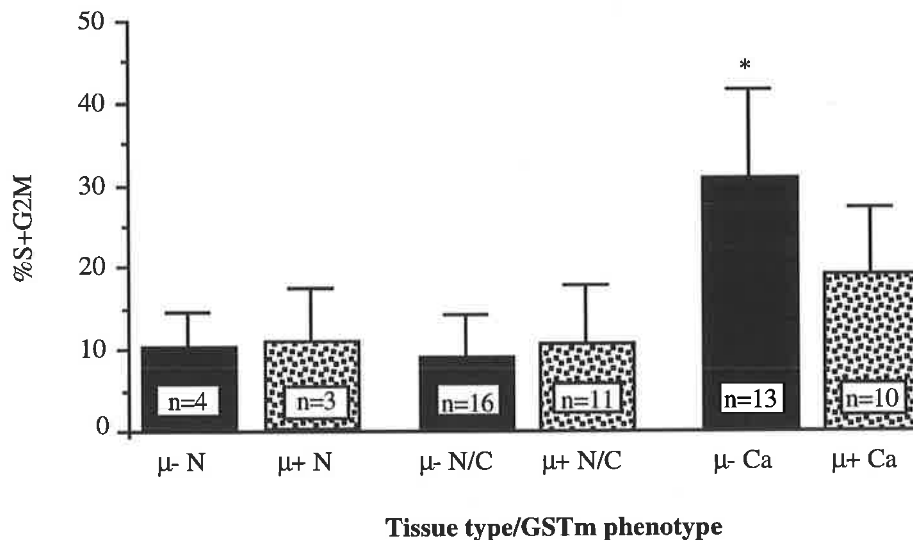


Fig. 5.10 Proliferative index (PI, expressed as mean{SD} in normal tissue(N), normal tissues from carcinoma patients(N/C) or carcinoma(Ca) tissues, between GSTμ positive and null phenotype individuals. PI was significantly higher in carcinoma than normal tissues. Carcinoma tissue from GSTμ null individuals had significantly higher PI than GSTμ positive individuals(p*=0.05, Student's t test).

Proliferative index and colonic lavage

Colonic lavage had no influence on the PI in this study(table 5.9). There were 57 biopsies from patients who has had no colonic lavage; 15 (26.3%) in the Normal group, 14 (24.6%) in the Adenoma group and 28 (49.1%) in the Carcinoma group. There were 118 biopsies from patients who had colonic lavage; 30 (25.4%) in the Normal group, 28 (23.7%) in the Adenoma group and 61 (51.7%) in the Carcinoma group. There was no difference in the proportion of biopsies from each group between those who had, or had not, any colonic lavage.

Table 5.9 PI between those with or without colonic lavage

	No lavage	With lavage
Number	57	118
Mean	8.47	8.66
SD	4.27	4.66

5.3 Summary

This study showed that PI, as measured by flow cytometry, was not significantly correlated to age, sex, site of carcinoma or colonic lavage. Patients in this study were considered "urban" and thus, results were consistent with a report by Fireman et al who found sex differences only in a rural, but not in an urban group of individuals. This study also corrobarated the results of Fireman et al (Fireman *et al.*, 1989b) in finding no correlation between cellular proliferation and colonic lavage. There was a significantly higher PI in adenomas and carcinomas than in normal tissues, consistent with hyperproliferation in neoplastic tissue. Aneuploid neoplastic tissues showed a significantly higher PI than diploid neoplastic tissues. These results support PI, as measured by flow cytometry, to be comparable with other methods for showing increased cellular proliferation in colonic neoplasms.

PI showed no correlation with adenoma size, probably because of the small numbers studied. However, PI was significantly higher in tubulovillous and villous adenomas than in tubular adenomas, and in moderately-differentiated than well-differentiated carcinomas. There were no differences in the PI between Duke's stages in normal or carcinoma tissue or with the site of carcinoma. Other studies have shown no association between ploidy and either grade or stage of the carcinoma. There were also no significant differences in the frequency of aneuploid carcinomas between the different Duke's stages, carcinoma grade or site of carcinoma. The above findings indicate that PI are poorly correlated with the histological features in adenoma or carcinoma that are associated with a poorer prognosis.

There were no significant differences in PI between the uninvolved mucosa of the Normal, Adenoma or Carcinoma groups, indicating that PI, as measured by flow cytometry, cannot be used as a biomarker of risk for colorectal neoplasms. This may reflect the low sensitivity of the method.

There were no significant differences in PI between slow and fast acetylators in the Normal, Adenoma or Carcinoma groups. PI was not correlated with tissue NAT activity, GST activity or GSH content. The acetylator status and the tissue enzyme activities were, therefore, not associated with abnormal tissue proliferation as measured by flow cytometry. However, the PI was significantly higher in GSTμ null phenotype individuals than in the GSTμ positive individuals in carcinoma tissue. This observation is unexplained. One possibility is that a significant association has arisen by chance, particularly as numbers in each group were relatively small. If the association is confirmed by larger studies, it is conceivable that failure to detoxify putative carcinogens (as GSTμ null individuals) might be associated with enhanced proliferation. However, as shown previously, the GSTμ null phenotype is not associated with an increased risk for colorectal neoplasia.

CHAPTER 6 MEAT CONSUMPTION AND ACETYLATOR PHENOTYPE IN COLON CANCER.

6.1 Introduction

The development of colorectal carcinoma is believed to be the result of an interaction between environmental and genetic factors. There is evidence to show that a high meat intake is an environmental factor which promotes colorectal carcinoma, and previous observations indicate that fast acetylators are at higher risk than slow acetylators. The following study explores the hypothesis that meat intake and acetylator status were additive risks for the development of colonic carcinoma.

6.2 Results

Dietary booklets were completed and returned by 185 individuals. However, 8 (4.3%) booklets (6 in the normal and 2 in the cancer groups), were poorly completed and were rejected as unsuitable for analysis. Therefore, satisfactory dietary information was available in 177 individuals, 90 controls and 87 cancer patients. Descriptive statistics of their dietary intake is shown in Table 6.1 below.

Table 6.1 Dietary intake (in grams) of all (Normal and Cancer) patients.

	Protein	Fat	Carbohydrate	Fibre
mean	78.1	79	250.3	24.1
SD	32.4	37.1	103.5	11.7
range	10.8-246.8	7.3-262.8	8.2-676.6	0.9-65.9

There was no significant difference in the protein intake between normal and colon cancer patients(table 6.2).

Table 6.2 Dietary intake (in grams) of protein between normal and cancer patients.

	Normal	Cancer
Mean	81.7	74.4
SD	40.4	20.6
Range	23.6-246.8	10.8-128.8

When patients were divided into low and high meat consumers as below and above, the mean protein intake of the normal controls (i.e. 81.7g) respectively, the distribution of slow and fast acetylators in these groups are shown below(table 6.3).

Table 6.3 The number of slow and fast acetylators in Normal and Cancer patients, in low and high meat consumers.

	Normal		Cancer	
Acetylator phenotype	Slow	Fast	Slow	Fast
Low meat (<81.7g)	36	18	33	26
High meat (>81.7g)	26	10	16	12

Low meat: Controls; Fast/Slow=18/36 (1) Cancer; Fast/Slow=26/33 (2)

Odds ratio = (2)/(1) = 1.6

High meat: Controls; Fast/Slow=10/26 (1) Cancer; Fast/Slow=12/16 (2)

Odds ratio = (2)/(1) = 2.0

There were no significant differences in the number of fast acetylators between normal and cancer patients, either in the low or high meat consumer groups. However, the odds ratio of fast to slow acetylators between the normal and cancer patients is higher in the high meat

consumer group(i.e.2) than in the low meat consumer group of patients(i.e.1.6)(fig 6.1, the odds ratio of slow acetylators is arbitrarily taken as 1).

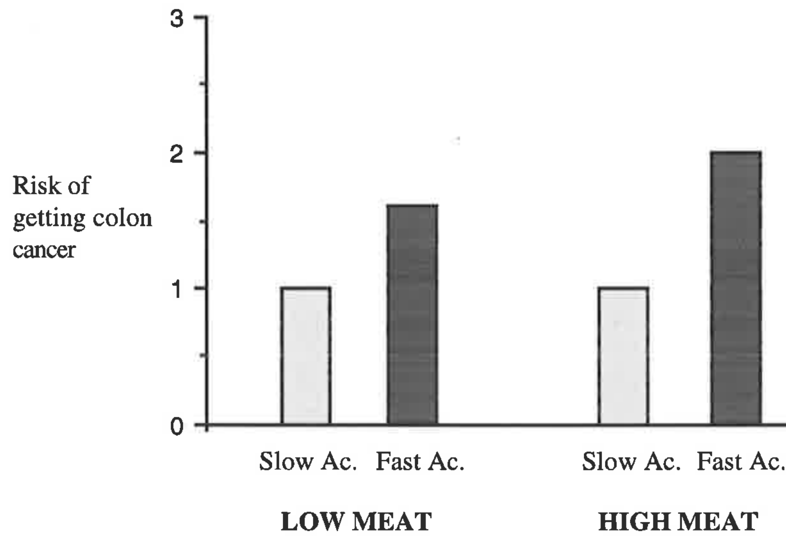


Fig 6.1 The relative risk(odds ratio) of getting colon cancer between slow and fast acetylators(Ac.) in low and high meat consumers.

In our sample of subjects, the higher odds ratio of fast to slow acetylators for developing colorectal carcinoma is restricted to female patients, and this is higher in high meat(4.5) than low meat(2.37) female consumers(table 6.4, fig.6.2).

Table 6.4 The number of slow and fast acetylators in Normal and Cancer patients, in low and high meat consumers, and in male and female patients.

For MALES:

	Normal		Cancer	
Acetylator phenotype	Slow	Fast	Slow	Fast
Low meat (<81.7g)	16	9	17	12
High meat (>81.7g)	17	8	10	5

Low meat (<81.7g):
Controls; Fast/Slow=9/16 (1) — Cancer; Fast/Slow=12/17 (2)
Odds ratio = (2)/(1) = 1.25

High meat (>81.7g):
Controls; Fast/Slow=8/17 (1) — Cancer; Fast/Slow=5/10 (2)
Odds ratio = (2)/(1) = 1.06

For FEMALES:

	Normal		Cancer	
Acetylator phenotype	Slow	Fast	Slow	Fast
Low meat (<81.7g)	20	9	15	16
High meat (>81.7g)	9	2	15	16

Low meat (<81.7g):
Controls; Fast/Slow=9/20 (1) — Cancer; Fast/Slow=16/15 (2)
Odds ratio = (2)/(1) = 2.37

High meat (>81.7g):
Controls; Fast/Slow=2/9 (1) — Cancer; Fast/Slow=16/15 (2)
Odds ratio = (2)/(1) = 4.5

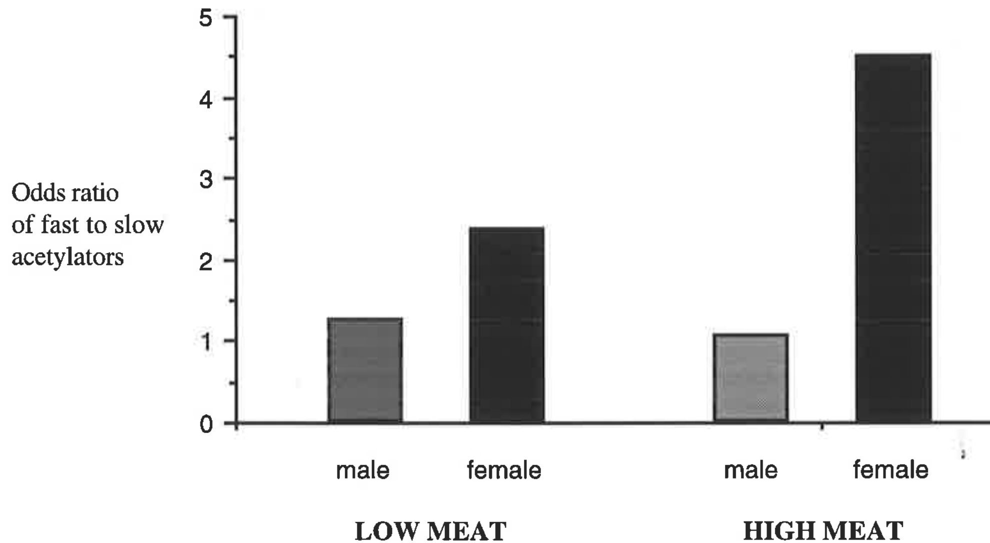


Fig. 6.2 The odds ratio of fast to slow acetylators in developing colon cancer in males and females between low and high meat consumers.

There was a positive correlation between protein and fat consumption(r=0.8), protein and carbohydrate consumption(r=0.69), fat and carbohydrate consumption(r=0.73), protein and fibre consumption(r=0.57), fat and fibre consumption(r=0.5) and carbohydrate and fibre consumption(r=0.74).

6.3 Conclusions

As shown in Chapter 3, the risk of developing colorectal carcinoma is higher in fast than in slow acetylators. Dietary analysis of patients indicate that this increased risk between fast and slow acetylators is more marked in high meat than in low meat consumers.. Segregating patients by sex further indicates this risk is even greater in females than males, although the numbers involved in this study were relatively small.

CHAPTER 7 CONCLUSIONS

A major strength of this study was the selection of the control group from patients with a normal barium enema x-ray or colonoscopy. Thus, it is most unlikely that the control group would have included patients with asymptomatic adenomas or carcinomas. By using a different control group, a study might simply determine risk factors for colonoscopy and not for colonic neoplasia.

On the other hand, a potential drawback of our control group is that the individuals were not totally healthy and asymptomatic, as indicated by their need for these lower GI investigations. They are hospital-based controls and the observed associations may therefore not be true for totally asymptomatic community-based controls. For example, one possibility relevant to this study is that hospital-based controls may have a lower percentage of fast acetylators than community-based controls.

Several studies have suggested that acetylator phenotype has a role in the aetiology of colonic carcinomas (Ilett *et al.*, 1987; Lang, Chu and Hunter, 1986). They suggest that the fast acetylator is more predisposed to develop colorectal carcinoma than the slow acetylator. This is supported by this study which showed a significantly higher percentage of fast acetylators in those with colorectal carcinomas(42.9%) than in controls(30%). However, the strength of this observation is reduced below the significant level of $p<0.05$ if the Yates continuity correction is applied to the results of the Chi squared tests. There was also a study from Spain(Ladero, Gonzalez and Benitez, 1991) which showed no significant relationship between acetylator status and colorectal carcinoma. Nevertheless, when data from all studies were pooled, the association between colorectal carcinoma and the fast acetylator phenotype is highly significant($p<0.001$). However, the power of individual studies to detect differences in acetylator phenotype was relatively low and this issue may only be resolved by a large

multicentre study.

Dietary data were collected by a self-administered retrospective dietary food frequency questionnaire. There are some problems with the method including poor recall of dietary habits and recent chances in diet in an attempt to ameliorate gastrointestinal symptoms. The odds ratio of fast to slow acetylators between the normal and cancer patients is higher in the high meat consumer group than in the low meat consumer group of patients. Therefore, there may be an effect of meat consumption among fast acetylators which is more pronounced in females. However, caution must be exercised in this interpretation as the numbers of patients studied here was small. Further studies are needed to confirm and explain the observed gender differences. This study is consistent with the hypothesis that the combination of fast acetylator phenotype with a high consumption of meat substantially increases the risk of colonic carcinomas. It supports Lang's paper showing that the amount of food-borne heterocyclic amine carcinogens may have a different risk potential for colonic neoplasia between slow and fast acetylators (Lang *et. al.*, 1994).

Previous studies have not explored the relationship of acetylator status with colonic adenomas. This study showed that patients with colonic adenomas have a frequency of fast acetylators(32.6%) intermediate between controls and those with carcinoma. It therefore appears that there could be an association between fast acetylators and increased cellular proliferation, consistent with the colonic adenoma-carcinoma sequence.

In this study, the polymorphic distribution of NAT activity in the colonic tissue corresponded to the systemic acetylator phenotype. Thus, sulphamethazine acetylation in colonic tissue was significantly higher in fast acetylators than in slow acetylators. This indicates that acetylator phenotype can be expressed in colonic tissue as well as in hepatic tissue although polymorphic NAT activity only accounts for a minority of NAT activity. At present, the substrate specificity

of carcinogens for the two forms of NAT remain unclear.

A method of genotyping individuals for their acetylator status has been devised in this study. It is based on amplification of part of the NAT gene by PCR and subsequent digestion with restriction endonucleases, to ascertain the presence or otherwise of different NAT restriction sites that are unique to the different NAT alleles. This study demonstrates good concordance between biochemical phenotyping and genotyping for the acetylator phenotype. It also demonstrates that there is a higher frequency of the fast allele(F1) in those with colorectal carcinoma than in controls, although the numbers studied are too small to achieve statistical significance. The ability to determine acetylator genotype from leucocyte DNA in blood should facilitate further epidemiological studies to assess the role of acetylator genotype in various diseases including colon cancer.

This study corroborated previous reports of higher GST activity in colonic cancer. Furthermore, concentrations of tissue GSH were significantly higher in adenomas and cancer. The reasons for these changes remain unclear but they may account, in part, for the resistance of colonic neoplasms to chemotherapeutic drugs which are effective in other cancers.

As expected, colonic adenomas and carcinomas showed significantly higher cell proliferation than uninvolved mucosa. However, correlations of proliferative activity with GST activity, GSH concentration and NAT activity in the normal and carcinoma tissues and with acetylator phenotype were not statistically significant. Furthermore, there was no significant difference in the proliferative index(PI), as measured by flow cytometry, in the uninvolved mucosa of normal controls when compared to "normal" mucosa associated with adenomas or carcinomas, indicating that this measurement of PI cannot be used as a biomarker of risk for the development of colonic neoplasia. The PI and total GST were significantly higher in GSTμ null phenotype individuals than in GSTμ positive individuals in the carcinoma tissue. This maybe a chance

finding due to the small numbers studied since individuals of the GSTμ null phenotype were not at higher risk for the development of colonic neoplasia.

REFERENCES

Alldrick, A. J. and Lutz, W. K. (1989). Covalent binding of [2-14C] 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) to mouse DNA in vivo. *Carcinogenesis*, **10**; 1419-1423.

Arai, T. and Kino, I. (1989). Morphometrical and cell kinetic studies of normal human colorectal mucosa. *Acta Pathol Jpn,* **39**; 725-730.

Armitage, N. C., Robins, R. A., Evans, D. F., Turner, D. R., Baldwin, R. W. and Hardcastle, J. D. (1985). The influence of tumour cell DNA abnormalities on survival in colorectal cancer. *Br J Surg*, **72**; 828-830.

Armstrong, B. K. (1988). The Epidemiology and Prevention of Cancer in Australia. *Aust NZ J Surg,* **58**; 179-187.

Arrick, B. A. and Nathan, C. F. (1984). Glutathione metabolism as a determinant of therapeutic efficacy: a review. *Cancer Res,* **44**; 4224-4232.

Baghurst, K. I., Crawford, D. A., Worsley, A. and Record, S. J. (1988a). The Victorian Nutrition Survey - intakes and sources of dietary fats and cholesterol. *Med J Aust,* **149**; 12-20.

Baghurst, K. I., Crawford, D. A., Worsley, F. A., Syrett, J. A., Record, S. J. and Baghurst, P. A. (1988b). The Victorian Nutrition Survey: a profile of the energy , macronutrient and sodium intakes of th population. *Comm Health Stud*, **XII**; 42-54.

Baghurst, K. I. and Record, S. J. (1983). Intake and sources, in selected Australian subpopulations, of dietary constituents implicated in the etiology of chronic diseases. *J Food Nut,* **40**; 1-15.

Baghurst, K.I. and Record, S.J. (1984). A computerised dietary analysis system for use with diet diaries or food frequency questionaires. *Comm Health Stud*, **8**; 11-18.

Baghurst, P. A., McMichael, A. J., Slavotinek, A. H., Baghurst, K. I., Boyle, P. and Walker, A. M. (1991). A case-control study of diet and cancer of the pancreas. *Am J Epidemiol*, 167-179.

Bakke, J and Gustafsson, J-A (1984). Mercapturic acid pathway metabolites of xenobiotics: generation of potentially toxic metabolites during enterohepatic circulation. *Trends in Pharmacol Sci*, **5**; 517-521.

Banner, B. F., Chacho, M. S., Roseman, D. L. and Coon, J. S. (1987). Multiparameter flow cytometric analysis of colon polyps. *Am J Clin Pathol,* **87**; 313-318.

Banner, B. F., Tomas De Le Vega, J. E., Roseman, D. L. and Coon, J. S. (1985). Should flow cytometric DNA analysis precede definitive surgery for colon carcinoma? *Ann Surg*, **202**; 740-744.

Batchelor, J.R., Welsh, K.I., Mansilla-Tinoco, R., Dollery, C.T., Hughes, G.R.V., Bernstein, R., Ryan, P., Naish, P.F., Aber, G.M., Bing, R.F., et al. (1980). Hydrallazine-induced syestemic lupus erythematosus: influence of HLA-DR and sex on susceptibility. *Lancet,* **1**; 1107-1109.

Batist, G., Woo, A. and Tsoa, M-S. (1991). Effect of proliferative state on glutathione S-transferase isoenzyme expression in cultured rat liver epithelial cells. *Carcinogenesis*, **12**; 2031-2034.

Bauer, K. D., Lincoln, S. T., Vera-Roman, J. M., Wallemark, C. B., Chmiel, J. S., Madurski, M. L., Muraq, T. and Scarpelli, D. G. (1987). Prognostic implications of proliferative activity and DNA aneuploidy in colonic adenocarcinomas. *Lab Invest*, **57**; 329-335.

Benito, E., Obrador, A, Stiggelbout, A. and al., et (1990). A population-based case-control study of colorectal cancer in Majorca. 1 Dietary factors. *Int J Cancer,* **45;** 69-76.

Bhargava, M. M., Listowsky, I. and Arias, I. M. (1978). Ligandin. Bilirubin binding and glutathione-s-transferase activity are independent processes. *J Biol Chem*, **253**; 4112.

Bjorkje, B., Hostmark, J., Skagen, D. W., Schrumpf, E.andLaerum, O. D. Flow cytometry of biopsy specimens from ulcerative colitis, colorectal adenomas and carcinomas. *Scand J Gastroenterol,* **22**; 1231-1237.

Blelke, E. (1980). Epidemiology of colorectal cancer, with emphasis on diet. W. Davis, K. R. Harrap and G. Stathopoulos (Eds.), *Human cancer: its characterization and treatment. International Congress Series no. 484* . Amsterdam: Excerpta Medica.

Blum, M, Grant, D. M., McBride, W., Heim, M. and Meyer, U. A. (1990). Human arylamine N-acetyltransferase genes: isolation, chromoasomal localization and functional expression. *DNA Cell Biol,* **9**; 193-203.

Board, P. G. (1981). Biochemical genetics of glutathione S-transferase in man. *Am J Hum Genet*, **33**; 36-43.

Board, P.G. and Webb, G. C. (1987). Isolation of a cDNA clone and localization of glutathione S-transferase 2 genes to chromosome band 6p12. *Proc Natl Acad Sci USA*, **84;** 2377-2381.

Board, P.G., Webb, G.C. and Coggan, M.C. (1989). Isolation of a cDNA clone and localization of the human flutathione S-transferase 3 genes to chromosome bands11q13 and12q13-14. *Ann Hum Genet,* **53**; 205-213.

Bone, E., Tamm, A. and Hill, M. (1976). The production of urinary phenols by gut bacteria and their possible role in the causation of large bowel cancer. *Am J Clin Nutr*, **29**; 1448-1454.

Buller, A. L., Clapper, M. L. and Tew, K. D. (1987). Glutathione S-transferases in nitrogen mustard-resistant and sensitive cell lines. *Molec Pharmac*, **31**; 575-578.

Cannon-Albright, L.A., Skolnick, M.H., Bishop, T., Lee, R.G. and Burt, R.W. (1988). Common Inheritance of Susceptibility to Colonic Adenomatous Polyps and Associated Colorectral Cancers. *N Engl J Med*, **319**; 533-537.

Cartwright, R. A., Glashan, R. W., Rogers, H. J., Ahmad, R. A., Barham-Hall, D., Higgins, E. and Khan, M. A. (1982). Role of N-acetyltransferase phenotypes in bladder carcinogenesis: A pharmagenetic epidemiological approach to bladder cancer. *Lancet*, **2**; 842-846.

Cerniglia, C.E., Freeman, J.P., Franklin, W. and Pack, L.D. (1982). Metabolism of azodyes derived from benzidine, 3,3'-dimethybenzidine and 3,3'-dimethoxybenzidine to potentially carcinogenic aromatic amines by intestinal bacteria. *Carcinogenesis*, **3**; 1255-1260.

Comstock, K. E.., Sanderson, B. J. S., Claflin, G. and Henner, W. D. (1990). GST1 gene deletion determined by polymerase chain reaction. *Nucl Acid Res*, **18**; 3670.

Cook, J. A., Pass, H. I. and Iype, S. N. (1991). Cellular glutathione and thiol measurements from surgically resected human lung tumor and normal lung tissue. *Cancer Res*, **51**; 4287-4294.

Costa, A., Massini, G., Del Bino, G. and Silvsetrini, R. (1981). DNA content and kinetic characteristics of non-Hodgkin's lymphoma determined by flow cytometry and autoradiography. *Cytometry*, **2**; 185-188.

De Jong, J. L., Chang, C. M., Whang-Peng, J., Knutsen, T. and Tu, C.P.D. (1988). The human liver glutathione S-transferase gene superfamily: expression and chromosome mapping of an Hb subunit cDNA. *Nucl Acids Res*, **16**; 8541-8554.

Deguchi, T., Mashimo, M. and Suzuki, T. (1990). Correlation between acetylator phenotypes and genotypes pf polymorphic arylamine N-acetyltransferase in human liver. *J Biol Chem*, **265**; 12757-12760.

Desai, M., Jariwala, G., Khorkhani, B., Desai, N. K. and Sheith, U. K. (1973). Isoniazid inactivation in Indian children. *Indian Paediatr,* **10**; 373-376.

Deschner, E. E., Godbold, J. and Lynch, H. T. (1988). Rectal epithelial cell proliferation in a group of young adults. *Cancer*, **61**; 2286-2290.

Devadatta, S., Gangadharam, P.R.J., Andrews, R.H., Fox, W., C.V., Ramakrishnan, Selkon, J.B. and Vela, S. (1960). Peripheral neuritis due to isoniazid. *Bull Wld Hlth Org*, **23**; 587-598.

Digiovanni, J., Slaga, T. J. and Boutwell, R. K. (1980). Comparison of tumor-initiating activity of 7,12-dimethylbenz(a)anthracene and benzo(a)pyrene in female SENCAR and CD-1 mice. *Carcinogenesis,* **1**; 381-389.

Doll, R and Peto, R (1981). The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst*, **66**; 1192-1308.

Dulik, D. M., Freselau, C. and Hilton, J. (1986). Characterization of melphalan-glutathione adducts whose formation is catalysed by glutathione transferases. *Biochem Pharmacol,* **35**; 3405-3409.

Dwyer, T., Calvert, G. D., Baghurst, K. and Leitch, D. (1981). Diet, other lifestyle factors and HDL choloesterol in a population of Australian male service recruits. *Am J Epidemiol,* **114**; 683-696.

Farah, F., Taylor, W., Rawlins, M. D. and James, O. (1977). Hepatic drug acetylation and oxidation: effects of ageing in man. *Br Med J,* **2**; 155-156.

Fath, R. B. and Winawer, S. J. (1983). Early diagnosis of colorectal cancer. *Ann Rev Med*, **34**; 501-517.

Felton, J. S., Knize, M. G., Shen, H. S., Andrewen, B. D. and Bjeldanes, L. F. (1986). Identification of the Mutagens in Cooked Beef. *Environ Health Perspect*, **67**; 17-24.

Felton, J. S., Knize, M. G., Shen, N. H., Lewis, P. R., Andresen, B. D., Happe, J. and Hatch, F. T. (1986) *Carcinogenesis*, **7;** 1081-1086.

Finan, P. J., Quirke, P., Dixon, M. F., Dyson, J. E. D., Giles, G. R. and Bird, C. C. (1986). Is DNA aneuploidy a good prognostic indicator in patients with advanced colorectal cancer? *Br J Cancer*, **54**; 327-330.

Fireman, Z., Rozen, P., Fine, N. and Chetrit, A. (1989a). Influence of demographic parameters on rectal epithelial proliferation. *Cancer Lett*, **47**; 133-140.

Fireman, Z., Rozen, P., Fine, N. and Chetrit, A. (1989b). Reproducibility studies and effects of bowel preparations on measurements of rectal epithelial proliferation. *Cancer Lett,* **45**; 59-64.

Flammang, T.J., Westra, J.G., Kadlubar, F.F. and Beland, F.A. (1985). DNA adducts formed from the probable proximate carcinogen, N-hydroxy-3,2'-dimethy-4-aminobiphenyl, by acid catalysis or S-acetyl coenzyme A-dependent enzymatic esterification. *Carcinogenesis*, **6**; 251-258.

Flammang, T.J., Yamazoe, Y., Guengerich, F.P. and Kadlubar, F.F. (1987). The S-acetyl coenzyme A-dependent metabolic activation of the carcinogen N-hydroxy-2-aminofluorene by human liver cytosol and its relationship to the aromatic amine N-acetyltranferase phenotype. *Carcinogenesis*, **8**; 1967-1970.

Friedlander, M., Hedley, D. W. and Taylor, I. W. (1984). Clinical and biological significance of aneuploidy in human tumours. *J Clin Pathol*, **37**; 961-974.

Gachalyi, B., Hajos, V.P. and Kaldor, A. (1984). Acetylator Phenotypes: Effect of Age. *Eur J Clin Pharmacol*, 43-45.

Gerhardsson, M., Floderus, B. and Norell, S. E. (1988). Physical activity and colon cancer risk. *Int J Epidemiol*, **17**; 743-746.

Giaretti, W. and Santi, L. (1990). Tumor progression by DNA flow cytometry in human colorectal cancer. *Int J Cancer*, **45**; 597-603.

Giovannucci, E., Stampfer, M.J., Colditz, G., Rimm, E.B. and Willett, W.C. (1992). Relationship of Diet to Risk of Colorectal Adenoma in Men. *J Natl Cancer Inst,* **84**; 91-98.

Goh, H. S. and Jass, J. R. (1986). DNA content and the adenoma-carcinoma sequence in the colorectum. *J Clin Pathol*, **39**; 387-392.

Griffith, O.W. (1980). Determination of glutathione disulphide using glutathione reductase and 2-vinylpyridine. *Anal Biochem*, **106**; 207-212.

Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1974). Glutathione S Transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem*, **249**; 7130-7139.

Haenszel, W., Berg, J. W., Segi, M., Kurihara, M. and Locke, F. B. (1973). Large bowel cancer in Hawaiian Japanese. *J Natl Cancer Inst*, **51**; 1765-1779.

Haenszel, W, Locke, F. B. and Segi, M. (1980). A case-control study of large bowel cancer in Japan. *J Natl Cancer Inst*, **64;** 17-22.

Hayatsu, H., Hayatsu, T. and Wataya, Y. (1986). Use of blue cotton for detection of mutagenicity in human feces excreted after ingestion of cooked meat. *Environ Health Perpect,* **67**; 31-34.

Hayes, J. D., Kerr, L. A. and Cronshaw, A. G. (1989). Evidence that glutathione transferases B1B1 and B2B2 are the products of separate genes and their expression in human liver is subject to interindividual variation. *Biochem J*, **264**; 437-445.

Hayes, J. D. and Wolf, C. R. (1988). Role of glutathione transferase in drug resistance. H. sies and B. Ketterer (Eds.), *Glutathione Conjugation Mechanisms and Biological Significance* . London: Academic Press.

Heagerty, A. H. M., Fitzgerald, D., Smith, A., Bowers, B., Jones, P., Fryer, A. A., Zhao, L., Alldersea, J. and Strange, R. C. (1994). Glutathione S-transferase GSTM1 phenotypes and protection against cutaneous tumours. *Lancet,* **343**; 266-268.

Heckbert, S. R., Weiss, N. S. , Hornung, S. K., Eaton, D. L. and Motulsky, A. G. (1992). Glutathione S-Transferase and Epoxide Hydrolase Activity in Human Leukocytes in Relation to Risk of Lung Cancer and Other Smoking-Related Cancers. *J Natl Cancer Inst*, **84**; 414-422.

Hedley, D. W., Friedlander, M. L., Taylor, I. W., Rugg, C. A. and Musgrove, E. A. (1983). Method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. *J Histochem Cytochem*, **31**; 1333-1335.

Hein, D. W. (1988). Acetylator genotype and arylamine-induced carcinogenesis. *Biochem Biophys Acta*, **948**; 37-66.

Hickman, D. and Sim, E. (1991). N-acetyltransferase polymorphism. Comparison of phenotype and genotype in humans. *Biochem Pharmacol,* **42**; 1007-1014.

Hickman, D. , Risch, A., Camilleri, J. P. and Sim, E. (1992). Genotyping human polymorphic arylamine N-acetyltransferase: identification of new slow allotypic variants. *Pharmacogenet.* **2**; 217-226.

Homma, H. and Listowsky, I. (1985). Identification of Yb-flutathione S-transferase as a major rat liver protein labelled with dexamethasone 21-methanesulfonate. *Proc Natl Acad Sci*, **82**; 7165-7169.

Howard, A. and Pelc, S. R. (1951). Nuclear incorporation of P-32 as demonstrated by autoradiographs. *Exp Cell Res*, **2**; 178-187.

Howie, A. F., Forrester, L. M. and Glancey, M. J. (1990). Glutathione S-transferase and glutathione peroxidase expression in normal and tumor human tissues. *Carcinogenesis*, **11**; 451-458.

Hughes, H.B., Biehl, J.P., Jones, A.P. and Schmidt, L.H. (1954). Metabolism of isonaizid in as related to the occurence of peripheral neuritis. *Am Rev Tuberc*, **70**; 266-273.

Ilett, K.F., David, B.M., Detchon, P., Castledon, W.M. and Rose, K. (1987). Acetylation Phenotype in Colorectal Carcinoma. *Cancer Res*, **47**; 1466-1469.

Jagerstad, M, Olsson, K., Grivas, S., Negishi, C., Wakabayashi, K., Tsuda, M., Sato, S. and Sugimura, T. (1984). Formation of 2-amino3,8-dimethylimidazo[4,5-f]quinoxaline in a midel system by heating creatinine , glycine and glucose. *Mutat Res*, **126**; 239-244.

Jones, D. J., Moore, M. and Schofield, P. F. (1988). Prognostic significance of DNA ploidy in colorectal cancer: a prospective flow cytometric study. *Br J Surg*, **75**; 28-33.

Kaderlik, K., Minchin, R. F., Mulder, G. J., Ilett, K. F., Daugaard-Jensen, M., Teitel, C. H. and Kadlubar, F. F. (1994) Metabolic activation pathway for the formation of DNA adducts of the carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in rat extrahepatic tissues. *Carcinogenesis*, **8**; 1703-1709.

Kaderlik, K., Mulder, G. J., Turesky, R. J., Lang, N.P. and Kadlubar, F.F. (1994). Glucuronidation of N-hydroxy heterocyclic amines by human and rat microsomes and formation of two distinct N-glucuronides of N-OH-PhIP in vivo and in vitro in the rat and dog. *Carcinogenesis,* **15**; 1695-1701.

Kaderlik, K., Turesky, R.J., Teitel, C.H., Lang, N.P. and Kadlubar, F.F. (1991). Metabolic pathways for heterocyclic amines in humans in relation to colon carcinogenesis. *Proceedins of the American Association for Cancer Research*, **32**; A712

Kakiuchi, H., Watanabe, M., Ushijima, T., Toyota, M., Imai, K., Weisburger, J. H., Sugimura, T. and Nagao, M. (1995). Specific 5'-GGGA-3' to %'-GGA-3' mutation of the Apc gene in rat colon tumors induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, *Proc. Natl Acad. Sci. USA,* **92**; 910-914.

Kantor, R. R. S., Giardina, S.L., Bartolazzi, A., Townsend, A. J., Myers, C. E., Cowan, K. H., Longo, D. L. and Natali, P. G. (1991). Monoclonal Antibodies to Glutathione S-Transferase pi-Immunohistochemical Analysis of Human Tissues and Cancer. *Int J Cancer*, **47**; 193-201.

Kaplowitz, N., Percy Robb, I. W. and Javitt, N. B. (1973). Role of hepatic anion binding protein in bromosulphthalein conjugation. *J Exp Med*, **138**; 483-487.

Kasai, H., Yamaizumi, Z., Wakabayashi, K., Nagao, M., Sugimura, T., Yokoyama, S., Miyazawa, T and Nishimura, S. (1980). Structure and chemical synthesis of MeIQ, a potent mutagen isolate from broiled fish. *Chem Letters* 1980, 1391-1394.

Kasai, H., Yamaizumi, Z., Wakabayashi, K., Nagao, M., Sugimura, T., Yokoyama, S., Miyazawa, T and Nishimura, S. (1981). Structure of a potent mutagen isolated from fried beef. *Chem Letters*, 1981; 485-488.

Kergueris, M. F., Bourin, M. and Larousse, C. (1986). Pharmacokinetics of Isoniazid: Influence of age. *Eur J clin Pharmac,* **30**; 335-340.

Kirlin, W.G., Ogolla, F., Andrews, A.F., Trinidad, A., Ferguson, R.J., Yerokun, T., Mpezo, M. and Nein, D.H. (1991). Acetylator Genotype-dependent Expression of Arylamine N-Acetyltransferase in Human Colon Cytosol from Non-Cancer and Colorectal Cancer Patients. *Cancer Res*, **51**; 549-555.

Kirlin, W.G., Trinidad, A., Yerokun, T., Ogolla, F., Ferguson, R.J., Andrews, A.F., Brady, P.K. and Hein, D.W. (1989). Polymorphic Expression of Aacetyl Coenzyme A-dependent Arylamine N-Acetyltransferase and Acetyl Coenzyme A-dependent O-Acetyltransferase-mediated Activation of N-Hydroxylamines by Human Bladder Cytosol. *Cancer Res*, **49**; 2448-2454.

Knize, H. G., Andresen, B. D., Healey, S. K., Shen, H. J., Lewis, P. R., Bjeldanes, L. F., Hatch, F. T. and Felton, J. S. (1985). Effects of temperature, patty thickness and fat content on the production of mutagens in fried ground beef. *Food Chem Toxicol*, **23**; 1035-1040.

Kokal, W., Sheibani, K., Terz, J. and Harada, R. (1986). Tumor DNA content in the prognosis of colorectal carcinoma. *J Am Med Assoc*, **255**; 3123-3127.

Kwok, S. and Higuchi, R. (1989). Avoiding false positives with PCR. *Nature*, **339**; 237-238.

La Vecchia, C., Negri, E., Decarli, A. and al., et (1988). A case-control study ofdiet and colorectal cancer in northern Italy. *Int J Cancer*, **41**; 492-498.

Ladero, J.M., Gonzalez, J.F. and Benitez, J. (1991). Acetylator Polymorphism in Human Colorectal Carcinoma. *Cancer Res*, **51**; 2098-2100.

Laisney, V., Van Cong, N., Cross, M. S. and Frezal, J. (1984). Human genes for glutathione S-transferases. *Hum Genet*, **68**; 221-227.

Lang, N. P., Butler, M. A., Maggengill, J., Lawson, M., Craig Scotts, R., Hauer-Jensen, M., and Kadlubar, F. F. (1994) Rapid Metabolic Phenotypes for Acetyltransferase and Cytochrome P4501A2 and Putative Exposure to Food-boorne Heterocyclic Amines Increase the Risk for Colorectal Cancer or Polyps. *Cancer Epidemiol Biomarkers and Prev,* **3**; 675-682.

Lang, N.P., Chu, K.J. and Hunter, C.F. (1986). Role of Aromatic Amine Acetylytransferase in Human Colorectal Cancer. *Arch Surg*, **121**; 1259-1261.

Lewis, A. D., Hickson, I. D., Robson, C. N., Harris, A. L., Hayes, J. D., Griffith, S. A., Manson, A. M., Hall, A. E., Moss, J. E. and Wolf, C. R. (1988). Amplification and increased expression of alpha class glutathione S-transferase-encoding genes associated with resistance to nitrogen mustards. *Proc Natl Acad Sci USA*, **85**; 8511-8515.

Lowry, O.H., Rosebrough, N.J., Farr, A.J. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *J Biol Chem,* **193**; 265-275.

MacFarlane, G. T., Gibson, G. R. and Cummings, J. H. (1992). Comparison of fermentation reactions in different regions of the human colon. *J Appl-Bacteriol*, **72**; 57-64.

Mannervik, B. (1985). The isoenzymes of glutathione S-transferase. *Adv Enzymol*, **57**; 357-417.

Mannervik, B. and Danielson, H (1988). Glutathione Transferases-Structure and Catalytic Activity. *CRC Crit Rev Biochem*, **23**; 283-337.

Manousos, O. , Day, N. E., Trichopoulos, D., Gerovassilis, F., Tzonou, A. and Polychronopoulou, A. (1983). Diet and colorectal cancer: a case-control study in Greece. *Int J Cancer*, **32**; 1-5.

McDivitt, R. W., Stone, K. R., Craig, R. B. and Meyer, J. S. (1985). A comparison of human breast cancer cell kinetics measured by flow cytometry and thymidine labelling. *Lab Invest*, **52**; 287-291.

McMahon, T. F., Beierschmitt, W. P. and Weiner, M. (1987). Changes in phase I and phase II biotransformation with age in male Fisher 344 rat colon: relationship to colon carcinogenesis. *Cancer Lett*, **36**; 273-282.

McQueen, C.A., Maslansky, C.J., Glowinski, I.B., Crescenzi, S.B., Weber, W.W. and Williams, G.M. (1982). Relationship between the genetically determined acetylator phenotype and DNA damage induced by hydralazine and 2-aminofluorene in cultured rabbit hepatocytes. *Proc Natl Acad Sci USA*, **79**; 1269-1272.

McQueen, C.A., Maslansky, C.J. and Williams, G.M. (1983). Role of acetylation polymorphism in determining susceptibility of cultured rabbit hepatocytes to DNA damage by aromatic amines. *Cancer Res*, **43**; 3120-3123.

Mekhail-Ishak, K., Hudson, N., Tsao, M-S. and Batist, G. (1989). Implications for therapy of drug-metabolizing enzymes in human colon cancer. *Cancer Res,* **49**; 4866-4869.

Melamed, M. R., Enker, W. E., Banner, P., Janov, A. J., Kessler, G. and Darzynkiewicz, Z. (1985). Flow cytometry of colorectal carcinoma with three-year follow-up. *Dis Colon Rectum*, **29**; 184-186.

Morgenstein, R., De Pierre, J. W. and Jornvall, H. (1985). Microsomal glutathione transferase: primary structure. *J Biol Chem*, **260**; 13976-13983.

Morson, B. (1974). President's address: The polyp-cancer sequence in the large bowel. *Proc R Soc Med*, **67**; 451-457.

Muir, C. S. (1990). Epidemiology, Basic Science and the Prevention of Cancer: Implications for the Future. *Cancer Res*, **50**; 6441-6448.

Negishi, C., Wakabayashi, K., Yamaizumi, J., Saito, H., Sato, S., Sugimura, T. and Jagerstad, M. (1985). Identification of 4,8-DiMeIQx, new mutagen. *Mutat Res*, **147**; 267-268.

Ohgaki, H., Hasegawa, H., Kato, T., Suenaga, M., Ubukata, M., Sato, S., Takayama, S. and Sugimura, T. (1986). Carcinogenicity in mice and rats of heterocyclic amines in cooked foods. *Environ Health Perspect*, **67**; 129-134.

Ohsako, S. and Deguchi, T. (1990). Cloning and expression of cDNAs for polymorphic and monomorphic arylamine N-acetyltransferases from human liver. *J Biol Chem*, **265**; 4630-4634.

Ostlund Farrants, A. K., Meyer, D. J., Coles, B., Southan, C., Aikten, A., Johnson, P.J. and Ketterer, B. (1987). The separation of glutathione transferase subunits by using reverse-phase high pressure liquid chromatography. *Biochem J,* **245**; 423-428.

Patrianakos, C. and Hoffman, D. (1979). Chemical studies on tobacco smoke. On the analysis of aromatic amines in cigarette smoke. *J Anal Toxicol*, **3**; 150-154.

Paul, A.A., Southgate, D.A.T., McCance and Widdowson (1978). The composition of foods. *HMSO*, 4th edition, London, .

Paulsen, G. and Nilsson, L.G. (1985). Distyribution of Acaetylator Phenotype in Relation to Age and Sex in Swedish patients. *Eu J Clin Pharmacol*, **28**; 311-315.

Perry, H.H., Tan, E.M., Carmody, S. and Sakamoto, A. (1970). Relationship pf acetyltransferase activity to antinuclear antibodies and toxic symptoms in hypertensive patients treated with hydralazine. *J LabClinMed*, **76**; 114-125.

Peters, W. H. M., Kock, L., Nagengast, F. M. and Roelofs, H. M. J. (1990). Immunodetection with a monoclonal antibody of glutathione s-transferase mu in patients with and without carcinomas. *Biochem Pharmacol*, **39**; 591-597.

Peters, W. H. M., Nagengast, F. M. and Wobbes, Theo. (1989). Glutathione S-transferases in normal and cancerous human colon tissue. *Carcinogenesis*, **10**; 2371-2374.

Peters, W. H. M., Wormskamp, N. G. M. and Thies, E. (1990). Expression of glutathione S-transferases in normal gastric mucosa and in gastric tumors. *Carcinogenesis*, **11**; 1593-1596.

Pontiroli, A. E., De Pasqua, A., Bonisolli, L. and Pozza, G. (1985). Ageing and acetylator phenotype as adetermined by administration of sulphadimidine. *Eur J Clin Pharmac*, **28**; 485-486.

Ponz de Leon, M., Roncucci, L., Di Donato, P., Tassi, L., Smerieri, O., Amorico, M. G., Malagoli, G., De Maria, D., Antonioli, A., Chahin, N. J., et al. (1988). Pattern of epithelial cell proliferation in colorectal mucosa ofnormal subjects and of patients with adenomatous polyps or cancer of the large bowel. *Cancer Res*, **61**; 2286-2290.

Potter, J. D. and McMichael, A. J. (1986). Diet and cancer of the colon and rectum: A case-control study. *J Natl Cancer Inst*, **76**; 557-569.

Preston-Martin, S., Pike, M. C., Ross, R. K., Jones, P. A. and Henderson, B. E. (1990). Increased cell division as a cause of human cancer. *Cancer Res*, **50**; 7415-7421.

Preussman, R. A. W. (1985). The role or food contaminants and additives in human carcinogenesis. J. V. Joosens, M. J. Hill and J. Geboers (Eds.), *Diet and human carcinogenesis* . Amsterdam: Excerpta Medica.

Price Evans, D. A. (1989). N-Acetyltransferase. *Pharmac Ther*, **42**; 157-234.

Puchalski, R. B. and Fahl, W. E. (1990). Expression of recombinant glutathione S-transferase pi, Ya or Yb1 confers resistance to alkylating agents. USA*Proc Natl Acad Sci USA*, **87**; 2443-2447.

Quirke, P., Dyson, J. E .D., Dixon, M. F., Bird, C. C. and Joslin, C. A. F. (1985). Heterogeneity of colorectal adenocarcinomas evaluated by flow cytometry and histopathology. *Br J Cancer*, **51**; 99-106.

Quirke, P., Fozard, J. B. J., Dixon, M. F., Dyson, J. E. D., Giles, E. R. and Bird, C. C. (1986). DNA aneuploidy in colorectal adenomas. *Br J Cancer*, 313-318.

Radulovic, L. L. and Kulkarni, A. P. (1986). H.P.L.C. separation and study of charge isomers of human placental glutathione transferase. *Biochem J*, **239**; 53-57.

Rhoads, D. M., Zarlergo, R. P. and Tu, C. P. (1987). The basic glutathione S-transferase from human livers are products of separate genes. *Biochem biophys Res Commun*, **145**; 474-481.

Risio, M., Lipkin, M., Candelaresi, G. L., Bertone, A., Coverlizza, S. and Rossini, G. P. (1991). Correlations between rectal mucosa cell proliferation and the clinical and pathological features of nonfamilial neoplasia of the large intestine. *Cancer Res*, **51**; 1917-1921.

Rognum, T. O., Thorud, E. and Lund, E. (1987). Survival of large bowel carcinoma patients with different DNA ploidy. *Br J Cancer*, **56**; 633-636.

Rohan, T., Record, S. and Cook, M. (1987). Repeatability of estimates of nutrient and energy intakes - the quantitative food frequency approach. *Nutr Res*, **7**; 125-137.

Rohan, T. E., McMichael, A. J. and Baghurst, P. A. (1988). A case control study of diet and breast cancer in women. *Am J Epidemiol*, **128**; 478-489.

Rohan, T. E. and Potter, J. D. (1984). Retrospective assessment of dietary intake. *Am J Epidemiol*, **120**; 876-887.

Roncucci, L., Ponz de Leon, M., Scalmati, A., Malagoli, G., Pratissoli, S., Perini, M. and Chahin, N. J. (1988). The influence of age of colonic epithelial cell proliferation. *Cancer*, **62**; 2373-2377.

Rose, D. P., Boyar, A. P. and Wynder, E. L. (1986). International comparisons of mortality rates for cancer of the breast, ovary, prostate and colon, and per capita food consumption. *Cancer*, **58**; 2363-2371.

Scheutzle, D. (1983). Sampling of vehicle emissions for chemical analysis and biological testing. *Environ Health Persp*, **47**; 65-80.

Schutte, B., Reynders, M. M. J., Wiggers, T., Arends, J. W., Volovics, L, Bosman, F. T. and Blijham, G. H. (1987). Retrospective analysis of the prognostic significance of DNA content and proliferative activity in large bowel carcinoma. *Cancer Res*, **47**; 5494-5496.

Sciallero, S., Bruno, S., Di Vinci, A., Geido, E., Aste, H. and Giaretti, W. (1988). Flow cytometric DNA ploidy in colorectal adenomas and family history of colorectal cancer. *Cancer*, **61**; 114-120.

Scott, N. A., Rainwater, L. M., Wieand, H. S., Weiland, L. H., Pemberton, J. H., Beart, R. W. and Lieber, M. M. (1987). The relative prognostic value of flow cytometric DNA analysis and conventional clinicopathologic criteria in patients with operable rectal carcinoma. *Dis Colon Rectum*, **30**; 513-520.

Seidegard, J., Vorachek, W. R., Pero, R. W. and Pearson, W. R. (1988). Hereditary differences in the expression of the human glutathione transferase active on trans-stilbene oxide are due to a gene deletion. *Proc Natl Acad Sci USA*, **85**; 7293-7297.

Shea, T., Claflin, G., Comstock, K. E., Sanderson, B. J. S., Burstein, N. A., Keenan, E. J., Mannervik, B. and Henner, W. D. (1990). Glutathione Transferase Activity and Isoenzyme Composition in Primary Human Breast Cancers. *Cancer Res,* **50**; 6848-6853.

Shearman, D. J. C. and Finlayson, N. D. C. (1989). Diseases of the gastrointestinal tract and liver. . Edinburgh: Churchill Livingstone.

Siedegard, J., De Pierre, J. W. and Pero, R. W. (1985). Hereditary interindividual differences in the glutathione transferase activity towards trans-stilbene oxide in resting human mononuclear leukocytes are due to a particular isoenzyme(s). *Carcinogenesis*, **6**; 1669-1673.

Siedegard, J., Pero, R. W., Markowitz, M. M., Roush, G., Miller, D. G. and Beattie, E. J. (1990). Isoenzyme(s) of glutathione transferase(class Mu) as a marker for the susceptibility to lung cancer: a follow up study. *Carcinogenesis*, **11**; 33-36.

Siedegard, J., Pero, R. W., Miller, D. G. and Beattie, E. J. (1986). A glutathione transferase in human leukocytes as a marker for the susceptibility to lung cancer. *Carcinogenesis*, **7**; 751-753.

Siegers, C. P., Bose-Younes, H., Thies, E., Hoppenkamps, R. and Younes, M. (1984). Glutathione and GSH-dependent Enzymes in the Tumorous and Nontumorous Mucosa of the Human Colon and Rectum. *J Cancer Res Clin Oncol*, **107**; 238-241.

Strandberg, I., Boman, G., Hassler, L. and Sjovist, F. (1976). Acetylator phenotype in patients with hydrallazine-induced lupoid syndrome. *Acta Med Scand*, **200**; 367-371.

Strange, R. C., Faulder, C. G., Davis, B. A., Hume, R., Brown, J. A. H., Cotton, W. and Hopkinson, D. A. (1984a). The human glutathione S-transferases: studies on the distribution and genetic variation of GST-1, GST-2, GST-3 isoenzymes. *Ann Hum Genet*, **48**; 11-20.

Strange, R. C., Faulder, C. G., Davis, B. A., Hume, R., Brown, J. A. H., Cotton, W. and Hopkinson, D. A. (1984b). The human glutathione S-transferases: studies on the distribution and genetic variation of GST-1, GST-2, GST-3 isoenzymes. *Ann Hum Genet*, **48**; 11-20.

Sugarbaker, J. P. , Gunderson, L. L and Wittes, R. E. (1985). Colorectal cancer. V. T. De Vita, S. Hellman and S. A. Rosenberg (Eds.), *Cancer: principles and practices of oncology* . Philadelphia: Lioppincott, J. B.

Suzuki, T., Coggan, M., Shaw, D. C. and Board, P. G (1987). Electrophoretic and immunological analysis of human glutathione S-transferase isenzymes. *Ann Hum Genet*, **51**; 95-106.

Tajima, K. and Tominaga, S. (1985). Dietary habits and gastro-intestinal cancer: a comparative case-control study of stomach and large intestinal cancaers in Nagoya, Japan. *Jpn J Cancer Res*, **76**; 705-716.

Terpstra, O. T., Blandenstein, M-V., Dees, J. and Eilers, G. A. M. (1987). Abnormal pattern of cell proliferation in the entire colonic mucosa of patients with colon adenom or cancer. *Gastroenterology*, **92**; 704-708.

Tietz, F. (1969). Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: application to mammalian blood and other tissues. *Anal Biochem*, **27**; 502-522.

Tomita, I, Kinae, N., Nakamura, Y. and Takenaka, H. (1984). Mutagenicity of various Japanese foodstuffs treated with nitrite. II. Directly-acating mutagens produced from N-containing compounds in foodstuffs. *IARC Sci Pub*, **57**; 33-41.

Trock, B., Lanza, E. and Greenwald, P. (1990). Dietary fiber, vegetables and colon cancer: Critical review and meta-analyses of the epidemiologic evidence. *J Natl Cancer Inst*, **82**; 650-661.

Turesky, R.J., Lang, N.P., Butler, M.A., Teitel, C.H. and Kadlubar, F.F. (1991). Metabolic activation of carcinogenic heterocyclic aromatic amines by human liver and colon. *Carncer Res*, **12**; 1839-1845.

Van den Ingh, H. F., Griffioen, G. and Cornelisse, J. (1985). Flow cytometric detection of aneuploidy in colorectal adenomas,. *Cancer Res*, **45**; 3392-3397.

Vinels, P., Bartsch, H., Caporaso, N., Harringyon, A. M., Kadlubar, F. F., Landi, M. T., Malaveille, C., Sheilds, P. G., Skipper, P., Talaska, G. and Tannenbaum, S. R. (994). Genetically based N-acetyltrransferase metabolic polymorphism and low-level environmental exposure to carcinogens. *Nature*, **369**; 154-156.

Visek, W. J. (1978). Diet and cell growth modulation by ammonia. *Am J Clin Nutr*, **31**; S216-20.

Wakabayashi, K., Nagao, M., Esumi, H. and Sugimura, T. (1992). *Cancer Res*. **52**; 2092s-2098s.

Watne, A. L. (1982). Syndromes of polyposis coli and cancer. *Curr Prob Cancer*, **7**; 1-31.

Weber, W.W. and Hein, D.W. (1985). N-Acetylation Pharmacogenetics. *Pharmac Rev*, **37**; 25-77.

Wei, E. T. and Shu, H. P. (1983). Nitroaromatic carcinogens in diesel soot: a review of laboratory findings. *Am J Public Health*, **73**; 1085-1088.

Welberg, J. W. M., de Vries, E. G. E., Hardonk, M. J., Mulder, N. H., Harms, G., Grond, J., Zwart, N., Koudstaal, J., de Ley, L. and Kleibeuker, J. H. (1990). Proliferation rate of colonic mucosa in normal subjects and patients with colonic neoplasms: a refined immunochemical method. *J Clin Pathol*, **43**; 453-456.

Wersto, R. P., Liblit, R. L., Deitch, D. and Koss, L. G. (1990). Variability in DNA measurements in multiple tumor samples in human colonic carcinoma. *Cancer*, **67**; 106-115.

Westra, J.G., Flammang, T.J., Fullerton, N.F., Beland, F.A., Weis, C.C. and Kadlubar, F.F. (1985). Formation of DNA adducts in vivo in rat liver and intetinal epithelium after administration of the carcinogen 3,s-dimethy-4-aminobiphenyl and its hydroxamic acid. *Carcinogenesis (Lond)*, **6**; 37-44.

Whelpton, R., Watkins, G. and Curry, S. H. (1981). Bratton-Marshall and liquid-chromatographic methods compared for determination of sulfamethazine acetylator status. *Clin Chem*, **27**; 1911-1914.

Willett, W. (1989). The search foe the caused of breast and colon cancer. *Nature*, **338**; 389-394.

Willett, W. C., Stampfer, M. J., Colditz, G. A., Rosner, B. A. and Speizer, F. E. (1990). Relation of meat, fat and fiber intake to the risk of colon cencer in a prospective study among women. *N Engl J Med*, **323**; 1664-1672.

Wilson, R. G., Smith, A. N. and Bird, C. C. (1990). Immunohistochemical detection of abnormal cell proliferation in colonic mucosa of subjects with polyps. *J Clin Pathol*, **43**; 744-747.

Wohlleb, J. C., Hunter, C. F., Blass, B., Kadlubar, F. F., Chu, D. Z. J. and Lang, N. P. (1990). Aromatic amine acetyltransferase as a marker for colorectal cancer: environmental and demographic associations. *Int J Cancer*, **46**; 22-30.

Wooley, R. C., Schreiber, K., Koss, L. G., Karas, M. and Sherman, A. (1982). DNA distribution in human colon carcinomas and its relationship to clinical behaviour. *J Natl Cancer Inst*, **69**; 15-22.

APPENDIX Ai

HPLC acetylator phenotyping (on serum)

Procedure

Patients drink 20mg SDZ/kg body weight and blood is taken at 1 hour (& 4 hours).

1. Aliquot 100ul. plasma standard or patient sample into Eppendorf tubes.
2. Add 100ul. of 20ug/ml SDZ (internal standard) to tubes and vortex for 10 sec.
3. Stand 15 mins at room temperature.
4. Centrifuge 3 mins. in Eppendorf at high speed.
5. Inject 15 ul of supernatant into HPLC.

Plasma standards

Conc (ug/ml)	ul of standard solution			plasma ul
	SMZ	AcSMZ	Solution used	
100	100	100	1000ug/ml	800
50	50	50	1000ug/ml	900
25	50	50	500ug/ml	900
12.5	50	50	250ug/ml	900
0	0	0	0	1000

E.g. Add 100ul SMZ(col.2) + 100ul AcMZ(col.3) of stock (1000ug/ml)(col.4) to 800ul plasma(col.5), to give 100ug/ml of standard SMZ and AcSMZ.
Then take 100ul of standard plasma for assay and add 100ul SDZ (20ug/ml) internal standard to construct a standard concentration curve.

AUTO HPLC

COLUMN:	2.2X 220 mm brownlee column
MOBILE PHASE:	10%
TEMP:	35 ^{0}C
PUMP:	0.5 ml/min (1.8 ml/min mamual)
PRESSURE:	3000
WAVE LENGHT:	254 nm
ATT. RED:	100 mv (100mv-manual)
ATT. BLACK:	200 mv (50mv- manual)
ABSORB RANGE:	0.05
CHART SPEED:	0.25 cm/min (10cm/hr manual)

INJ VOL:	15 ul	
RETENTION TIMES: (approx)	SDZ	5 min
	SMZ	15 min
	AcSMZ	18min

Phenotyping reagents
Ref: Bratton-Marshall. Clin Chem 1981 27 1191-4.

1) 10mM Sodium acetate buffer pH4

0.82 g/l anhydrous NaAc
Adjust pH with glacial acetic acid and 0.1M NaOH

2) 10% Nanograde (HPLC) Acetonitrile (CH_3CN))

MOBILE PHASE: 90% NaAC + 10% CH_3CN
Add 1800ml NaAc + 200ml CH_3CN or to 5L NaAC add 555ml CH_3CN. Filter daily

3) Sulphadiazine (SDZ-internal standard) mw 250.3

a) Stock solution = 1mg/ml
To 25mg SDZ add 10ml of glased distilled water, 5ml NaOH (0.1M) and make vol. up to 25ml with CH_3CN

b) 20ug/ml SDZ- make fresh daily
Rinse out flask with CH_3CN and add 200ul SDZ (1mg/ml stock) to 9.8 ml CH_3CN. Vortex.

4) Sulphamethazine (SMZ)

a) Stock solution = 1mg/ml
To 25mg SMZ add 10ml of glassed distilled water, 5ml NaOH (0.1M) and make vol. up to 25ml with water.

b) Dilute to give 500 and 250 ug/ml solutions with water

5) Acetyl sulphamethazine (AcSMZ)

To 1g SMZ add 4ml acetic acid:4ml acetic anhydride (V:V). heat to 100° for 10mins
Recrystalise with dioxane:water (v:v), cool, then filter and dry overnight in dessicator
a) Stock solution = 1mg/ml (make as SMZ)
b) Dilute to give 500 and 250 ug/ml solutions with water

APPENDIX Aii

Tissue N-Acetyl Transferase Assay

Ref: Hearse DJ Biochem 1973 132 519-526
Weber WW Methods in Enzymology 1981 77 274
Mattano SS Carcinogenesis 1987 8(1) 133-137

<u>Procedure</u>

1. Set water bath 37°C and label Eppendorf tubes 1-10 normal, 11-20 tumour.

2. Place in Eppendorf tubes: 10ul PABA (0.4mM)
   10ul SMZ (0.4mM)
   9ul Ac carnitine (150mM)
   5ul Ac CoA (8mM)
   6.7ul carnitine A T (27.18U/ml)
   place in waterbath 37°C.

3. Add 50ul tissue supernatant to incubation solutions at the required time
   Incubate 0, 1, 2.5, 5, 10, 15, 45, 60, 75, and 90 mins. Vortex.

4. Stop the reaction with 40ul SDZ (20ug/ml) in CH_3CN, vortex, centrifuge, store at -70°C.

5. For assay , thaw, centrifuge at 12000rpm for 3 min. and use 50ul. for HPLC tube

6. Assay using auto HPLC with 8% mobile phase (NaAC:CH_3CN) with std curve.

COLUMN:	5um X 220 mm brownlee column with 3cm c-18 cartridge
MOBILE PHASE:	8%
TEMP:	35 °C
PUMP:	2 ml/min
PRESSURE:	3000
WAVE LENGHT:	254 nm
ATT. RED:	20 mv

ATT. BLACK:`		50 mv
ABSORB RANGE:		0.01
CHART SPEED:		0.25 cm/min
INJ VOL:		15 ul
RETENTION TIMES:	PABA	4 min
(variable)	SDZ	6-7min
	AcPABA	7-8 min
	SMZ	23-24 min
	AcSMZ	34 min

<u>NAT Reagents</u>

1) 0.1M Potassium phosphate (KH_2PO_4) buffer pH 7.4 mw 136.09 Store 4°C
0.1M = 13.6g/l
6.8g/500ml water, adjust pH to 7.4 with orthophosphoric acid/KOH

2) 0.4mM p-amino benzoic acid (PABA) mw 159.1 Store 4°C
0.4mM = 63.64 mg/L
6.36mg/100ml water

3) 0.4mM Sulphamethazine (SMZ) mw 278.3 Store 4°C
0.4mM =111.32 mg/L water

4) 150mM Acetyl Carnitine mw 239.7 Store 4°C
150mM =35.955 g/L
35.955mg/ml 0.1M KH_2PO_4 pH 7.4

5) 8mM Acetyl CoA-Sodium salt (PABA) mw 809.6 make fresh daily
97% purity: 1M = 809.6/0.97 = 834.64
8mM = 6.677 mg/ml in water

6) 20ug/ml Sulphadiazine (SDZ) mw 250.3 make dilution fresh daily

a) Stock solution = 1mg/ml
To 25mg SDZ add 10ml of glassed distilled water, 5ml NaOH (0.1m) and make vol. up to 25ml with CH_3CN

b) 20ug/ml SDZ- make fresh daily
Rinse out flask with CH_3CN and add 200ul SDZ (1mg/ml stock) to 9.8 ml CH_3CN. Vortex

7) 27.18 u/ml carntine acetyl transferase (CAT) **
Make up diluted enzyme fresh daily. Store at 4°C

Stock Suspension Contains: . 38 mg protein/ml
93 U/mg protein
353.4 U/ ml

Dilute 1 in 13 to give 27.18 U/ml (approx 26U/ml as previous bottle)
20ul CAT + 240ul 0.1M KH_2PO_4 buffer pH 7.4
** Value changes with each bottle

HPLC NAT reagents

1) 10mM Sodium acetate ,anhydrous (NaAC)

10mM= 0.8203 g/L glass distilled water
1.64g/2L water
4.1g /5L water
and adjust to pH 4 with glacial acetic acid/0.1M NaOH

2) 8% Mobile phase Filter before use

920 ml NaAc : 80 ml CH_3CN (acetilnitrile) -> 1 L MP
3680 ml NaAc : 320ml CH_3CN -> 4L MP or

To 5L NaAC pH 4 add 435 ml CH3CN -> 8%
To 1L NaAC pH 4 add 174 ml CH3CN -> 8%
(6% = 940 ml NaCA : 60 ml CH_3CN)

Standard curve

100ul of 0.2M AcPABA in 2.4ml buffer or water -> 0.008mM AcPABA

10 nmol: (a) Take 200ul PABA (0.2mM), 200ul MZ (0.2mM), 200ul AcSMZ (0.2mM) and 200ul AcPABA(0.008mM)

8 nmol : Take 160 ul of A (10 nmol) and add 40ul KH_2PO_4 buffer (0.1M) pH7.4

6 nmol : Take 120 ul of A (10 nmol) and add 80ul KH_2PO_4 buffer (0.1M) pH7.4

4 nmol : Take 80 ul of A (10 nmol) and add 120ul KH_2PO_4 buffer (0.1M) pH7.4

2 nmol : Take 40 ul of A (10 nmol) and add 160ul KH_2PO_4 buffer (0.1M) pH7.4

Take 50ul of each concentration and add 50ul HPLC water and 40 ul SDZ (20ug/ml), 100ul (1mg/ml) SDZ to 4.9ml CH_3CN) into Eppendorf tubes.
Spin and add 5ul to HPLC tubes, tap to remove air bubbles

APPENDIX Aiii

Protein assay

<u>Procedure</u>

BSA mg	Added ul	water ul	working solution ul	1/2 dil Folins reagent ul
0	0	1000	1000	200
0.02	20	980	1000	200
0.04	40	960	1000	200
0.08	80	920	1000	200
Samples	20	980		

(1) Make duplicates in macrocurvettes

(2) Do 1/2 or 1/5 dilution with distilled water

(3) Add reagents in above order

Make sure FOLINS is 1/2 dilution with water

(4) Read absorbance at 30 mins at 750 nm, slit width 1nm

(5) Draw standard curve(scatter plot, simple)

(6) Calculate mg protein in samples from standard curve

(7) Calculate <u>mg protein/ml</u>

=mg protein x 50 x dilution factor of cytosol

Protein assay reagents

1) Bovine serum albumin (BSA) store at -20°C
1mg/ml water

2) 2%Na_2CO_3/0.1M NaOH store in refrigerator
Weight 20g Na_2CO_3 and dissolve in 1L 0.1M NaOH

3) 0.5% $CuSO_4.5H_2O$/1% trisodium citrate store in fridge
Weigh 500mg $CuSO_4.5H_2O$ + 1g trisodium citrate and make up to 100ml with water

4) WORKING SOLUTION make fresh
Mix 2%Na_2CO_3/0.1M NaOH and 0.5% $CuSO_4.5H_2O$/1% trisodium citrate in proporton 50:1
ie 24.5ml : 0.5ml

5) Folins reagent (keep in foil)
Dilute 2M folins with water to give 1m folins reagent ie 2mls folins :2 ml water

6) Cytosol
Might need to be diluted 1/2 or 1/5 with Kh2PO4 buffer or water

APPENDIX Bi

Lymphocyte isolation from peripheral blood

1. Dilute the red blood cells and buffy coat with saline or Hanks BSS to twice the original volume.

2. Layer Ficoll (Histopaque 1077 Sigma, St. Louis, USA) under the diluted cells carefully to ensure a good interface. Use about 2 mls. of Ficaoll per 8 mls. of cells.

3. Spin 15minutes at 1800 rpm. (20 mins. for 50 ml. tubes).

4. Skim no more than 5 mls. of lymphocytes from the interface with a pasteur pipette. Bring volume to 10 mls. with saline and count. Use Hanks instead of saline when viability is important e.g. freezing viable cells.

5. Spin down 10 minutes at 1600 rpm.

6. Aspirate supernatant and resuspend cells in 10 mls of Hanks or saline. Saline should be used prior to PCR.

APPENDIX Bii

DNA preparation

If the samples are to be used in the PCR, extreme care must be taken not to introduce any cross-contamination. A new tip should be used for each sample.

1. Resuspend harvested cells at $20x10^6$/ ml or less in (phosphate buffered) saline and transfer into Eppendorf or centrifuge tubes. (If only one 10 ml. tube of blood was obtained, there are generally no more than 20 million cells and the cells can be transferred to an Eppendorf without counting).

2. Spin on microfuge at 6500 g/ 30 seconds or benchfuge 1600 rpm/ 5minutes.

3. Resuspend pellet in 500 ul. TEN 1 buffer(50mM Tris-HCL pH 8, 2mM EDTA, 400mM NaCl). 500 ul. can be approximated by the point at which an Eppendorf bends. Add 1 drop/Eppendorf of proteinase K and mix by inversion. Add 1 drop/Eppendorf of 20% SDS and mix by inversion.

4. Incubate at 37°C for 4 hrs to overnight. It is a good idea to check the tube 10-60 minutes after beginning the incubation and gently mixing by inversion to resuspend any large pieces of material.

5. Add approximately 500ul of the organic phase of TE saturated phenol, mix gently by inversion and put on rotating wheel for at least 15 minutes.

6. Microfuge at 13500 rpm for 2 minutes. During this time, prepare tube(s) with approximately 500 ul. of the organic phase of TE saturated 1:1 phenol:isoamyl alcohol-chloroform (PIAC).

7. Collect supernatant and any interphase material, and add to the PIAC. Mix gently by inversion and put on rotating wheel for at least 15 minutes.

8. Microfuge at 13500 rpm for 1 minute. During this time, prepare tube(s) with approximately 500 ul. of isoamyl alcohol-chloroform (IAC).

9. Collect supernatant and add to the IAC. If there is any material at the interface, it must be left behind or collected and re-extracted with the PIAC. Mix gently by inversion and put on the rotating wheel for at least 15 minutes.

10. Microfuge at 13500 rpm. for 1 minute. Collect supernatant (no more than 0.5 mls.) and top up with reagent grade ethanol (2 volumes). Allow to precipitate overnight at -20°C. For rapid work, precipitation may be done using liquid nitrogen. The DNA may be left as an ethanol precipitate indefinitely but may take longer to solvate if it has beeen in ethanol for a long time.

11. To solvate, spin at 13500 rpm. for 5 minutes and remove ethanol. Alternatively, the precipitate may be directly picked out into a fresh tube. This may help to minimise RNA contamination.

12. Add 500 ul. of 75% ethanol. Rinse all tubes and spin at 5 minutes at 13500 rpm.

13. Remove ethanol using a tip. Be careful not to dislogde the pellet. Allow the pellet to dry at room temperature or using the speedvac..

14. Resuspend pellet in 400 ul. of TE (10mM Tris-HCL, pH 8, 1mM EDTA) and allow to solvate at 4°C for several days after which time the optical density can be done.

Reagents

-Sterile (distilled) water
-100mM Tris base- 3.03g in 250ml sterile water, pH8 -c.HCL (1ml)
-10mM EDTA- 73mg in 250ml sterile water, pH 8-NaOH (2M-pH7 and 1M-pH8)
-4M NaCl-58.4g/250ml sterile water

TEN1, pH8 (10mM Tris-HCL (pH8), 1mM EDTA, 0.4M NaCl)
5ml Tris base(100mM pH 8 with HCL)+5ml EDTA(10mM)+5ml NaCl(4M) in 35ml sterile water, pH8 (total vol=50ml with 1/10 dils=5:45->molarity)

20% SDS, pH7.2 {use a mask}
20g laurylsulphate(SDS)/100ml sterilewater. ph 7.2 with 1M & 0.1M HCL (heat to help dissolve) aliquot into glass bottle

Proteinase K
100mg (pellet) in 10 mls sterile water, aliquot in 1ml Eppendorfs(sterile) and freeze

75% EtOH
750ml 100% EtOH and 300ml sterile water

3M Sodium acetate, pH 4, 8
24.6g anhydrous Na Ac in 50ml sterile water, pH to 5.2 with glacial acetic acid, make volume up to 100mls.

PBS, pH 7.4
5(PBS):45mls sterile water, pH to 7.4 wth HCL

1M Tris Cl. pH 8
121.1g trizma base in 1 L sterile water, pH 8 with conc. HCL (approx. 2ml)

0.1M Tris Cl. pH 8 = 1/10 dil of 1M- 100ml 1M and 900ml sterile water

Chlorofrom:isoamyl alcohol {use mask and fume hood}
96 mls chloroform and 4 mls isoamyl alcohol

TE buffer, pH 8(10mM Tris-HCl pH 8, 1mM EDTA, pH 8)
5ml Tris base(100mM. pH 8 with HCL)+5ml EDTA(10mM) in 40ml sterile water, pH8 with HCL

Phenol- {use mask,laboratory coat, double gloves and fume hood}
In baked brown jar (160°C for 2 hr), add 100g Phenol crystals, melt at 68°C in hot water beaker (vol=100ml) and add 3 crystals of hydroxyl quinolone (0.1%). Mix. Put phenol in funnel and add equal vol (100ml) of 1M Tris.Cl., pH 8, mix, leave 15 minutes to separate into 2 clear solutions. Pour Tris into sink.
Wash with equal vol. (100 ml) of 0.1M Tris Cl., pH 8 and 0.2% (200ul) ß-mercaptoethanol until pH of aqueous phase is >7.6 or phenol is pH>8. (2-3 washes). Store with Tris buffer in refrigerator for up to 2 months.

APPENDIX Ci

Tissue Glutathione S Transferase Activity

Procedure

Cytosol- homogenise mucosa in 3ml KH_2PO_4 0.1M, pH 7.4, centrifuge 20 000g (15 000 rpm) 20 min usin JA-21 rotor for Beckman centrifuge.

1. Duplicates with macrocurvettes and blank (substitue with 200ul water)
2. 1/10 dilution of cytosol (100ul:900ul KH_2PO_4 0.1M, pH 7.4)
3. Add 200ul diluted cytosol
   1.65ml KH_2PO_4 0.121M, pH 6.5)
   50ul CDNB (0.04M)
   100ul GSH (0.02M)- to initiate the reaction

4.Record change in absorbance at 340nm for 20 mins
5. Do protein assay

Calculate GST activity using the molar extinction coefficient
96{Change in absorbance (OD) / min} x 96 x 5(dilution) = U/ml
Divide by protein concentration per ml= U/mg protein

GST reagents

1) 0.1M KH_2PO_4 , pH 7.4
mw 136.09 store at 4^oC
0.1M= 13.609g/l H_2O Adjust pH 7.4 with orthophosphoric acid/KOH

2) 0.121M KH_2PO_4, pH 6.5
mw 136.09 Store at 4^oC
0.121M= 16.467g/l H_2O Adjust pH 6.5 with orthophosphoric acid/KOH

3) 0.04M CDNB (1,chloro-2,4-dinitrobenzene)
mw 202.6 Store at 4^oC, in foil, (unstable in light)
0.04M= 8.104g/l or 0.8104g/100 ml Methanol

4) 0.02M Glutathione - GSH reduced
mw 307.3 Make fresh daily
0.02M= 6.146mg/ml or 61.46mg/10ml H_2O

APPENDIX Cii

Tissue Total Glutathione

Cytosol- homogenise mucosa in 3ml KH_2PO_4 0.1M, pH 7.4, centrifuge 20 000g (15 000 rpm) 20 min usin JA-21 rotor for Beckman centrifuge.

1 Mix 500ul of cytosol with 500ul 5%TCA, vortex,
spin on microfuge-high, 5' remove supernatant(can store in -70°C until use).

2. ASSAY: 700ul 0.3 mM NADPH (made fresh)
100ul 6 mM DTNB
200ul TCA/supernatant
Incubate 5min room temp

3. Initiate reaction with 20ul GSSG reductase (25U/ml) (make enzyme fresh)

4. Record change in absorbance at 412nm
Calculate nmol/ml regression equation

5. Do protein assay

Calculate GSH
{change in optical density/min= slope} x 2(dil) / mg protein =nmol/mg protein

<u>Standards-duplicates</u>
Make up 0.02M GSH reduced (6.146mg/ml water)
20ul of 0.02M GSH reduced in 10mls 0.1M KH_2PO_4, pH 7.4 ->40nmol/ml
Do 1/2 dilution : 20nmol/ml
Do 1/2 dilution : 10nmol/ml
Do 1/2 dilution : 5nmol/ml

GSH reagents

1) 0.125M KH_2PO_4/6.3mM EDTA buffer pH7.5

KH_2PO_4	mw 136.09	EDTA	mw 372.24
0.125M=	17g/l	6.3mM=	2.34g/l
	1.7g/100ml		234.5mg/100ml

Adjust pH 7.5 with orthophosphoric acid/KOH

2) 0.3mM NADPH in KH_2PO_4/EDTA buffer

mw 833.4	make fresh daily
0.3mM=	250g/l
	25mg/10ml
	62.5mg/25ml KH_2PO_4/EDTA buffer

3) 6mM DTNB

mw 396.4

6mM=	2.378 g/l
	9.512mg/4ml KH_2PO_4/EDTA buffer

4) GSSG reductase (approx 25U/ml) KH_2PO_4/EDTA buffer

Stock

1.9mg protein/ml 155U/mg protein 294.5U/ml

Dilute 1/10 to give 29.45 U/ml

20ul enzyme stock + 180ul KH_2PO_4/EDTA buffer

80ul enzyme stock + 720ul KH_2PO_4/EDTA buffer

Stock

12.6mg protein/ml 175U/mg protein 2205U/ml

Dilute 1/180 to give 27.5 U/ml

10ul enzyme stock + 790ul KH_2PO_4/EDTA buffer

5ul enzyme stock + 395ul KH_2PO_4/EDTA buffer