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THE ROLE OF FOLATE IN THE DEVELOPMENT OF COLORECTAL CANCER

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Submitted for the degree of Philosophy (Ph.D)

Dublin University

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March 2004

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DECLARATION

This thesis is entirely the work of the author with the exception of any Comet assay work; which was performed on material prepared in Dublin as part of a conjoint study with the University of Ulster, Coleraine. All comet assay work was performed by Dr. Angela P. McGlynn and Dr. Gillian R.Wasson.

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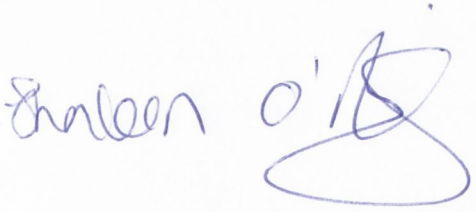

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Dublin
March 1st 2004

SUMMARY

Colorectal cancer is a leading cause of morbidity and mortality in Europe and the USA each year. Most large bowel cancers arise within pre-existing adenomatous polyps or adenomas. Several case-control and prospective studies have investigated the relationship between dietary folate intake and the risk of colorectal adenoma development, which is a well-established precursor of colorectal cancer. This project focused on establishing a method that would allow two techniques, colonic tissue folate calculation and the Comet assay, to be combined in a complementary fashion to investigate the relationship between purported DNA biomarkers, systemic and localised folate status and their role in the development of colorectal cancer.

The comet assay allows the microelectrophorectic direct visualization of DNA damage in individual cells, the damaged cells appear like a comet consisting of brightly fluorescent head and a tail whose length and fluorescent intensity will relate to the number of DNA-strand breaks. This technique was modified to examine uracil misincorporation and hypomethylation, the major pathways through which folate has been implicated in colorectal cancer development.

An ion-chelation method was used to isolate colonocytes/epithelial cells from human tissue biopsies and a folate extraction method was developed to allow quantification of the folate content of this tissue. This method was compared and contrasted against a whole biopsy folate analysis method commonly used. It would appear that the epithelial cell folate is the more accurate marker within the study group examined and that it is a reproducible and valid assay for looking at the relationship between folate status and colorectal cancer.

The role of folate was examined in relation to systemic markers, epithelial cell folate concentrations and modified comet ratios in a case-control setting using individuals with and without polyps. Results showed that there was a higher level of DNA damage in the polyp tissue, the level of epithelial cell folate was found to be lowest in polyp tissue compared to normal and the case group also had an overall lower mean concentration of epithelial cell folate. Serum folate and red cell folate were positively related in the case group, while homocysteine was negatively related. In the control group a negative relationship was only seen between homocysteine and epithelial cell folate when below the median was examined. The polyp group was sub-divided into hyperplastic and adenomas, this revealed uracil misincorporation was significantly higher in hyperplastic polyps while hypomethylation was nearly significantly higher in adenomatous polyps. This supports recent suggestions that uracil misincorporation is an earlier event in the adenoma-to-carcinoma sequence than hypomethylation.

The double-blind, placebo-controlled intervention trial yielded some interesting results. The placebo group, on the whole, remained unchanged over the six-month follow-up period; the supplemented group showed the expected changed in systemic folate status, which proves compliance was maintained. The epithelial cell folate was significantly increased in the polyp site and displayed a trend towards significance in the adjacent to polyp site, this was not seen in the placebo group. Uracil misincorporation decreased significantly in the polyp and adjacent site while hypomethylation decreased significantly in the adjacent to polyp site with a trend towards significance in the polyp site for the supplemented group. These preliminary results indicate folic acid supplementation may have a role in the prevention of colorectal cancer.

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McGlynn, A.P., Wasson, G.R., **O'Reilly, S.L.**, McKelvey-Martin, V.J., McNulty, H., Strain, J.J., McKerr, G., Mullan, F., Mahmud, N., Scott, J.M., Weir, D.G., Downes, C.S. 2003. Detection of replicative integrity in small colonic biopsies using the BrdUrd comet assay. *British Journal of Cancer* 88, 895-901.

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ABBREVIATIONS

ACF	aberrant crypt foci
AD	Alzheimer disease
ADH_3	alcohol dehydrogenase 3
AICAR	5-amino-imidiazole-4-carboxamide-ribonuclueotide
APC	adenomatous polyposis coli
BAF	bulky adduct forming
BMI	body mass index
BrdUrd	bromodeoxyuridine
CAD	coronary artery disease
CBS	cystathionine β-synthase
CEA	carcinoembryonic antigen
CHD	coronary heart disease
CLA	conjugated linoleic acid
COMT	catechol-O-methyltransferases
COX-2	cyclooxygenase-2
CPC	chicken pancreas conjugase
CpG	cytosine guanine dinucleotide
CRC	colorectal cancer
CVD	cardiovascular disease
CYP	cytochrome P450
DAPI	4,6-diamindino-2-phenylindole
DFE	dietary folate equivalents
dH_2O	distilled water
DMH	dimethylhydrazine
DRV	dietary reference values
dTTP	thymidylate
DTT	dithiothreitol
dUMP	deoxyuridine monophosphate
EB	ethidium bromide
EPIC	European prospective investigation into cancer and nutrition
FAP	familial adenomatous polyposis
FIGLU	formimino glutamic acid
FISH	fluorescent in-situ hybridization
FOB	faecal occult blood
FPIA	fluorescence polarization immuno assay
GAR	glycinamide ribonucleotide
HAA	heterocyclic amines
HBSS	Hanks' balanced salt solution
Hcy	homocysteine
HH	HpaII and HhaI methylation specific endonucleases
HHB	buffer only treated HH slides
HHR	ratio between HH and HHB slides
hMLH1	human Mut-L homologue 1
hMSH2	human Mut-S homologue 2
HNE	4-hydroxy-2-nonenal
HNPCC	hereditary non-polyposis colorectal cancer
H_2O_2	hydrogen peroxide

HPLC	high performance liquid chromatography
HPV	human papilloma virus
IBD	inflammatory bowel disease
IF	intrinsic factor
IFA	intrinsic factor antibody
IGF-1	insulin growth factor-1
IGF-BP3	insulin growth factor-binding factor 3
LIs	labeling indices
LMP	low melting point
MeCP2	methyl-CpG-binding protein 2
MI	myocardial infarction
MMA	methyl malonic acid
MMR	mismatch repair
MS	methionine synthase
MSI	microsatellite instability
5-methyl THF	5-methyltetrahydrofolate
5,10-MTHF	5,10-methyltetrahydrofolate
MTHFR	methylenetetrahydrofolate reductase
NaCn	sodium cyanide
NAT	N-aetyltransferase
NER	nucleotide excision repair
NMP	normal melting point
NTDs	neural tube defects
PA	pernicious anaemia
PABA	p-aminobenzoic acid
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
PI	propidium iodide
PLP	pyridoxal-5'-phosphate
PUFAs	polyunsaturated fatty acids
PVD	peripheral vascular disease
RBC	red blood cell
RDA	recommended daily amount
SAH	s-adenosylhomocysteine
SAM	s-adenosylmethionine
s.c.	subcutaneous
SCGE	single cell gel electrophoresis or Comet
SSBs	single strand breaks
SMHT	serine hydroxymethyltransferase
TC	transcobalamin
tHcy	total homocysteine
THF	tetrahydrofolate
TNM	tumour nodes metastasis
UC	ulcerative colitis
UG	uracil glycoslase
UGB	buffer only uracil glycosylase slides
UGR	ratio of UG to UGB slides
UK	United Kingdom
US	United States of America

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CHAPTER 1

INTRODUCTION

1. COLORECTAL CANCER

i. Morbidity and Mortality

Colorectal cancer (CRC) is a leading cause of morbidity and mortality with about 300,000 new cases and 200,000 deaths in Europe and the USA each year. CRC was among the top 4 cancers in five racial / ethnic populations during 1992 through 1998 in the US. It accounted for 10.5% of all cancer deaths and 11.6% of all cases reported. Long-term incidence rates have only increased in the ascending colon in females (1.5% annual increase 1992 through 1998) while overall death rates have decreased with increased levels of screening and treatment (Howe *et al.* 2001).In Ireland, on average each year there are 1185 new cases of malignant CRC diagnosed in females, 1439 in males, in Ireland as a whole. European-age-standardised rates were significantly higher among males than females, by about 57%. Colon cancer was the third most common site for females after skin and breast, while in males it was the fourth most common after skin, prostrate and lung. Anorectal cancer is less common (7th most common in males, 9th in females). CRC is very rarely found in individuals below age 35 (<1% of all cases). On average, females are estimated to have a 1-in-32 chance of developing CRC by the age of 74, males have a 1-in-20 chance. The median age of diagnosis is 72 years for females and 69years for males. Annual averages for CRC attributed deaths in females are 630 and 739 in males; just over 5 deaths for every 10 incidence cases. Mortality rates are significantly higher for males than females, by about 64%. On average, females are estimated to have a 1-in-73 chance, males a 1-in-42 chance of dying from CRC by the age of 74 (National Cancer Registry Ireland 2001). Estimated 5 year relative survival rates (from diagnosis date) were broadly similar for colon (about 48% for both sexes) and anorectal cancers (about 44%), based on European data (Gatta *et al.* 2000). Irish figures from 1997 estimated a 54% probability of 5 year survival for males and 58% for females (National Cancer Registry Ireland 1998).One of the most important factors that influence survival rates was the

stage of the cancer at diagnosis. The most commonly used methods of treatment are surgical resection either alone or alongside adjuvant chemotherapy and/or radiotherapy (National Cancer Registry Ireland 1999).

ii. Colorectal Polyps

Most large bowel cancers arise within pre-existing adenomatous polyps or adenomas. These lesions are common, the true incidence was difficult to estimate in symptom-free and unselected populations. Necropsy studies have shown a prevalence of about 35% in Europe and the USA but lower levels of about 10-15% in Africa and Asia. A polyp can be defined as an elevation above the mucosal surface. Polyps range in size from a few millimeters to 10cm in diameter. They may be single or multiple, in some syndromes hundreds. Dysplasia is the term used for unequivocal epithelial proliferation in colorectal (and other) epithelium (Riddell *et al.* 1983). Dysplastic epithelium is characterized by increased cellularity and abnormal morphology, including the development of spindle-shaped nuclei with hyperchromasia, nuclear stratification and loss of nuclear polarity. Aberrant Crypt Foci are characterized by groups of crypts with enlarged diameter, thickened hypercellular epithelium and altered mucin pattern. Adenomas were grossly visible non-malignant clonal lesions characterized by dysplasia (adenomatous epithelium). Adenomas often connotated as a polyp, however, most colorectal polyps are not adenomas but rather hyperplastic (metaplastic) polyps. Most polyps are asymptomatic and found by chance while investigating symptoms of altered bowel habit, pain, blood present in the stools and anaemia of unknown cause.

Hyperplastic polyps are generally regarded as having no malignant potential or indication of risk for metachronous cancer (Winawer *et al.* 1997). However, intraepithelial dysplasia has now been seen as the important histological precursor known to take several morphologic forms in addition to the polypoid adenoma eg. Hamatomous polyps in Peutz-

Jeghers syndrome, in serrated adenomas and mixed adenomatous-hyperplastic polyps, in flat and depressed adenomas and in dysplastic aberrant crypt foci (Hamilton *et al.* 2001;Takayama *et al.* 1998). Microsatellite instability (MSI) is seen in 10-15% of sporadic CRCs mostly in the proximal colon (Ashktorab *et al.* 2003). These cancers are generally poorly differentiated, have mucinous histology and histopathologic evidence of host immune response in the form of numerous intratumoral lymphocytes and peritumoral lymphoid modules (Boland *et al.* 1998). Patients with large or numerous hyperplastic polyps, termed "hyperplastic polyposis syndrome" have a predisposition to right-sided colon cancer with MSI (Jass *et al.* 2000;Rashid *et al.* 2000a). Genetic alterations of the type seen in neoplasms have been reported in hyperplastic polyps, including mutations of *K-ras* (Otori *et al.* 1997) and MSI (Rashid *et al.* 2000a).It has also been recognized that some otherwise typical hyperplastic lesions can show focal dysplasia, a phenomenon known as a serrated adenoma or as mixed hyperplastic / adenomatous polyps (Longacre and Fenoglio-Preiser 1990a). More recently, right-sided hyperplastic polyps have been suggested as a potential precursor of sporadic cancers showing MSI (Jass 2001a). In addition, epidemiological studies have shown that the risk factors for adenomas and carcinomas were also risk factors for hyperplastic polyps (Martinez *et al.* 1997b;Morimoto *et al.* 2002b). It seems that right-sided hyperplastic polyps and large hyperplastic polyps appear to be the ones of concern as some may give rise to sporadic colorectal carcinomas with MSI. Methylation of the human mut-L homologue 1 (hMLH1) gene promoter within neoplastic cell subpopulations may be a critical step in the progression to carcinoma. The frequency with which benign lesions progress to cancer with MSI is unclear (Hawkins and Ward 2001b).

a. Clinical Presentation

The presenting symptoms of patients with CRC were seen as a function of the anatomical location and the nature of the lesion and were quite variable. Tumours may be

asymptomatic for long periods. Symptoms, which may result from obstruction, perforation and bleeding, include abdominal pain, melena (bloody stools), weight loss, anaemia, diarrhoea, constipation or a change in bowel habit, an abdominal mass and, occasionally, nausea and vomiting.

Right-sided colonic lesions often cause vague epigastric pain, altered bowel habit and chronic blood loss leading to fatigue. Angina pectoris and microcytic hypochromic anaemia are occasionally associated. As the lumen is smaller on the left side of the colon and the faeces have become more solid, patients often present with altered bowel habit, constipation or obstruction. Cancer involving the rectosigmoid region is associated with tenesmus (crampy rectal pain), narrowing of the stool width and bleeding per rectum (PR). Except for patients with obstructive or perforative cancers, the duration of symptoms did not correlate with prognosis (McDermott *et al.* 1981) but prognosis was strongly associated with stage at presentation.

b. Non-Neoplastic Polyps

Hamartomatous polyps are usually large and stalked. They are either juvenile or Peutz-Jeghers in type. Juvenile polyps (occurring in children and teenagers) are confined mainly to the colon and histologically display mucus-retention cysts. They are autosomal dominant and cause bleeding and intussusception, often in the first year of life. With higher numbers of polyps (>10) there is an increased risk of developing colorectal cancer. Peutz-Jeghers polyps are usually multiple and histologically had a characteristic fibromuscular fronds radiating between disorganized mucosal crypts. They are a cause of chronic anaemia. Cronkhite-Canada syndrome is similar to Peutz-Jegher but it is also associated with ectodermal abnormalities.

c. Neoplastic Polyps

Adenomas are classified by histological architecture as tubular, tubulovillous or villous. Villous adenomas are associated with a higher malignant potential, as are large polyps (up to

25% of adenomas are >1cm in diameter) and a high grade dysplasia in the epithelium (5-10% adenomatous polyps display severe dysplasia). About 5% of adenomatous polyps are estimated to become malignant, which can take 5-10 years. In adults, 2-5cm polyps are often found in the rectum and 90% of these are non-malignant hyperplastic ones. Larger polyps in the rectum and 70-80% of all other polyps found are adenomas, 5% (20% of those 2cm or greater in diameter) will contain invasive carcinoma at discovery.

Table 1.i Types of Polyps

TYPE	SOLITARY	MULTIPLE POLYPOSIS
Hamartomas	Peutz-Jeugers Juvenile	Peutz-Jeghers syndrome Juvenile Cronkhite-Canada syndrome
Inflammatory	Inflammatory eg Crohns disease, Ulcerative Colitis, Schistosomiasis	Inflammatory Polyposis
Neoplastic	Adenomas – Tubular Tubulovillous Villous	Familial Adenomatous Polyposis (FAP) Heriditary Non-Polyposis Colorectal Cancer (HNPCC)
Miscellaneous	Hyperplastic Lymphoid	Benign Lymphoid Polyposis

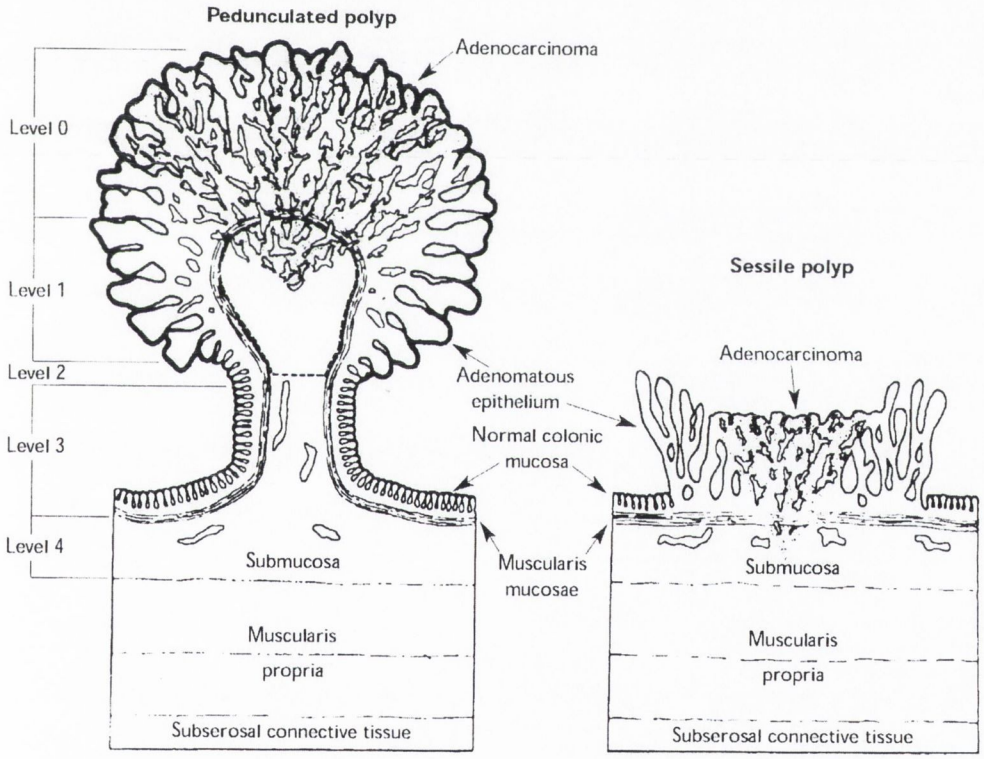


Figure 1.i Anatomic landmarks of colorectal adenomas (levels of invasion of carcinoma depicted) (Haggitt *et al.* 1985)

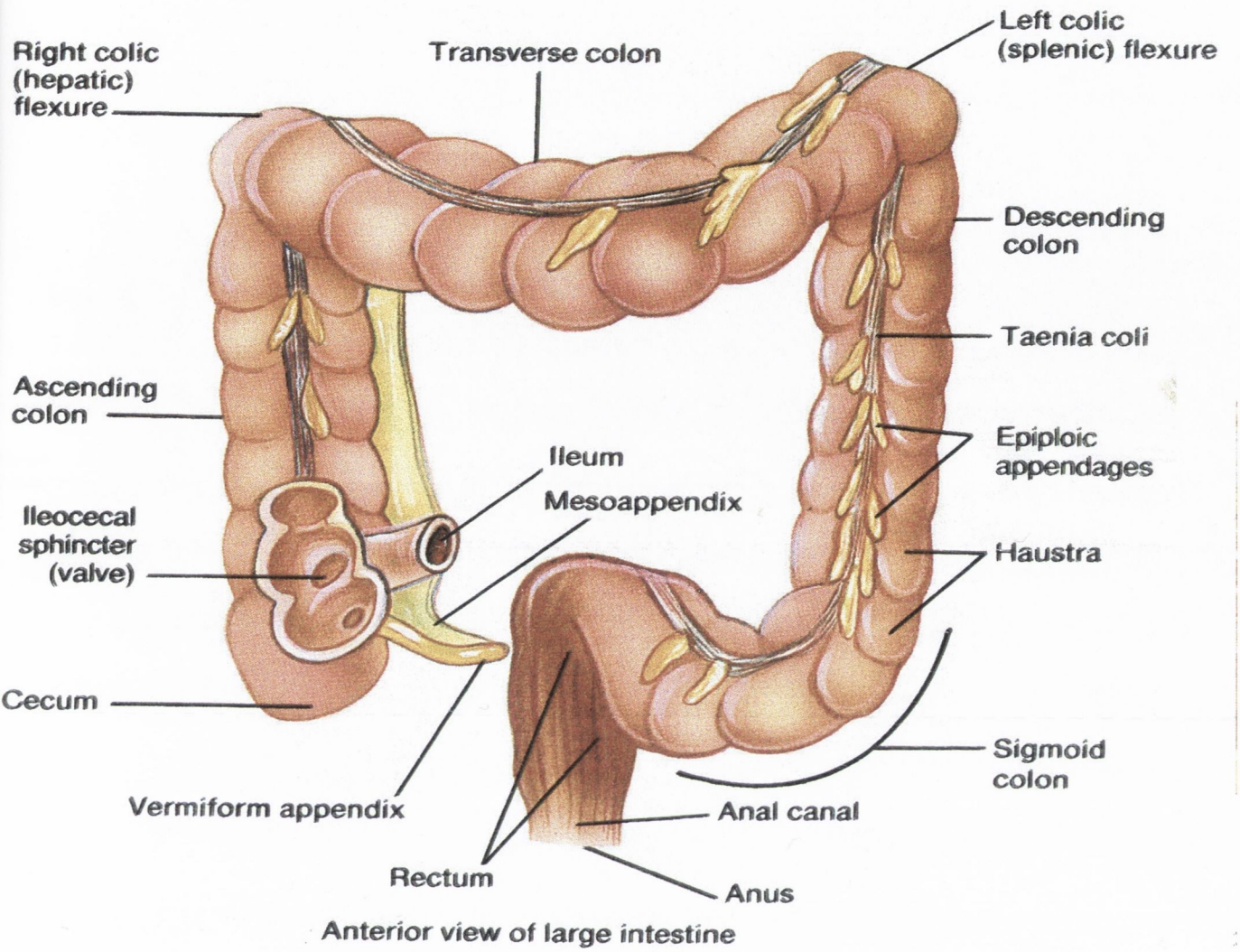


Figure 1.ii Anatomy of the Large Intestine (Tortora and Grabowski 1993)

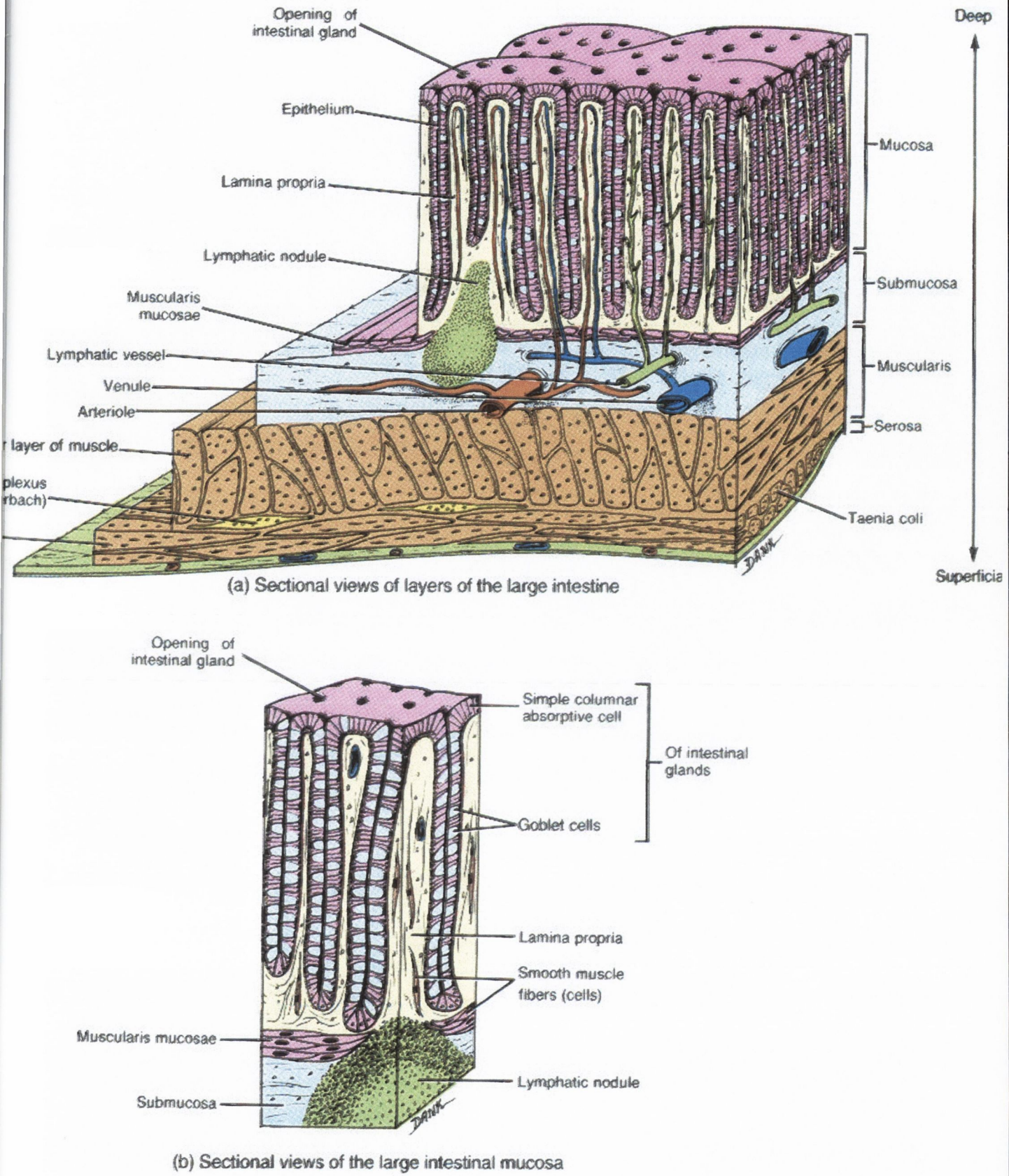


Figure 1.iii Histological Presentation of the Large Intestine (Tortora and Grabowski 1993)



Figure 1.iv Normal Descending Colonic Mucosa



Figure 1.v Small Stalked Tubular Adenoma

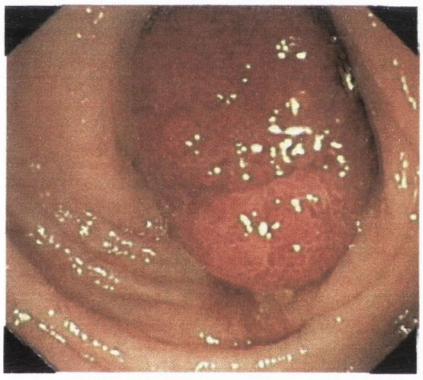


Figure 1.vi Large Tubulovillous Adenoma

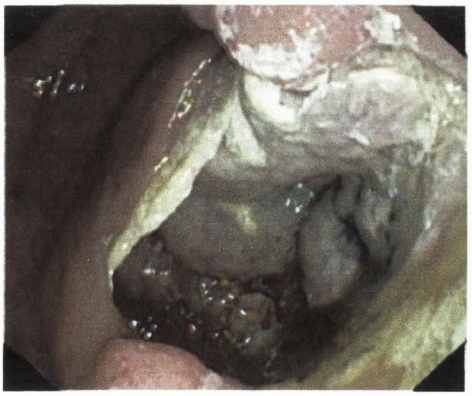


Figure 1.vii Colonic Carcinoma

iii. Colorectal Carcinoma – Diagnosis and Prognosis

Adenocarcinoma of the large bowel is the second most common tumour in the UK with a lifetime incidence of about 1 in 27 (both male and female). The incidence of the disease increased with age, the average age at diagnosis having been 60-65 years. The disease is rare in Africa and Asia, this difference was thought to be mainly environmental rather than genetic. Ulcerative Colitis and FAP are predisposing factors. CRC has been classified on the basis of epidemiological pattern (Lynch *et al.* 1992;Burn *et al.* 2001):

i. Sporadic - Occurred in the absence of a family history of CRC in a first degree relative (>75%).

ii. Familial - Occurred when at least one first degree relative has CRC (5-10% cases).

iii. Hereditary - Occurred where there was a suggestion of an autosomal dominant pattern of inheritance (5-8% cases).

About 90% of CRC is sporadic and occurs in individuals who do not have a strong incidence of the disease. Over half of these have tumours in the rectosigmoid area. The tumour, usually a polypoid mass with ulceration, spreads by direct infiltration through the bowel wall. It then invades the lymphatics and blood vessels with subsequent spread; the most common site is the liver. Synchronous tumours are present in 2% of cases. Siblings and parents of patients with adenomatous polyps are at an increased risk of CRC, particularly when the adenoma is diagnosed before the age of 60 or, in the case of siblings, when a parent had been diagnosed with CRC (Winawer *et al.* 1996).

In 1932, Dukes first proposed a classification system for staging CRC. It still has clinical value in predicting prognosis (Dukes 1932). To more accurately predict prognosis, many variations of the original Dukes classification system have been proposed. In 1988, the American Joint Committee on Cancer and the Union Internationale Contre le Cancer agreed to standardize the staging to allow for the production of uniformly comparable data (American

Joint Committee on Cancer 1988). Several factors in addition to the disease stage affect prognosis and survival. Survival in patients younger than 40 years of age is lower than overall survival due to features indicated an aggressive cancer (high histological grading, mucinous histology and a higher stage of presentation) (Cusack *et al.* 1996). Obstruction and perforation have also been shown to reduce survival (Willett *et al.* 1985). In aggregate, those patients presenting with obstruction or perforation have a worse overall 5-year survival rate, 30% versus 40% in patients without. Surprisingly, rectal bleeding has been associated with more favourable prognosis (Steinberg *et al.* 1986). Rectal bleeding, which indicates mucosal erosion, leaded to earlier diagnosis and surgical intervention. Several large studies have revealed an improved survival rate for women with CRC (Landis *et al.* 1998). Recent data further confirmed a greater decline in mortality in women than in men over the past 50 years (Ries *et al.* 2000); however, the reasons for this difference were unknown.

Location and configuration of a tumour also affected prognosis. Tumours located below the rectosigmoid junction carried a poorer 5-year survival than those located more proximally (Godwin and Brown 1975). The pre-operative level of the tumour marker carcinoembryonic antigen (CEA) is related to the stage of disease and is reflective of tumour burden in CEA-producing tumours. CEA values greater than 5ng/ml have been associated with a poorer prognosis, independent of pathological staging. The CEA level also played a role in predicting tumour recurrence and as a monitor of response to treatment. Increasing CEA values may predate clinical or radiological evidence of recurrence (Aabo *et al.* 1986).

Table 1.ii *TNM Classification of CRC*

Primary Tumour	Lymph Nodes	Distant Metastasis
T0 No evidence of tumour	**N0** No lymph node involvement	**M0** No distant metastasis
Tis Carcinoma in situ		
T1 Tumour invades submucosa	**N1** 1-3 perirectal or pericolic nodes involved	**M1** Distant Metastasis
T2 Tumour invades muscularis Propria	**N2** 4 or more perirectal or pericolic nodes involved	
T3 Tumour invades subserosal, pericolic or perirectal tissues	**N3** Lymph node involvement along major vascular trunk	
T4 Tumour directly invades other organs or structures		

Table 1.iii *Staging Systems for CRC*

AJCC[1]	Dukes[2]	TNM[1]	% Cases[3]	% 5-yr Survival[3]
0		Tis		
I	A	T1 N0 M0 T2 N0 M0	10	90-100
II	B	T3 N0 M0 T4 N0 M0	35	65-75
III	C C2	Any T N1 M0 Any T N2 M0 Any T N3 M0	30	30-40
IV	D	Any T Any N M1	25	<5

[1] Based on American Joint Committee on Cancer : Manual for Staging of Cancer, 4th edition Philadelphia: JB lippincott, 1992.

[2] Based on Dukes CE : The classification of cancer of the rectum. *J Pathol* 1932;35:323-332 and Turnbull RB, Kyle K, Watson FB, et al : Influence of the no touch isolation technique on survival rates, *Ann Surg* 1967; 1967:420.

[3] Based on Winawer SJ(1997): Colorectal cancer screening: clinical guidelines and rationale. *Gastroenterology* 112:594-642.

iv. Screening

A major focus in CRC is prevention and early detection as this can have a significant impact on mortality and morbidity figures. General population screening has been instigated in some countries to this end (Frame *et al.* 1997). Faecal Occult Blood (FOB) testing can lead to a reduction in cancer-related mortality of 15-33% (Rennert *et al.* 2001). A once-in-a-lifetime flexible sigmoidoscopy at 55 years has, also, been recommended. The test is quick, simple, has high sensitivity and could potentially reduce mortality by 50% but has the disadvantage that only 65% of adenomas and early cancers can be visualized due to the limited access (Newcomb *et al.* 1992). Also, over recent decades, the location of CRC has been shifting from the left side to the right (McCallion *et al.* 2001). One point to note was that there has been an increase in the age of the general longevity of the population and in the elderly there is an increase in the proportion of right-sided CRC (Saltzstein *et al.* 1998). It has also been demonstrated that aspirin and non-steroidal anti-inflammatory users experience a reduction in cancer mortality in the range of 40% even for relatively occasional users (Giovannucci *et al.* 1995a) so if these 2 factors were considered then this might go towards explaining the left to right shift in CRC . Full colonoscopy is useful in screening families with a history of CRC as it can decrease mortality by 70%(Bennett and Hardcastle 1996). It may be sufficient to perform this test only every 5-10 years to achieve the above outcome but it is considered too expensive and carries a degree of morbidity risk, which has to be considered when considering a population screening tool. Other imaging modalities that are being examined are Fast Spiral Computed Tomography, which cut 2mm slices and enabled an endoluminal view using a 3-dimensional colon imaging program. However, this still required bowel preparation and air insufflation like a colonoscopy. It has been suggested that this may become a screening approach and the colonoscopy procedure used for treatment of any abnormality seen (Vallett 2001).

Another approach that may be used in the future was stool DNA-based detection. Recently Dong *et al* were able to extract sufficient DNA to allow diagnosis of the mutations already existed in excised tumours (Dong *et al.* 2001). This study focused on mutations *K-ras* oncogene and the *TP53* tumour suppressor gene (each of which is mutated in 40-60% CRCs) as well as microsatellite instability (MSI) marker *BAT26* (which is altered in 15% sporadic CRC cases). The basis of the technique was that neoplasms and tumours shed cells into the stools, which should be identifiable through PCR. It has been a consistent finding that the quantity and quality of DNA extracted from stools were superior in the presence of neoplasm (Villa *et al.* 1996), to such an extent that the amount of extracted DNA per gram of stool has been used as a marker of malignancy (Loktionov *et al.* 1998). The problem with the test was its high false-positive rate (up to 14% for *K-ras* in normal colons (Dong *et al.* 2001) and the markers examined were markers for tumours (*TP53* and *BAT26* have no role in the detection in adenomas except perhaps those with severe dysplasia) and *K-ras* lacked specificity for site. Genes that may be of more relevance for screening may be *APC*, a gene mutated early in the development of adenomas or L-DNA; DNA strands longer than 200 base pairs that are a marker of repressed apoptosis. These markers may be of more importance in screening and detection of adenomas, in a previous study they detected 73% of adenomas 1cm or larger (Alquist *et al.* 2001).

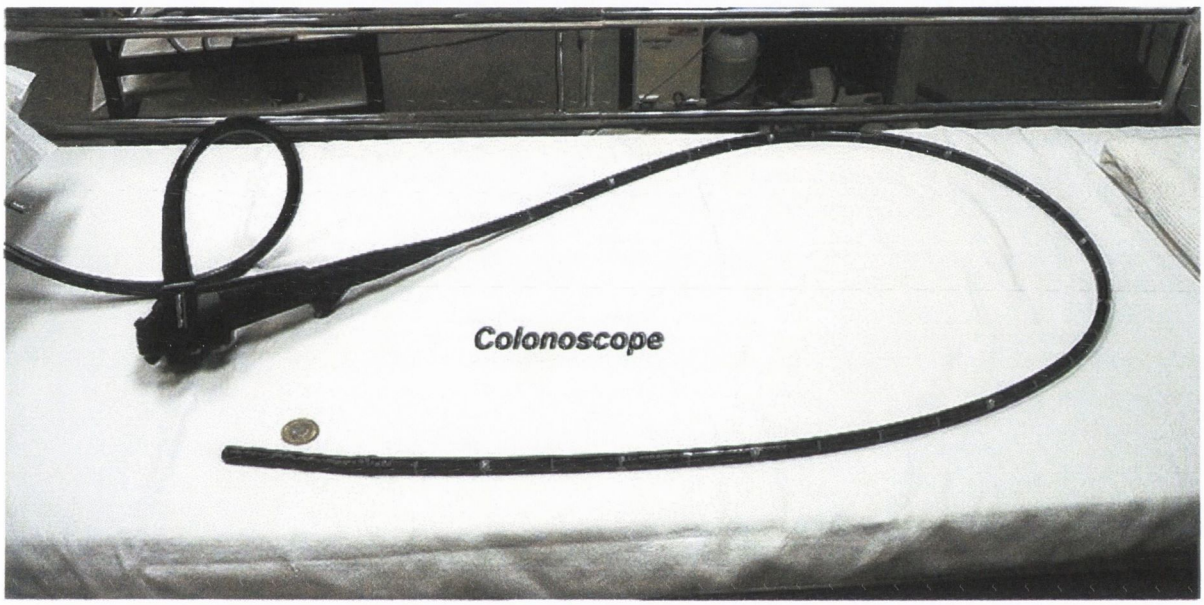


Figure 1.viii Colonoscope used in screening and diagnostic procedures

2. DIET AND COLORECTAL CANCER

i. Introduction

There has been an approximately 15-fold range in age-standardised incidence of CRC across the world and good epidemiological evidence that much of this variation could be attributed to variation in food intake patterns (Department of Health 1998). The associations were distinguished into convincing, probable and possible, and could be either positive (increased intakes would decrease risk) or inverse (increased intakes would increase risk) (World Cancer Research Fund and American Institute for Cancer Prevention 1997;Willett and Trichopoulos 1996). Other non-nutrients and nutrition-related indicators have also been looked at – alcohol, height, obesity and physical activity; these were also thought to have a role to play in cancer of the large bowel. The UK Working Group on Diet and Cancer concluded that there was "moderately consistent evidence that diets with less red and processed meat and with more vegetables and fibre are associated with lower risk of colorectal cancer. Evidence is inconsistent for vitamins A, C and E, and β-carotene".

A person's probability of acquiring cancer has been stated to be heavily influenced by dietary habits (Hill *et al.* 1994). Many studies have shown that overall dietary patterns, dietary components consumed or mode of food preparation were all possibly relevant in either reducing or increasing the risk of developing cancer e.g. it has been suggested that adoption of the Mediterranean style of diet could reduce overall mortality from CRC in Northern Europe and North America by about 25% (Trichopoulou *et al.* 2000). A recent evaluation of the Dietary Guidelines for Americans found that adherence to these may be associated with lower cancer risk (Harnack *et al.* 2002). It has been suggested that dietary intervention studies or chemoprevention trials, tend to fail because of gaps in knowledge about the specific mechanism of action of that food component in the individual and also the ideal time frame

required to be protective or exert the desired effect may be unknown. Each individual will have a variety of genetic traits, which would define the reaction to biological components in their diet. It would only be prudent to assume each individual could have a different reaction to the same exposure. In 1981, Doll and Peto state that while research looks at components of food, very little was known about the combined magnitude of cancer risk or protection conveyed by a mixed diet. They published an estimate for cancer mortality attributed to diet, which was 70% (Doll and Peto 1981). A variety of agents has been studied in relation to cancer risk such as fruit and vegetables, their vitamins and pre-vitamins with antioxidant activity, fibre and other ingredients such as dietary fats. Some of these agents have been found to be protective while others have been found to be hazardous.

The influence of prevalent genetic polymorphisms such as those coding for phase I enzymes (involved in activating steps in the liver) and II enzymes (involved in facilitating the attachment of polar groups to increase solubility and thereby facilitate elimination of compounds) may play a role in how our nutrition influences our health (Patterson *et al.* 1999). While familial background and specific mutations in tumour suppressor genes or in oncogenes are established risk factors for a variety of cancers, these specific mutations are relatively rare and account for relatively small proportions of all cancer cases. Other, often very common, polymorphisms in genes encoding for metabolic enzymes could be of much greater importance to the overall probability of cancer in a large population. These metabolic enzymes include mainly phase I e.g. cytochrome *P450 1A2* (CYP1A2) and II enzyme systems e.g. *N-acetyltranferase 2* (NAT2), as well as genes encoding electrophile scavenging and DNA repair mechanisms. These systems are mechanisms for reducing the harmful effects of environmental carcinogens, including other enzyme systems relevant to cancer risk, such as those controlling the hormonal pathways, and are controlled by genes that are polymorphic in man. Metabolic enzyme activity and response to carcinogens are dependent on the structure of the gene that

encodes them, but could also often have been modified by diet as well as other behaviours such as smoking.

Table 1.iv Dietary components related to CRC risk

Food Component	CRC Risk
Fibre	↓
Saturated Fat	↑
Proteins	↑
Fruits	↓
Vegetables	⇊
Red Meat	⇈
Alcohol	↑
Vitamin A and carotenoids	↓
Folate	↓
Vitamin D	↓
Vitamin E	↓
Calcium	↓

↓ = Possibly Decreases Risk

⇊ = Probably Decreases Risk

↑ = Possibly Increases Risk

⇈ = Probably Increases Risk

There has been considerable disagreement in the results of epidemiological studies of specific cancers. A lot of effort has been put into trying to explain such differences by means of geographic variation. It is logical to expect interaction between consumption levels of a specific dietary component and the polymorphic gene status, to determine whether or not this food item would contribute to the formation of cancer in a given individual or population.

ii. Meat

Based on epidemiological studies it is assumed that meat, especially red meat, enhances risk for cancer, particularly of the colon, breast and prostrate. CRC incidence has risen sharply in Shanghai, China since the early 1970's and diet may have contributed to this. In a population based case-control study, 931 cases and 1552 controls had their dietary practises and food preferences analysed using a food frequency questionnaire. CRC risk was the highest for red meat and preserved foods, these being foods that in China were not the staples they have become in the modern diet (Chiu *et al.* 2003). Meat protein was reported to contribute to cancer formation (Biesalski 2002). Diets high in meat and fat but low in dietary fibre and vegetables have been shown to increase the genotoxic potential of 'faecal water' of human volunteers (Rieger *et al.* 1999). Heterocyclic amines (HAA) were suspected carcinogens that were formed in meat when it was cooked at high temperature for long periods. These compounds required metabolic activation by CYP1A2 and NAT2 or NAT1 before they could bind to DNA. It has been hypothesized that well-done meat increased the risk for CRC, especially in individuals with the rapid phenotype for CYP1A2 and NAT2. This association may be particularly strong in smokers because smoking was known to induce CYP1A2. Previously high red meat consumption has been shown to be hazardous only to NAT2 fast metabolisers (Chen *et al.* 1998b). Le Marchard *et al* conducted a study looking at CYP1A2 and NAT1/2 phenotypes and diet in particular red meat consumption in 349 CRC patients and 467

population controls. In "ever-smokers" a preference for well-done red meat was associated with an 8.8 fold increase in risk of CRC (95% CI, 1.7-44.9) among subjects with NAT2 and CYP1A2 rapid phenotypes, compared to smokers with low NAT2 and CYP1A2 activities who preferred rare to medium red meat. This data supported the hypothesis that exposure to HAAs increased risk for CRC, particularly in individuals that were genetically susceptible and that smoking facilitated this by inducing CYP1A2 (Le Marchand *et al.* 2001). *N*-nitroso compounds were suspected in CRC, they are carcinogens that individuals consuming a diet high in red meat were exposed. Many of these compounds underwent an α-hydroxylation by cytochrome *P450 2E1* (CYP2E1) to form DNA adducts. The gene encoding for this enzyme was polymorphic and may have been susceptible to alteration. In a recent study that looked at two functional polymorphisms of CYP2E1 (G1259C *RsaI* substitution and a 5'96-bp insertion variant) in relation to CRC and the association of red meat and processed meat. 521 patients with CRC and 639 controls were examined and subjects with the 5' insert variant were found to be at a 60% increased risk (95%CI 1.1-2.5) for rectal cancer. Subjects who were exposed to high levels of nitrosoamines and the insert variation were at a 2 to 3 fold increased risk for red and processed meat respectively (Le Marchand *et al.* 2002). Another aspect of red meat consumption was the higher haem iron content, this has been looked at in some experimental studies where they were able to show a negative relationship (Sesink *et al.* 1999;Tavani *et al.* 2000).

iii. Fruit and Vegetables

Epidemiological evidence has overwhelmingly supported the apparent inverse association between consumption of vegetables and fruits and cancer risk, as demonstrated in a review of more than 250 case-control and cohort studies (Smith-Warner and Giovannucci 1999). Convincing evidence exists for inverse risk associations with cancer of the orofacial

region, oesophagus, lung (Heber 2000), stomach and colorectum. This inverse association has also been shown in the area of cardiovascular disease and all-cause mortality in the US population (Bazzano *et al.* 2002). Broekmans *et al* examined the affect on biomarkers through comparing the effects of 100g and 500g fruit and vegetables daily in 47 healthy volunteers over a four-week period. Concentrations of lutein were higher by 46%, lycopene by 22%, β-carotene by 45% and vitamin C by 64%. The group on the higher amount of fruit and vegetables had 15% higher serum folate and an 11% lower serum homocysteine (Broekmans *et al.* 2000). Data from the EPIC-Italy study showed that strong negative associations existed between levels of leucocyte DNA adducts (biological marker that might be predictive of cancer risk) and consumption of fresh vegetables and fruits, particularly green leafy vegetables (Palli *et al.* 2000). In addition, evidence existed that increased vegetable and fruit consumption could reduce oxidative damage to DNA (Thompson *et al.* 1999). It has been shown that the protective effect of isothiocyanate in cruciferous vegetables on the risk of CRC was highly dependent on glutathione transferase *M1* polymorphic status (Lin *et al.* 1998).

Intermediate analyses of the relationship between the consumption of fruits, vegetables and fibre and the risk of developing CRC, stratified by polymorphism of the NAT2 gene, were carried out using the Molecular Epidemiology of Colorectal Cancer (MECC) study database. The MECC was a population-based study of all invasive colorectal cancers diagnosed in Northern Israel since 1998 (1200 CRC cases and 1300 controls). Population-based, age, gender and religion controls were matched with cancer cases. All study participants underwent an extensive interview, including a validated semi-quantitative food frequency questionnaire and donated venous blood. Daily consumption levels of fruit and vegetables were calculated. NAT2 status was determined in the DNA analysis. In this study, vegetables not fruits were found to have a protective effect against CRC. A significant difference was seen in women who were fast metabolisers of NAT2 who had a much greater protective effect from vegetables

than those with a null or that were slow in metabolising NAT2 (Rennert 2003). In reality, numerous constituents found in vegetables and fruits – including fibre, micronutrients and various phytochemicals – and interactions among these constituents might have contributed to the ability of these foods to reduce cancer risk. It is the determination of which ones were the most effective and how they exerted their effects, which has posed the greatest challenge to research.

iv. Fibre

Thirty years ago fibre was examined in relation to CRC by Burkitt, who in 1969 hypothesized that colon cancer was caused by a lack of fibre in the diet (Burkitt 1969). The mechanism he proposed has since had various aspects of it confirmed by Hill and Fernandez (Hill and Ferndanez 1990). It was later shown that fibre from different sources was related differently to bowel cancer risk (Armstrong and Doll 1975). In 1997, it was concluded that there was good evidence that cereal fibre protects against CRC(Hill 1997;Le Leu *et al.* 2002). The European Cancer Prevention Consensus Group accepted this conclusion and expanded it to include breast cancer and possibly other cancer sites (European Cancer Prevention Consensus Panel 1998). There are other research groups who argue that fibre and cereal fibre have little to do with CRC prevention (Fuchs *et al.* 1999;Asano and McLeod 2002), while other groups have looked at it in combination with increased intakes of fruit and vegetables and reduced fat (Schatzkin *et al.* 2000). Another group have looked at total carbohydrate and non-fibrous carbohydrates as a dietary risk factor for CRC in 473 cases and 1192 controls. A significant association was seen between non-fibrous carbohydrate and risk of CRC in both men and women and a sex difference in risk by colorectal sub-site, with risk concentrated for women in the right colon (OR 6.5, 95%CI 2.4-18.4) and the rectum for men (OR 2.4, 95%CI 1.2-4.8) (Borugian *et al.* 2002). Recently, two large-scale studies have obtained positive results

on fibre's role in CRC. The first group, the Prostrate, Lung, Colorectal and Ovarian Cancer Screening Trial examined 43,611 participants and found the highest quintile of dietary fibre intake had a 27% lower risk of adenoma than those from the lowest (95%CI 14-38, P for trend= 0.002). The study only examined the distal colon, as the procedure used was a sigmoidoscopy. The risk for rectal adenoma was not significantly associated with fibre intake. The inverse association for fibre was strongest for cereal, grain and fruit fibre (Peters *et al.* 2003). In the EPIC cohort a population of 519,978 was recruited from 10 different European countries (Bingham *et al.* 2003). Dietary fibre was inversely correlated with the incidence of CRC (RR 0.58, 95%CI 0.41-0.85) for the highest versus the lowest intakes. The effect was most pronounced for the distal colon and non-significant for the rectum. No food source was seen to be more protective than others. The results of these trials suggest that an approximate doubling of dietary fibre could reduce the risk of CRC by 30-40%.

Butyrate has been examined as the main anti-cancer agent resulting from high fibre intakes. The action of butyrate in the colon has been thought to be through several potential mechanisms: anti-proliferative (Kruh *et al.* 1995), induction of apoptosis (Mahyar-Roemer and Roemer 2001), anti-inflammatory (Saemann *et al.* 2000), down regulation of angiogenesis (Pellizzaro *et al.* 2002) ,up regulation of immunosurveillance (Anderson and Mathers 2000) and providing antioxidant protection (Courtois *et al.* 2003).

The intervention studies to date have mainly used dietary fibre and various vitamins to analyse their potential chemopreventative roles. Most of these well designed trials failed to show the anticipated protection (Rennert 2002). Studies on fibre have not been successful in showing protection against CRC (Bonithon-Kopp *et al.* 2000;Bonithon-Kopp *et al.* 2000;Schatzkin *et al.* 2000;Alberts *et al.* 2000;Mai *et al.* 2003). The failure of intervention studies to show the predicted benefits of nutritional components could be explained by poor

choice of intervention products, wrong timing of duration of trial, wrong dosage, poor age group selection, wrong intervention end-points chosen or that possibly it may not have arole in CRC prevention. Several recent trials have used adenoma recurrence as their primary endpoint. As only 1 in 10 adenomas will become a carcinoma and the factors that caused progression to carcinoma may be different to those that initiated polyp growth, it was possible that increased intakes of dietary fibre and other nutrients could prevent carcinoma development, with such benefits having been difficult to detect in adenoma recurrence trials. It is also possible that the fundamental error could be due to the differences of the individual and population subgroups.

v. Fat

Most research in this area has been conducted on the association of total fat, specific fat-containing foods and the types of fat or fatty acids and cancer risk. Although data from animal and ecological studies suggested a direct relationship, intervention studies have not borne this out consistently (Zhou and Blackburn 1999). *n-6* polyunsaturated fatty acids (PUFAs), such as linoleic acid, appeared to enhance the promotional phase of carcinogenesis in *in vitro* models, whereas *n-3* PUFAs, such as α-linoleic acid, eicosapentanoic acid and docosahexanoic acid, seemed to exert inhibitory effects on cancer cells (Bartsch *et al.* 1999). Epidemiological and clinical data supported a possible inverse relationship between consumption of fish and *n-3* PUFAs and risk of CRC (de Deckere 1999). In a European multi-centre breast cancer study, the ratio of *n-3* to *n-6* PUFAs was inversely associated with breast cancer risk, indicating that it may be the balance that was of importance (Simonsen *et al.* 1998). These findings were in agreement with the mortality data for 24 European countries that show an inverse correlation between fish and fish oil consumption with CRC, expressed as a proportion of animal fat (Caygill *et al.* 1996).

Dairy products in the western world have acted as a dietary source of total and saturated fats. The major part of milk lipids was triglycerides (97-98%) or esters of fatty acids. The remainder consisted of phospholipids, sterols, free fatty acids and various quantities of fat-soluable vitamins A, D, E and K. Around two thirds of fatty acids were saturated. The main hypothesis for CRC risk was based on the intraluminal effect of products of fat digestion. It has been postulated that dietary fat may contribute to CRC risk by increasing bile acid and fatty acid excretion in the colonic lumen. The gut flora hydrolysed bile acids to become secondary bile acids. Free fatty acids and ionised secondary bile acids may damage the epithelial cells and thus induce a compensatory hyperproliferation of crypt cells (Van Der Meer *et al.* 1991). A more recent hypothesis was based on the circulatory effects of fat on increasing insulin concentrations. Evidence from animal studies suggested that insulin was an important growth factor of colonic epithelial cells and a mitogen of tumour cell growth *in vitro* (Giovannucci 1995). Some epidemiological evidence has been provided to support the theory that chronic hyperinsulinaemia as a result of insulin resistance increases CRC risk (Kono *et al.* 1998;Kaaks *et al.* 2000;Hu *et al.* 1999). In a recent case-control study nested in the Physicians' Health Study, circulating levels of insulin growth factor-I (IGF-I) and insulin growth factor-binding protein-3 (IGFBP-3) were assayed among 193 men who developed CRC during the 13 years of follow-up and 318 age- and smoking-matched cancer-free control men. Overall, there was a moderate but statistically nonsignificant inverse relationship between intake of low-fat milk or calcium from dairy foods and CRC risk. Intake of dairy products was associated with a modest increase in circulating IGF-I levels but intake of low-fat milk was associated with lower risk of CRC, especially in those with high IGF-I / IGFBP-3. The authors concluded that this sub-population group might have benefited from specific dietary intervention (Ma *et al.* 2001). Epidemiological studies have not generally supported the hypothesis that the total fat content

of the diet increased colon cancer risk. However, it may have been possible that some subtype of fat could be related to colorectal carcinogenesis (Giovannucci and Goldin 1997).

The other side of the dairy argument was that other lipid components might be beneficial, such as conjugated linoleic acid (CLA). Butyric acid may inhibit proliferation and induced differentiation in a wide range of tumour cell lines *in vitro* (Parodi 1999). Butyric acid in the lipid fraction of milk occurred excessively and it was rapidly absorbed by the intestine and was largely metabolised in the liver so it was unlikely that it was involved in colon carcinogenesis. Milk lipids constituted the principal dietary source of dietary CLA. Studies in cell lines have shown that CLA inhibits the proliferation of colorectal, breast and skin tumour cells (Parodi 1999) and mammary tumourigenesis in rats (Ip and Scimeca 1997). CLA could have a cancer-protective effect by modifying the cell membrane fluidity, by reducing the synthesis of prostaglandins and/or stimulating the immune response.

vi. Micronutrients

The most frequently studied nutrients were the anti-oxidants (vitamin A, C, E and selenium), calcium, vitamin D and folate. Epidemiological studies of diets high in vitamin C-containing fruits and vegetables indicated that vitamin C probably decreased risk for stomach cancer, this was plausible because of the role of oxidative damage from inflammation by helicobacter pylori infection (a main risk factor for gastric cancer)(Kono and Hirohata 1996). Vitamin C has also been possibly linked with a reduced risk of oropharyngeal, oesophagus, lung, pancreas and cervix. Apoptosis may lower the risk of neoplasia by removing genetically damaged or mutated cells. A high rate of apoptosis has been linked to a reduced risk of colorectal adenomas and antioxidants may protect cells from harmful oxidation processes but may interfere with apoptosis by protecting genetically damaged cells from reactive oxygen species-dependent cell death. In a recent study, 503 patients were examined to see if there was

a relationship between vitamin C and apoptosis in normal rectal mucosa. Among individuals with adenomas, there was a linear association between apoptosis and total vitamin C intakes. Therefore, given that high apoptosis may lower CRC risk, vitamin C supplements may be contraindicated for patients with a history of adenomas (Connelly *et al.* 2003), which would be at odds with the preventative role for vitamin C.

Epidemiological and experimental data suggest that calcium and vitamin D may influence the risk for prostate and CRC (Platz and Giovannucci 1999). Numerous epidemiological have suggested a weak association between calcium intake and decreased risk of CRC but the results are not conclusive; however, this may be due to the time frames chosen by the research groups. It has been proposed that ionised calcium phosphate might bind to secondary bile acids and free fatty acids, primarily deoxycholic and lithocolic acids, thereby reducing their toxic effects on the colonic epithelial cells and preventing their stimulatory effects on proliferation of the intestinal mucosa (Govers *et al.* 1996). Calcium has been shown to reduce the colonic content of diacylglycerol formed by bacteria which may activate cellular transduction pathways and has been postulated to increase cellular proliferation (Holt *et al.* 1996). A recent review of the epidemiological evidence from published case-control and cohort studies (Martinez and Willett 1998) concluded that calcium intake was not associated with a substantially lower risk of CRC or polyps. Studies published after this, however, supported the hypothesis that high levels of calcium may reduce the risk of CRC. A cohort study in Finland observed a significant 40% reduction in risk of colon cancer at high levels of calcium intake (Pietinen *et al.* 1999) as did a large multi-centre case-control study from the USA (Kampman *et al.* 2000). Recent trials on recurrent adenomas were suggestive of a beneficial effect of calcium supplementation, though modest. The Calcium Polyp Prevention Study, a large clinical controlled trial, found calcium carbonate supplementation to be associated with a small, significant reduction in adenoma recurrence (Baron *et al.* 1999).The European trial on

the prevention of adenoma recurrence in 655 patients resulted in a modest but not significant reduction from calcium supplementation (Bonithon-Kopp *et al.* 2000).

In an early study, Garland and co-workers found a significant reduction of the risk of developing CRC in subjects with higher levels of serum-25-hydroxyvitamin D (Garland *et al.* 1986). Experimental evidence suggests that vitamin D may inhibit cell proliferation (Taniyama *et al.* 2000;Holt *et al.* 2002). The cell growth inhibitory properties of the vitamin appear to be related to the concentrations of vitamin D receptors on the cell surface rather than vitamin D concentrations. The inhibition might also be due to the effect of vitamin D on calcium absorption. A study looking at vitamin D and calcium status alongside vitamin D receptors and colorectal adenomas found that there was an inverse association of serum 25-hydroxyvitamin D with colorectal adenoma. With each 10ng/ml increase in serum 25-hydroxyvitamin D, the risk of colorectal adenoma decreased by 26% (OR 0.74,95% CI 0.6-0.92) (Peters *et al.* 2001). The role of vitamin D has only been addressed in a small number of epidemiological studies. The significant risk reduction of high vitamin D consumption has not been confirmed by any prospective studies. As most studies that observed an increased association with vitamin D intake are prospective ones in which exposure is assessed many years before diagnosis, it is possible that vitamin D has a protective effect only on early stages of tumour development (Kampman *et al.* 2000).

vii. Lifestyle-Related Issues

a. Physical Activity

Physical activity plays an important role in the aetiology of colon cancer. Its significance seen by its consistent association as an independent predictor of colon cancer as well as by its impact on the odds ratios associated with other factors. Slattery and Potter looked at the associations between total energy intake, fibre, calcium, fruit and vegetables, red meat,

whole grains as well as dietary patterns along with cigarette smoking, alcohol consumption, body mass index (BMI) and the use of aspirin and/or Non-Steroidal Anti-Inflammatory Drugs and physical activity (Slattery and Potter 2002). They found the most statistically significant interactions between physical activity and high-risk dietary pattern and vegetable intake. In populations where activity levels are high, the estimated risk associated with high vegetable intake was 0.9 (95% C.I.0.6-1.3) while sedentary populations were 0.6 (95% C.I. 0.5-0.9). Given these observations, it is most probably that physical activity operates through multiple biological mechanisms that influence the carcinogenic process (Slattery and Potter 2002;Tavani *et al.* 1999). High levels of physical activity have consistently been identified as being associated with a reduced risk of colon cancer. It is estimated that 13% of colon cancer can be attributed to being physically inactive (Slattery 2000). A population-based case-control study of 1,447 cases of rectal cancer and 3,106 controls aged 20-76 years in Canada was used to assess the impact of physical activity and obesity on rectal cancer risk (Mao *et al.* 2003). They observed that the greatest increase was in those with high-energy intakes, low physical activity and high BMI. This observation has been seen in other cancers such as breast (Breslow *et al.* 2001;Kruk and Aboul-Enein 2003), testicular (Thorsen *et al.* 2003), and ovarian (Zhang *et al.* 2003).

b. Obesity

Several large studies of obesity and CRC risk have found no association among women but a reasonably consistent positive association among men. In both the American Cancer Society Cohort (Lew and Garfinkel 1979) and the Seventh Day Adventists' Cohort (Phillips and Snowdon 1985), a high BMI was associated with an increased risk of CRC among men. In women, a positive association that is stronger among or limited to those who are premenopausal has been suggested by studies that stratified analysis by age. Obesity was

associated with a two-fold increase in risk of developing CRC in pre-menopausal women but was not associated with altered risk in post-menopausal women (Terry *et al.* 2002). There is some interest in the possibility that a high relative body weight may be associated with an increased risk of colorectal cancer through the mitogenic effects of increased blood levels of insulin (Bruce *et al.* 2000). A recent study looking at the relationship between physical activity, energy intake, obesity and the risk of rectal cancer found that the greatest risk of rectal cancer was observed in men and women with a high energy intake, high BMI and low physical activity level. They suggest that there is a synergistic effect among the 3 risk factors for rectal cancer (Mao *et al.* 2003).

c. Cigarette Smoking

Carcinogens from tobacco reach the colorectal mucosa through either the alimentary tract or the circulatory system and could possibly damage or alter the expression of important cancer-related genes. Cigarette smoking has been seen to promote inflammation-associated adenoma/adenocarcinoma formation in mouse colon and the tumour development is associated with inhibition of cellular apoptosis and is supported by increased angiogenesis (Liu *et al.* 2003a). Tobacco when burned produces numerous genotoxic compounds, including aromatic amines, nitrosamines, aromatic hydrocarbons and heterocyclic amines (Hoffman and Hoffman 1997). Cigarette smoking has been definitively linked with many cancers such as pancreatic (Risch 2003), breast (Band 2003), lung (Hwang *et al.* 2003), cervical (Steckley *et al.* 2003) and liver (Wang *et al.* 2003). The diet, nutrient intakes and circulating levels of nutrients of smokers tend to differ from those of non-smokers. The intake of anti-oxidants is generally lower in smokers, this may make smokers more susceptible to oxidative damage (Margetts and Jackson 1994). It is generally assumed that there is a 30-40 year induction period between smoking and CRC risk as suggested by recent studies (Giovannucci *et al.* 1994a;Giovannucci

et al. 1994b;Slattery *et al.* 1997a). Meta-analysis of epidemiological studies have found that long-term heavy cigarette smokers have a 2-3 fold increased risk of developing colorectal adenomas. Risk of large adenomas, was shown to be elevated in 12 studies. The 27 studies from various countries show an association between tobacco use and CRC. In the USA, 15 out of 16 studies conducted after 1970 in men and in 1990s in women show a relationship. This pattern is consistent with an induction period of 3 to 4 decades between genotoxic exposure and the diagnosis of CRC and with men having started smoking several decades earlier than women. In a recent study looking at associations between smoking, passive smoking, NAT2 and rectal cancer it was found that men who smoked for >20pack years were at an increased risk (Slattery *et al.* 2003). Overall, accumulating evidence strongly supports the addition of CRC to the list of tobacco-associated malignancies and the possibility that up to 1 in 5 CRC cases in the US may be potentially attributable to tobacco use (Giovannucci 2001). Smoking has also been linked with adenoma recurrence and was found to be significant in long-term smokers (Reid *et al.* 2003). How much and how quickly the risk drops after quitting remains in question but most data suggests that at least some of the excess risk persists indefinitely in past smokers (Sturmer *et al.* 2000). Only one study suggests that the excess risk approaches zero after greater than 20 years after smoking cessation (Chao *et al.* 2000).

d. Alcohol

Epidemiological evidence strongly supports a direct association of alcohol intake with cancers at several sites – including maxillofacial and liver- and suggests a direct association with breast, colorectal and lung cancers (Longnecker and Tseng 1999;Seitz *et al.* 1998). However, the potential of dietary habits to confound the association between alcohol and health needs further study. A cross-sectional study of the French cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC) looked at 73,000 self-administered

food frequency questionnaires in women. Increased alcohol consumption was associated with higher consumption of animal products, cheese, potatoes, oil, bread and breakfast cereals and with lower intake of fruit and vegetables and dairy products. In this population of middle-aged, highly educated French women marked differences in dietary patterns and nutrient intakes were found according to alcohol consumption. Part of the detrimental effects of alcohol on health may be due to the less healthy dietary habits of drinkers (Kesse *et al.* 2001). The UNC Alumni Heart Study found that the health benefits of wine drinkers compared with other alcoholic beverages may be as a result of their confounding dietary habits and other lifestyle factors (Barefoot *et al.* 2002). Also, a prospective study of 7608 men showed that heavy alcohol drinkers ($\geq$ 30g/day) contributes directly to weight gain and obesity, irrespective of the type of alcohol consumed (Wannamethee and Shaper 2003). Epidemiological data linking alcohol and CRC may be dependent on, in part, folate and methionine dietary intake. In a large prospective trial, men in the highest quintiles of methionine and folate intakes who consumed more than 20g alcohol daily were not at increased risk for CRC (RR=0.79; 95%CI = 0.38-1.64, RR=1.03; 95%CI =0.52-2.06 respectively) (Giovannucci *et al.* 1995b).

Possible mechanisms by which alcohol might increase overall cancer risk include carcinogenicity of acetaldehyde, a metabolite of alcohol; carcinogenicity of congeners in alcoholic beverages (preservatives and flavouring agents); effects on cell membrane integrity; increase in lipid peroxidase; effects on carcinogen metabolism; alterations in hormone levels, particularly oestrogen and impairment of nutrient metabolism (Blot 1992). For instance, in rats, high alcohol intake can lead to folate deficiency in the colon, this is thought to be as a result of folate breakdown by acetaldehyde produced by microbial oxidation of alcohol (Homann *et al.* 2000). In MIN (multiple intestinal neoplasia) mice, which are a genetic model of intestinal neoplasia, ethanol supplementation resulted in a significant increase in tumour number suggesting it may be a promoter of CRC (Roy *et al.* 2002). The effect of ethanol and

acetaldehyde on DNA in human lymphocytes, gastric mucosa and colonic mucosa have been examined and the results suggest that acetaldehyde may form crosslinks with DNA and that these were poorly repaired with the colonic mucosa cells showing the greatest sensitivity to ethanol (Blasiak *et al.* 2000). Chronic alcohol consumption produces genomic DNA hypomethylation in the colonic mucosa of rats but this was not specific to the *p53* gene (Choi *et al.* 1999). Chronic alcoholism can result in specific micronutrient deficiencies; this includes folate, B_{12} and B_6. The possible link between homocysteine (Hcy) and alcoholism stems from the fact that Hcy metabolism is closely linked to the metabolism of the above vitamins. Hcy stands at the intersection of two pathways: methylation and transsulfuration. In methylation, Hcy acquires a methyl group from 5-methyltetrahydrofolate in a vitamin B_{12} dependant reaction, whereas in the transsulfuration pathway, Hcy condenses with serine to form cystathionine in an irreversible reaction catalysed by the pyridoxal-5'-phosphate-containing enzyme, cystathionine-β-synthase. Due to these relationships, nutritional deficiency of one of these vitamins, because of chronic alcohol intake, could lead to metabolic disruption and potentially to hyperhomocysteinaemia. Consistent with an inference of alcohol in these metabolic pathways, a study on chronic alcoholics was able to demonstrate DNA hypomethylation in peripheral lymphocytes in whom hyperhomocysteineaemia was seen along with disturbed vitamin status (Cravo and Camilo 2000). A J-shaped effect of alcohol consumption on all-cause mortality has been shown, with the lowest mortality in those consuming moderate amounts (1-2 drinks/day). It appears that alcohol consumption has a protective effect on coronary heart disease but that non-cardiac mortality from stroke, cancer and alcoholic liver disease increases with alcohol consumption beyond moderate levels (Koehler *et al.* 2001).

e. Aspirin

Aspirin works in part through the inhibition of cyclooxygenase-2 (COX-2), an enzyme that is found in CRC tissue. Fibroblasts and endothelial cells have been shown to play an important role in polyp expansion by expressing COX-2, resulting in tumour angiogenesis (Sonoshita *et al.* 2002). It has been demonstrated, recently, that aspirin results in reduced microsatellite instability in CRC cell lines and that it acts also through COX-independent mechanisms by resulting in an increase is mismatch repair protein expression and subsequent apoptosis, which might serve as an additional means of growth inhibition in aspirin treated colon cancer cells (Goel *et al.* 2003). Epidemiological studies have shown a consistent 40-50% reduction in the risk of colorectal neoplasia, despite differences in the design of the studies, the populations studied, patterns of aspirin use and outcomes. In randomised trials involving patients with FAP, sulindac and celecoxib have reduced the number and size of adenomatous polyps. In another study, Sandler and colleagues (Sandler *et al.* 2003) were able to show that daily use of 325mg enteric-coated aspirin is associated with a significant reduction in the incidence in colorectal adenomas in 635 patients with previous CRC. The study was terminated after a median duration of treatment of 31 months because one or more adenomas were discovered in 17% of aspirin treatment group and 27% in the placebo. The hazard ratio for the detection of a new polyp in the aspirin group as compared with the placebo group was 0.64, indicating that aspirin delayed the development of adenomas.

A dose-finding study used rectal mucosal prostaglandin (PG) E_2 levels as a biomarker found that 81mg aspirin dose was as effective at suppressing the PGE_2 levels to an equivalent extent as did the 650mg dose in 60 subjects with prior sporadic colorectal adenomas (Sample *et al.* 2002). In a study of 1121 patients who recently had adenomas removed were assigned

randomly to placebo, 81mg or 325mg aspirin daily (Baron *et al.* 2003). After a mean duration of 33 months of treatment, advanced neoplasms were found in 12.9% patients in the placebo group, 7.7% patients in the 81mg group and 10.7% of the 325mg aspirin group. The rate of stroke and serious bleeding was higher in the aspirin groups but not statistically significant (Imperiale 2003). Cost-effective analysis of aspirin as a chemopreventative agent in CRC indicated that it saved fewer lives at higher costs than does screening for CRC by any method and is not beneficial among people at average risk who follow screening recommendations.

3. COMET ASSAY

i. Development

The comet assay, also called single cell gel electrophoresis (SCGE), was first introduced in 1984 by Ostling and Johanson. The technique was used for the microelectrophorectic direct visualization of DNA damage in individual cells (Ostling and Johanson 1984). They developed a simple technique that could yield valuable results on DNA damage. The technique was to suspend a small number of irradiated cells in a thin layer of agarose gel on a microscope slide. These were then lysed, electrophoresed and stained with a fluorescent DNA binding dye. During electrophoresis, the current pulled the charged DNA from the nucleus but relaxed and broken DNA fragments migrated further. They suggested that the relaxation of the supercoiled DNA by single-strand breaks (SSBs) allows DNA to be pulled out of the nucleus. The images generated resembled comets and this is the name most commonly used in current research. The "comets" were measured to determine the extent of DNA damage and it was discovered that the extent of DNA in the head of the comet was a function of the dose of irradiation. In 1988 Singh and co-workers, introduced alkaline conditions to assay, this made the relationship between single-strand breakage more obvious, so alkali-labile sites as well as frank breaks are revealed (Singh *et al.* 1988). The principle behind this was that only if DNA base pairing was disrupted by alkaline conditions that discontinuities in single strands be detected. At neutral pH the continuity of the long duplex molecule was not affected by an occasional SSB. SSBs can have a profound effect on the behaviour of DNA even without alkaline treatment as the DNA in the nucleus is supercoiled and tightly packed. Following staining with ethidium bromide, increased extension of the DNA from the nucleus towards the anode is observed with increased damage to the cells. The damaged cells appear like a comet consisting of brightly fluorescent head and a tail whose length and fluorescent intensity will relate to the number of DNA-strand breaks. Comets form

as broken ends of the negatively charged DNA become free to migrate to the negatively charged anode. Two main principles are believed to determine the pattern of comet formation. The ability of the DNA to migrate is a function of both the size of the DNA and the number of broken "ends" which may be still attached to larger pieces of DNA but they can still migrate a short distance from the head so, within the tail, the series of fragments appear to retain a higher order structure and these probably remain connected by single-stranded regions. Tail length initially increases with damage but reaches a maximum that is largely defined by the electrophoresis conditions, not the size of the fragments. Undamaged cells appear in the head of the comet as intact nuclei without tails (Collins 2002;Olive 2002)

The benefits of the comet assay are that it allows the detection of intercellular differences in DNA damage and repair in almost every eukaryote cell population, the assay itself requires only a small cell population (from 1 to 10,000 cells) and the results can be obtained in a single day at a low cost. The improved microscope technology and the development of powerful analysis software e.g. Komet has allowed the method to become more accurate and yield more detailed results for statistical analysis. The main drawback of comet sampling is the low cell density needed to prevent nucleus overlap after electrophoresis; this limits large-scale high throughput screening. Another problem is that there can be inconsistent comet focusing. A new technique tries to overcome these problems by using a vertical comet assay enabling three-dimensional confocal comet imaging of nuclei seeded at a very high density alongside dedicated software algorithms to retrieve quantitative data at a single cell level (Baert and Van Oostveldt 2003).

There are several different methodologies that can be employed in preparing single-cell populations for comet analysis. The most common is, following the isolation of the desired cells, the suspension of the cells in low melting point (LMP) agarose at a final concentration of 0.5-1.0% at 35-45°C and they are then cast onto a fully frosted slide covered in normal melting

point agarose (NMP). The benefit of using a single layer of LMP and NMP agarose is that it allows better interaction of the cells with any subsequent enzymes that might be used to detect specific DNA damage sites. Following slide preparation, the slides are immersed in a lysis buffer generally for a minimum of 1hour. Neutral lysis will detect only double strand breaks since at this pH DNA base pairing is not disrupted and thus discontinuities in SSBs cannot be detected, while alkaline will detect SSBs. The recommended lysis solution consists of 100mM EDTA, 2.5M sodium chloride and 10 mM Trizma base adjusted to pH 10, with 1% Triton X-100 added just prior to use. The addition of 10% DMSO to this may be useful to prevent potential radical-inducing DNA damage associated with the iron released during lysis from erythrocytes present in blood and tissue samples. The lysis solution is chilled prior to use, primarily to maintain the stability of the agarose gel. Overnight lysis has been argued to increase efficiency in the detection of strand breaks, probably due to allowing more time for DNA unwinding and diffusion before electrophoresis (Olive 2001). Prior to electrophoresis, the slides are allowed to equilibrate in the alkaline electrophoresis solution. It is recommended that the gels are rinsed in distilled water to remove residual detergents and salts prior to the alkali unwinding step since the concentration of sodium chloride in the electrophoresis buffer may have influence on the electrophoresis conditions (Klaude *et al.* 1996). The time spent on electrophoresing the cells will vary based on the levels of DNA damage expressed in the cells and the salt concentration of the electrophoresis buffer. Since DNA is required to migrate only a fraction of a millimeter for microscopic observation, significant DNA migration, which leads to comet formation, is possible with very short electrophoresis runs (5-30min) and low voltages (0.5-5V/cm) as compared with the most conventional DNA electrophoretic techniques. The same electrophoresis unit and power supply unit should be used throughout the same study. Slide-to-slide variation can be minimized by maintaining a constant temperature throughout the electrophoresis (generally between 2^0 and 20^0c although around 5^0c is

recommended) and by using a recirculating electrophoresis unit. Once the electrophoresis is complete, the slides can then be washed and stained with a fluorescent DNA binding stain for imaging. The comets are viewed by fluorescent microscopy.

Other experimental variables that need to be considered are the cell suspension preparation, slides, and gel size preparation, time of neutralization, dehydration of the slides, the DNA-specific dye used for visualization, the magnification used to examine the migrating DNA and the methods used for data collection and analysis. The method of isolation of the target cell population is important, as the method should not allow any additional repair or strand breaks to be created. Slide preparation can vary between research groups, some favor applying the LMP agarose containing the cell suspension directly to the slides while others prefer a "sandwich" technique of applying a base layer of NMP, then the LMP layer and afterwards a final layer of NMP to secure the cells. There are also considerable variations to these two methods: Singh and Khan in 1995 started dehydrating the primary NMP layer by incubating at 40-50^{0}c for a few minutes creating permanent slides, some authors use coverslips instead of fully frosted slides to reduce the amount of background intensity upon microscopy but the concentration and the amount of agarose can contribute to this also. The concentration of the LMP agarose and the dilution of the cells are important parameters, typically 1000 to 50000 cells are suspended in 0.5-1% LMP agarose at 35-45^{0}c. Too many cells make analysis difficult as comets will overlap and run into one another. In the microscopy step the general visualization range used is 200-250X but 160 to 400X have been used. The magnification that is most appropriate depends on the type of cell being evaluated, the range of migration and the constraints of the microscope and / or the imaging system used. Similarly, the selection of fluorescent dye depends to a large extent on the limitations of the equipment and the manner in which the data will be collected. The most frequently used dyes are ethidium bromide (EB),

propidium iodide (PI) and 4,6-Diamindino-2-phenylindole (DAPI). Antifade can be used for some fluorescent dyes to minimize the rate of signal quenching (Hartmann *et al.* 2001).

The methods used to analyse the comet images will vary. Singh and co-workers have used the tail length since 1988, where they found that it reflected the amount of breakage in the cell. However, this variable has been shown to change the most at low levels of DNA damage and it also depends on the sensitivity of the fluorochrome used. It can be used to measure a positive or negative response to a range of damage induced in cells. However, it has been demonstrated that the tail length plateaus after migrating a certain distance (Olive 1989). After this maximum is reached, the comet tail would not extend in length but grow in intensity as compared with the head. Percentage tail DNA produces a linear response but it too depends on the sensitivity of the fluorochrome used. The tail moment is one of the more commonly used units of expression. This shows a curved response and is defined as the product of the tail length and the fraction of the total DNA in the tail (tail moment = (% DNA in tail x tail length)/100). The tail moment incorporates a measure of both the smallest detectable size of migrating DNA (tail length) and the number of relaxed/broken pieces (intensity of DNA in the tail)(Fairbairn *et al.* 1995). However, the tail moment alone for inter-laboratory comparison is meaningless. The extent of tail moment reflects the extent of the tail multiplied by the % DNA in the tail. This aims to allow for the decrease in the sensitivity at the tail end, as there is limited detection of the camera and the human eye there. The olive tail moment measures the center of gravity of the head and the center of gravity of the tail. This can be considered the best tool for DNA damage detection as it involves a separate integration of the head and the tail. Another new method of analysis is the tail profile; the formula used is integrated form of the tail moment. Mathematically, the tail profile allows for a more accurate quantification of the DNA damage by averaging the intensity of each column of pixels generated by the digital image and multiplying it by the distance from the center of the head to that column, and then

dividing by the total intensity. The use of this integrated formula allows for the calculation to account for subtle changes in the tail's distribution (Bowden *et al.* 2003). Ejchart and Sadley-Sosnowska proposed another method for critical evaluation of the comet assay. The method was based on representing frequency histograms of tail moments and % DNA in the tail with the use of the unsymmetrical Weibull distribution. The distribution is fully characterized by two parameters: shape (α) and scale (β).Coefficients of variation and confidence intervals of the parameters have been determined. They also suggest that shifting of probability density plots could also serve as a qualitative visualization of DNA damage (Ejchart and Sadlej-Sosnowska 2003). Others would argue that for the tail moment, the mean of the log-transformed data is clearly superior to other measures, whereas the mean of the raw data without transformation can lead to very inefficient analyses. The 90th percentile, capturing the upper tail of the distribution, performs well for the tail length, with a slight improvement obtained by applying a log transformation prior to calculations. Other recommendations are that a suitably designed linear model (ANOVA) provided a relatively simple yet efficient means of detecting treatment effects and important factors can be accounted for within the model. It is also recommended that the model include factors such as electrophoresis and other experimental variables(Wiklund and Agurell 2003). However, these methods are still at the developmental stage and are far from widespread in their usage.

Other conditions that can effect the results obtained from a comet assay are the temperature of storage of the cells, work on cell cultures of V79 cells have shown no difference between storage of the cells 24 hours prior to processing at +4, -20, -80 or –198 degrees centigrade. Longer storage (48h or 168h) resulted in a significant increase in the length of DNA migration and decrease in cell viability, -80^{0}c was better for longer storage periods (Zhang *et al.* 1998). The temperature during the alkaline treatment and electrophoresis can also exert an effect. V79 Chinese hamster cells were analysed using 2 different temperatures. The

findings suggested that room temperature strongly enhanced the DNA migration compared with 4 degrees centigrade (Speit *et al.* 1999). To reduce or minimize experimental variation, the comet assay should be performed in a standardized manner. Variation can occur between electrophoresis sessions and also inter-experimenter if slides are analysed by more than one individual. The use of positive and negative controls has been examined, the use of a negative control (untreated slide) seems to be justifiable in most cases and the authors concluded that the internal standard (in their case untreated and EMS exposed K562 cells) was stable across electrophoresis sessions and among experimenters and the formula in which the measurements of each electrophoretic run are recalculated against the concurrent negative standard was suggested as a useful calibration model for the alkaline comet assay (De Boeck *et al.* 2000).

The comet technique is constantly being improved and other aspects of the assay have evolved. There has been an initial step made towards expanding the technique to look at different cell populations (Godard *et al.* 2002). In this technique a specific labeling of natural killer Kurloff cells with immunomagnetic beads, which are visible as grey-dull spheres against the bright-red staining of nuclear origin on the comet preparation of tumoral L2C target cells. The use of such labeled effector cells suggests the potential of the comet assay to visually identify different cell populations. Another new method involves the use of TdT-mediated fluorescein-cUTP nick end labeling (TUNEL) method. The labeling protocol revealed clumps and / or a series of stripes in the comet tail perpendicular to the direction of the electrophoresis; these sites may have accounted for the substructure seen in conventional comet assays. In a second variation, an opaque disk was passed into a field-con-charged-coupled device (ICCD) camera to gain an increase to a single photon detection level so that low levels of DNA damage in the tail could be revealed, this offered an improvement in sensitivity of the assay (Kindzelskii and Petty 1999).

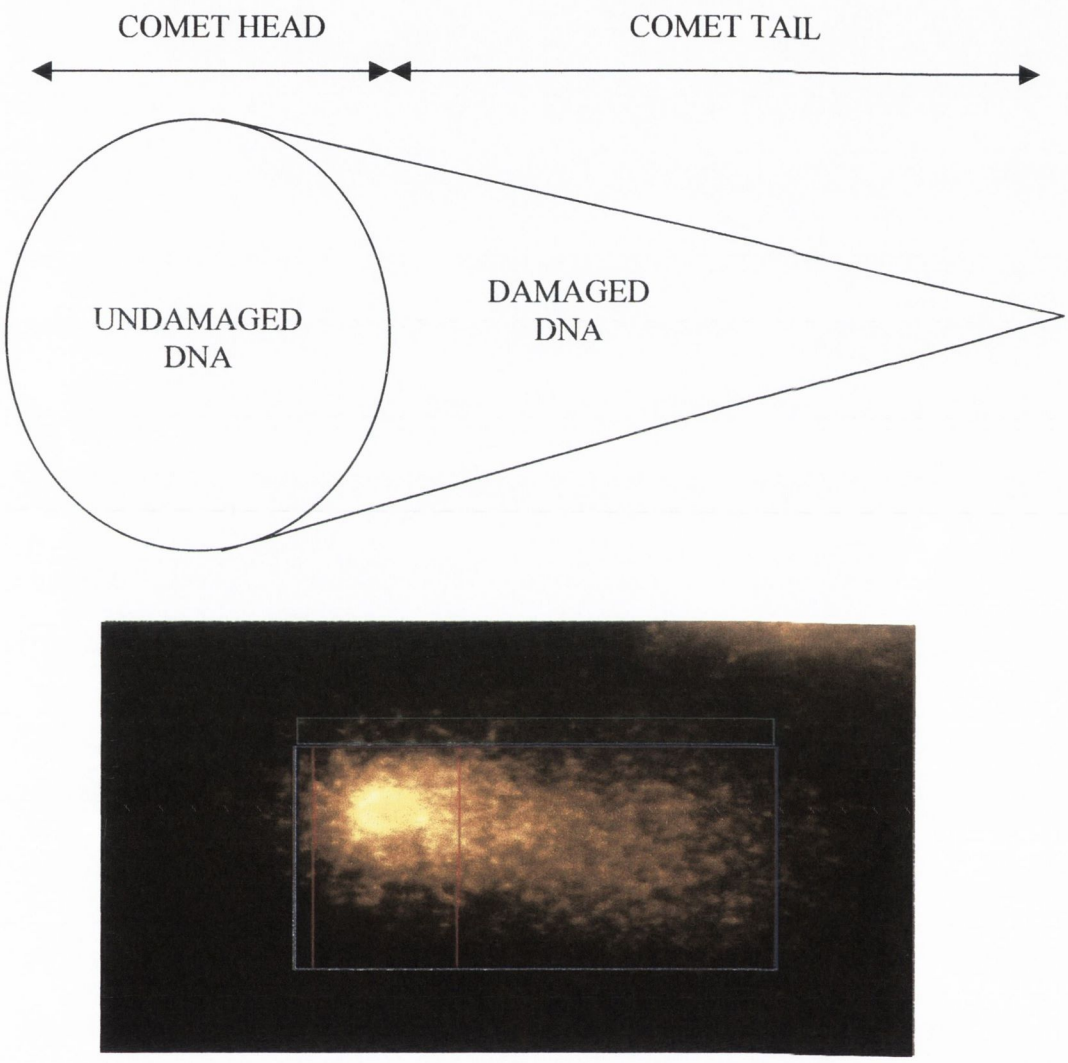


Figure 1.ix Diagramatic representation and actual photograph of a single cell Comet result. The comet head displays the region of undamaged DNA, while the tail shows the region of damaged DNA.

ii. Applications

The comet assay has numerous uses, one of which is in human biomonitoring and the assessment of genetic damage in exposed populations. The assay enables the detection of various forms of DNA damage in individual cells with ease and speed, and is therefore, well suited to the analysis of a large group in a population. Molecular biomarkers have been used in epidemiological studies whereby biomarkers of DNA damage or mutation are measured in subjects whose diet is supplemented with protective substances should be a reasonable alternative. In most reports in which bioavailability of antioxidants was determined, a significant increase was found in the supplemented compared to the placebo groups (Anderson *et al.* 1997;Pool-Zobel *et al.* 1998;Duthie *et al.* 1996;Hartman *et al.* 1995;Duthie and Dobson 1999). However, only two studies were able to show significant correlations between serum concentrations of the anti-oxidant and the levels of protection (Duthie *et al.* 1996;Hartman *et al.* 1995). Jenkinson and co-workers have been able to show that increasing dietary levels of vitamin E by 80mg/day ameliorated the damaging effects of polyunsaturated fatty acids through using the comet assay on human volunteer lymphocytes (Jenkinson *et al.* 1999).In a recent study Collins *et al.* looked at oxidation status, DNA repair and antioxidant levels in a randomized cross-over study on healthy humans given a supplement of kiwifruit (a naturally rich source of vitamin C) for 3-week periods at different "doses" with 2-week washout periods between doses. Endogenous oxidation of bases in lymphocyte DNA and the resistance of the DNA to oxidation *ex* vivo were assessed. The capacity to repair DNA base excision was measured with an *in vitro* method, a modified version of the comet assay. The assay depends on the incubation of extract with substrate DNA comprising gel-embedded nucleoids from cells treated previously with a specific DNA-damaging agent. The incision rate was estimated as the increase in comet score from 0 to 10 min of incubation (Collins *et al.* 2001). The level of expression of repair-related genes 8-oxoGua DNA glycosylase 1 and AP endonuclase 1 was

assessed by semi-quantitative real time PCR (RT-PCR). Concentrations of dietary antioxidants were measured in plasma. The antioxidant status of plasma and lymphocytes were increased by consumption of kiwifruit. Levels of endogenous oxidation of pyrimidines and purines in DNA were markedly decreased and DNA repair was substantially increased (Collins *et al.* 2003). Studies have looked at the effects of different diets on genotoxicity of human faecal water. Results from one such study showed that diets high in fat and meat but low in dietary fibre increased the genotoxicity of faecal water on HT29 colonic adenocarcinoma cell line (Rieger *et al.* 1999). Dietary flavinoids have also been examined using human colonocyte cell lines (Caco-2) and found that flavinoids, in particular quercetin which is the most abundant one in the diet, were likely to be important in defending human colonocytes against oxidative attack (Duthie and Dobson 1999). The comet assay has been applied to a number of clinical studies to investigate the consequences of certain physiological conditions or therapeutic exposure to chemicals at the cellular level. Oxidative stress as a result of hyperglycemia, local tissue damage or altered antioxidant status were incriminated as causes of increased damage in diabetic patients (Tangiguchi *et al.* 1996). The role of reactive oxygen species in chronic renal failure patients on dialysis and the subsequent effect of supplementation with vitamin E were examined. The DNA breakage observed in the lymphocytes of patients pre-supplementation was significantly higher than in the control group but the protective effect of the supplementation was only seen after 14 weeks of supplementation (Kan *et al.* 2002). The comet assay has also been employed to assess the effect of a radiotherapy or chemotherapy regimen (Gutierrez *et al.* 1997). A large interest has been shown in the area of biomonitoring occupation, lifestyle and environmental exposure to genotoxins as comet assays can identify low levels of exposure with great sensitivity (Lebailly *et al.* 1998). The levels of normal DNA damage in a healthy Indian population has been assessed, the extent of damage was significantly different in smokers versus non-smokers as well as vegetarians and non-

vegetarians. Significant differences were seen regarding the extent of smoking in smokers and an age-dependent effect was also observed (Dhawan *et al.* 2001). A recent comet study looking at oxidative stress in isolated gastric epithelium cells was able to show that the damage accumulates in older and more superficial cells (Everett *et al.* 2000). Another adaptation of the alkaline comet assay has been its use alongside bromodeoxyuridine labeling of DNA to examine the replicative integrity of small colonic biopsies, the preliminary results of which indicate that alterations in replicative integrity are an early event in neoplasia and suggested a possible use of the method in trials to investigate diet or environmental factors that may effect the health of the colon (McGlynn *et al.* 2003).

iii. Bromodeoxyuridine (BrdUrd) Comet Assay

BrdUrd sterically resembles thymine. Through the electronegative influence of the Bromine atom, BrdUrd frequently assumes a tautomeric form that base pairs with guanine instead of adenine. The Van Der Waals radius of the bromine atom makes it an excellent thymine analogue as it has a very close radius to that of the methyl group one. Bromine has a much heavier molecular weight than the methyl group; therefore it imparts an increased density to the substituted DNA and provides a physical basis for separating replicating from non-replicating DNA.

The combination of the alkaline comet method with pulse-labelling of replicating DNA with the thymidine analogue BrdUrd and the immunolocalisation of BrdUrd within the heads and tails of comets, allowed the assessment of replicative integrity on a single cell basis regarding the efficiency of completion of DNA replication (McGlynn *et al.* 1999). The method involved incorporating a label during a brief pulse of BrdUrd, which was initially close to strand discontinuities and could then be seen as a fluorescently labeled comet tail upon electrophoresis. The % BrdUrd in the tail indicated the proportion of recently replicated,

imperfectly matured DNA in the nucleus. In pulse-chase experiments, as the replicating forks moved away from the labeled regions, the label was incorporated into high molecular weight, continuous DNA and was then observed in the head of the comet. The assay has been shown to be suitable as an *ex vivo* molecular end point that can be easily measured in colonic epithelial cells collected through punch biopsy from a routine colonoscopy procedure and that it has the potential to facilitate trial investigations of diet or environmental factors that may affect the replicative integrity of the colon and it was a novel biomarker for colorectal carcinogenesis (McGlynn *et al.* 2003).

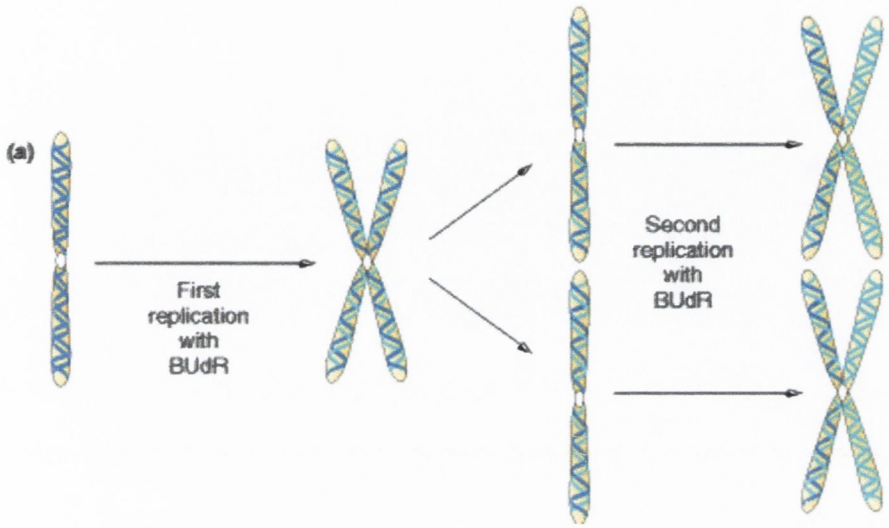


Figure 1.x A diagrammatic representation of harlequin chromosomes. In this procedure, the chromosomes go through 2 rounds of replication in the presence of BrdUrd/BudR, which replaces thymidine in the newly synthesised DNA. The chromosomes are then stained with a fluorescent dye and stain to produce the appearance shown (the light blue lines represent the BrdUrd-substituted strands) (Griffiths *et al.* 2000)

iv. Uracil Misincorporation Comet Assay

Genes methylated at specific locations are either not transcribed or are transcribed at a reduced level of expression. Decreased DNA methylation has been associated with some forms of cancer. Folate deficiency may deplete cellular S-adenosylmethionine (SAM) levels causing DNA hypomethylation and inappropriate activation of proto-oncogenes. Under conditions of folate depletion, a block in the methylation of dUMP to TMP leads to an increase in the cellular levels of deoxyuridine triphosphate and uracil misincorporation into the DNA molecule in the place of thymidine. Normal DNA repair processes remove the uracil. However, if conversion of dUMP to thymidine was continually limited by folate availability, uracil was misincorporated and removed in a catastrophic repair cycle, which may ultimately have induced double strand breakage, chromosome instability and cancer (Reidy 1987;Blount and Ames 1995).

By using the bacterial repair enzyme uracil DNA glycosylase, a novel variation of the comet assay has been developed to detect uracil misincorporation in human DNA specifically (Duthie and McMillan 1997). The effect of perturbing folic acid and deoxyuridine levels on uracil misincorporation in normal lymphocytes and cultured HeLa human tumour cells was examined by incubating the cells as agarose-embedded nucleoids (which contained low levels of uracil in their DNA) with 1 unit of uracil DNA glycolyase per microgram of DNA. The cells were grown in folate-deficient medium were found to be growth arrested. Incubating cells in folate-deficient medium showed a significant increase in the level of uracil detected compared with control cells. HeLa cells showed an increase in non-specific DNA damage. Deoxyuridine significantly increased the level of uracil detected in the DNA of both folate-deficient and control HeLa cells. This approach has been used by the same research group to look at folic acid depletion in human lymphocytes *in vitro* and its relationship with DNA instability (strand breakage, uracil misincorporation and defective

repair). They found that folate supply had no effect on the level of oxidized pyrimidines in lymphocyte DNA suggesting that folate deficiency increases uracil misincorporation relatively specifically. The results also helped determine the mechanism(s) through which folic acid maintains DNA stability (Duthie and Hawdon 1998). More recent work has looked at the role of moderate folate deficiency with DNA stability and oxidative base repair in the lymphocytes of Hooded Lister rats. Lymphocyte strand breakage increased successively over the time period of ten weeks but low folate status specifically and progressively induced uracil misincorporation throughout the study. Neither folate deficiency nor choline/methionine deficiency altered the DNA oxidative base damage (Duthie *et al.* 2000a). A study characterizing uracil DNA glycosylase activity and protein expression in a panel of 20 colorectal tumours and 6 colorectal cancer cell lines found that uracil DNA glycosylase activity was statistically higher in tumour tissue compared with normal bowel (Dusseau *et al.* 2001).

v. Fluorescent *In Situ* Hybridisation (FISH) Comet Assay

The idea of using tritiated thymidine probes to mark the cellular position of DNA, *in situ* hybridization to metaphases as target DNA, and probe detection using autoradiography, was exploited in the 1960's. Tritiated thymidine has been replaced by fluorochrome-labelled nucleotides, which can be used as fluorescent probes and be visualized by fluorescence microscopy. The FISH-comet assay uses fluorescently labeled DNA probes that hybridise to a specific gene sequence. Thus, the localization of a particular gene within a comet cell population can be established, offering the opportunity to follow gene-specific repair in response to DNA damage. The position of the fluorescent hybridization spots in the comet head or tail indicates whether the sequence of interest lies within or in the vicinity of a damaged region of DNA. The combination of the comet assay with FISH offers an excellent

opportunity to determine the exact location of DNA sequences of interest within a given cell. In particular, the FISH-comet is well suited to the study of repair relating to gene specific sequences following DNA damage. However, few studies have employed this technique. Previous studies have looked at using the FISH-comet to identify damaged and repaired DNA sequences in individual cells in bladder carcinoma (McKelvey-Martin *et al.* 1998), to detect chromosomal damage in response to UV-radiation (Bock *et al.* 1999;Menke *et al.* 2000) and to look at genetic damage in oligozoospermic males (Schmid *et al.* 2003). The method has also been used in primary human colon cells from surgical samples to look at putative risk factors for damage to global DNA and *TP53*. *TP53* is involved in the final mutations necessary to convert an adenoma to carcinoma. Migration of *TP53* signals into the comet tails were quantified, this proved to be a more sensitive measure than global DNA for genotoxicity induced by *trans*-2-hexenal and hydrogen peroxide (H_2O_2) (Schaeferhenrich *et al.* 2003b). Another study looked at the effect of oxidative stress and resulting lipid peroxidation in colonic cells by using 4-hydroxy-2-nonenal (HNE) and H_2O_2 on a recently established cell line from a differentiated microadenoma LT97 (it represents cells from frequent preneoplastic lesions in the colon). The results showed after HNE incubation, *TP53* migrated more efficiently into the comet tail than the global DNA, which suggests a higher susceptibility to HNE (Schaeferhenrich *et al.* 2003a).The FISH-comet for *TP53* was developed by McKenna and co-workers to demonstrate repair of this region in 2 human bladder carcinoma cell lines (McKenna *et al.* 2003).

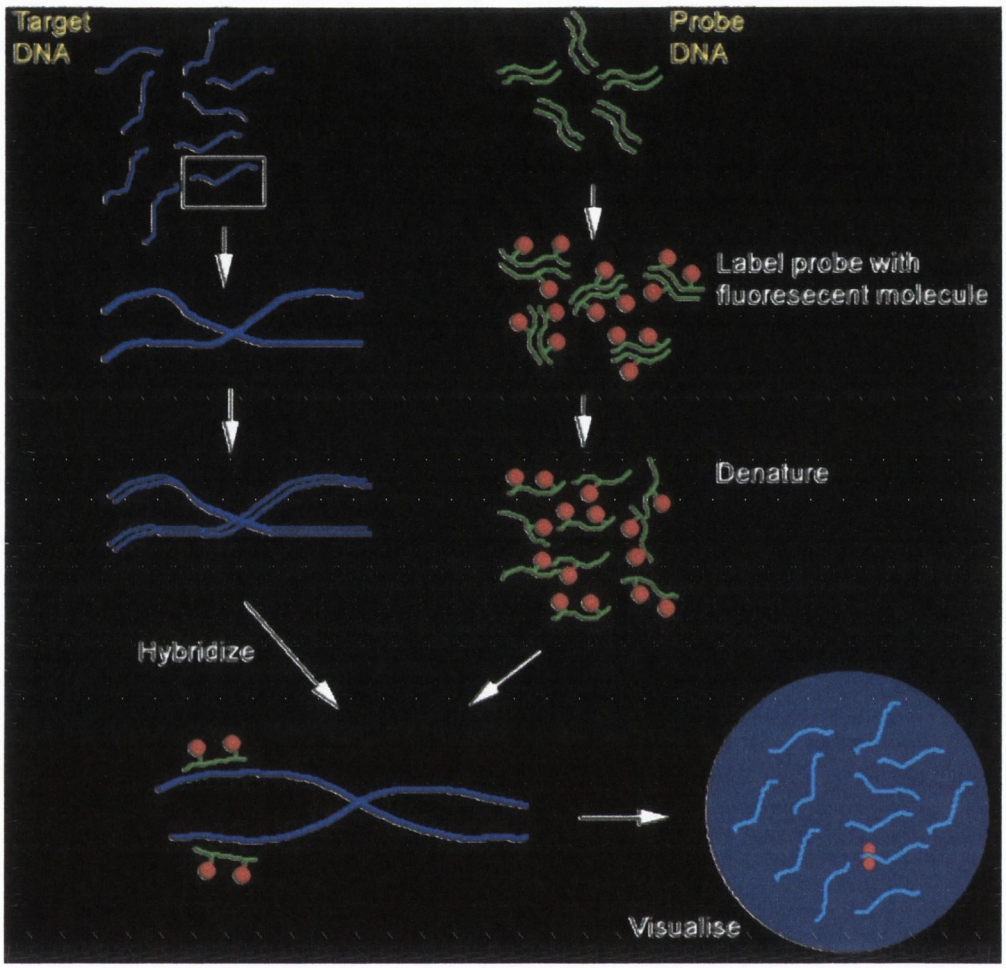


Figure 1.xi Diagrammatic representation of Fluorescent *in situ* hybridization steps involved in the FISH-Comet assay (Griffiths *et al.* 2000).

4. HOMOCYSTEINE AND DISEASE

i. Background

Methionine is an essential amino acid that is converted to S-adenosylmethionine (SAM) by methionine adenosyltransferase. S-adenosylmethionine is the main methyl donor. S-adenosylhomocysteine (SAH) is formed after donation of the methyl group. SAH is hydrolysed to homocysteine. Homocysteine (Hcy) can either be remethylated to methionine or transsulphurated to cysteine via cystathionine. The enzyme methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase) uses folate, in the 5-methyl-tetrahydrofolate form, as a methyl group donor for the remethylation of Hcy to methionine. Vitamin B_{12} (methylcobalamin) is involved in this remethylation reaction as a co-factor. Betaine-homocysteine methyltranferase is an enzyme involved in an alternative remethylation pathway. Betaine (trimethylglycine) is formed in cells as an oxidation product of choline and can be obtained externally from foodstuffs e.g. spinach, beets. The physiologic amount of betaine in man varies in different tissues. The concentration in human serum is 20-60μmol / L, whereas the kidney and liver greater concentrations can be found (Lever *et al.* 1994;Sakura *et al.* 1998). Betaine is converted to demetylglycine and serves as a methyl donor for the remethylation from Hcy to methionine by betaine-homocysteine methyltransferase. The latter enzyme is mainly found in the liver and only minor activity has been shown in the kidneys and adrenal glands (Finkelstein 1990). Vitamin B_6 is a co-factor in the transsulphuration of Hcy to cysteine. Hcy can first condense with serine to form cystathionine by the B_6-dependent enzyme cystathionine β-synthase (CBS). This reaction is irreversible. Cystathionine can then further metabolized to cysteine by the B_6-dependent enzyme γ-cystathionase (Ueland *et al.* 1993a).

A recent study looked at the effect on growth of isogenic wild-type and CBS deficient yeast in response to Hcy and SAH. CBS deficient yeast exported significantly more Hcy into

the media than the wild-type and had elevated internal pools of Hcy and SAH. Measurement of the intracellular SAM/SAH ratios indicated that CBS deficient yeast had reduced ratios relative to the wild-type and the ratio was further reduced by adding SAH to growth media. The growth inhibition was reversed by the addition of SAH; this indicated that it is the ratio and not the absolute level that is critical for cell growth. The overall results suggest that CBS plays a key role in the regulation of the SAM/SAH ratio inside the cell and that excessive perturbations of this ratio can inhibit growth so that elevated extracellular Hcy may reflect an altered intracellular SAM/SAH ratio (Christopher *et al.* 2002).

Serine is derived from diet and is biosynthesized from glycolysis via 3-phosphateglycerate. Serine plays a central role in intermediary metabolism as a contributor to phospholipid, sphingolipid and cysteine biosynthesis and as a primary source of glycine. As a consequence of its role in glycine production, serine also is the major donor of folate-linked one-carbon units, which are used in the biosynthesis of purines and 2'-deoxythymidine 5'-monophosphate and the remethylation of homocysteine to methioinine. The freely reversible interconversion of serine and glycine is catalysed by serine hydroxymethyltransferase (SHMT), a reaction that is both folate and pyridoxal 5-phosphate dependent (Schirch 1984).

In serum or plasma, Hcy exists in several interchangeable forms, among which protein-bound Hcy predominates followed by low molecular weight mixed disulfides. Only trace amounts of the reduced thiol have been detected. These Hcy forms are collectively measured as tHcy after quantitative reduction (Ueland 1995). A plethora of methods for tHcy have been published (Ueland *et al.* 1993b;Ubbink 2000). The methods will express the final value as μmol/L. 5-15μmol/L has been generally considered a normal fasting plasma level, 16-30μmol/L seen as moderately high while 31-100μmol/L has been seen as intermediate hyperhomocysteinaemia and > 100μmol/L severe hyperhomocysteinaemia (Malinow *et al.* 1999). It has been estimated that about 20% of the population have elevated Hcy levels.

Blood sampling and processing are important in obtaining reliable tHcy values(Ueland *et al.* 1993b). The release of Hcy from blood cells is time- and temperature-dependent, which in adults can cause an increase of about 10% per hour at room temperature. Therefore, it has been recommended that blood samples are put on ice before centrifugation or that the plasma or serum fraction must be separated within 30 minutes. Alternative strategies have been reported, these include blood sampling into acidic citrate (Willems *et al.* 1998), sodium fluoride (Ubbink *et al.* 1992) or 3-deazaadenosine (al-Khafaji *et al.* 1998). Preventing an artificial increase in tHcy has been paramount in research settings in the establishment of reference values or investigating associations between tHcy and disease.

Prandial status has also been identified as an important factor that must be considered. The Hordaland Homocysteine Study was used to examine this variable to see if it impacted on tHcy results. They found that in all groups, except older women, tHcy concentrations were generally higher with increasing time after a meal ($P < 0.01$). The findings suggested that fasting status and time since last meal be considered as it influences results and should be taken into account (Nurk *et al.* 2001).

Apart from genetic factors there are a number of other factors that can influence tHcy levels. These include:

a. Age

Hcy increases with age. This could be explained by a decrease in the production and/or the metabolic activity of the enzymes regulating its metabolism (Ubbink 1994). Furthermore, the bioavailability of vitamin B_6, B_{12} and folate, which function as co-factors, decline with aging (Selhub *et al.* 1993).

b. Gender

Hcy levels are generally higher in men than pre-menopausal women; this can be explained by the higher muscle mass in males. The rise in tHcy seen in women

postmenopausaly can be explained by hormonal differences (mainly 17 β-oestradiol) and oestrogen may also play a role in the lower levels seen before the menopause. Genetic defects that impact on Hcy metabolism are CBS deficiency, 5,10-methyltetrahydrofolate reductase (MTHFR) deficiency or methionine synthase deficiency.

c. Smoking

Smoking has been associated with higher Hcy and the effect seems to be in a gradient (Nygard *et al.* 1995). The exact mechanism has yet to be uncovered but apart from a direct action, smokers tend to have lower levels of folate as well as vitamin B_6 and B_{12}.

d. Medications

A number of mediations influence Hcy levels. Most will interact with folate or vitamin B_6. Methotrexate, carbamazepine and phenytoin have been shown to interact with folate while azaribine and similar drugs interact with B_6. Other drugs that interact with Hcy are corticosteroids, cholestyramine, colestipol, niacin, theophylline, oestrogen-containing oral contraceptives, metformin and cyclosporin A.

e. Diseases

Certain disease states can affect Hcy metabolism. Pernicious anaemia, atrophic gastritis, malabsorption syndromes can all decrease the availability of essential co-factors. Renal failure, hypothyroidism, psoriasis and malignancies can also influence Hcy metabolism.

f. Diet and Alcohol

Different components of the diet are thought to influence Hcy status. Coffee drinking has been reported to increase tHcy (Stolzenburg-Solomon *et al.* 1999b;Nygard *et al.* 1997b). A recent observational study showed a positive association between caffeine intake and Hcy concentrations (Jacques *et al.* 2001). Caffeine has been shown to be partly responsible for the Hcy raising effect. Coffee, but not caffeine, affects Hcy metabolism within hours after intake, although the effect was shown to be still substantial after an overnight fast (Verhoef *et al.*

2002). Chlorogenic acid, a major polyphenol found in coffee, was consumed in amounts of up to 1g per day in coffee drinkers. This compound has also been found to raise tHcy concentrations in plasma. This could be partly responsible for the higher Hcy values seen in this group (Olthof *et al.* 2001). Black tea has been investigated as it has been reported in some studies to increase tHcy (Olthof *et al.* 2001) while in others to form an inverse relationship (McPartlan *et al.* 1992) but overall the results have been inconclusive (Hodgson *et al.* 2003).

Alcohol must be considered when looking at tHcy. Recently there has been the suggestion that there is a J-shaped relation between alcohol consumption and tHcy. Koehler *et al* studied a cross-section of 278 healthy elderly adults in New Mexico and found that when the data was broken down into quartiles, tHcy was significantly higher in those consuming >2 drinks/day than those consuming <1 drink/day. This relationship suggests a tHcy-lowering mechanism for cardiovascular protection with moderate alcohol consumption. This relationship is supported by the Nurses' Health Study, which showed that the protective effect of a high folate diet was augmented in those consuming ≤2 alcoholic drinks per day (Rimm *et al.* 1998).A common finding among excessive alcohol consumers generally show marked elevations in tHcy (Cravo and Camilo 2000), this is thought to be due to the effects of alcohol on the methionine cycle (Trimble *et al.* 1993;Halsted *et al.* 1996).

It has also been suggested that the type of alcohol will exert an effect. In an experimental study (van der Gaag *et al.* 2000) showed that red wine and spirits increased tHcy but beer consumption had no such effect. It has been suggested that it was the vitamin B_6 ,B_{12} and folate content of beer, which are essential co-factors in the metabolism of Hcy that may be counteracting the possible detrimental effect of alcohol on Hcy. Studies that have been able to show an inverse association with Hcy have been predominantly beer-drinking populations (UK and Netherlands). Betaine is a methyl donor and in sufficient amounts can decrease Hcy (Mar and Zeisel 1999), wine has been shown to contain around 10mg betaine/L but this is probably

too low to counteract the negative effects of alcohol. The French Supplementation with Antioxidant Vitamins and Minerals Study examined the relation between Hcy and the consumption of different alcoholic beverages. They found that wine consumption may increase tHcy, whereas beer showed no true effect (Mennen *et al.* 2003).

ii. Disease Associations

Total plasma homocysteine (tHcy) is associated with mortality. In a prospective study looking at 2127 men and 2639 women aged 65-67 years in 1992-1993 from the Hordaland County, Norway, 162 men and 97 women died during a median 4.1 year of follow-up. Those in the quintile group with a concentration of <9.0μmol/L had a highly significant risk of mortality from non-vascular and cardiovascular causes. In other groups, an increase of 5μmol/L in tHcy was associated with an increase of 49% in all-cause mortality, 50% increase in cardiovascular mortality, 26% increase in cancer mortality and 104% increase in non-cancer, non-cardiovascular mortality. Thus, tHcy can be seen to be a strong predictor of mortality (Vollset *et al.* 2001). The value of tHcy as a prognostic value has previously reported in the United States and Israel (Bostom *et al.* 1999b;Kark *et al.* 1999).

a. Cardiovascular Disease

Hcy's significance was recognized in the 1960's in children with a genetic disorder of the enzymes that regulate Hcy metabolism called homocystinuria. High levels of Hcy are detected in the bloodstream as well as the excretion of Hcy metabolites in the urine. This disorder was associated with severe atherosclerosis and thromboembolism (Mudd *et al.* 1995). However, it was only in the last 10 years that it became appreciated that mild elevations in Hcy were associated with vascular disease. Hyperhomocysteinaemia can result from a deficiency in CBS, methionine synthase, MTHFR or a deficiency in folate, vitamin B_6 and B_{12}. Many epidemiological studies have shown that an elevation in tHcy as an independent risk factor for

coronary artery disease (CAD), peripheral vascular disease (PVD) and venous thrombosis (Refsum *et al.* 1998;Mangoni and Jackson 2002).The familial role of elevated tHcy has been examined within a study of twins (Berg *et al.* 1992), family members (Franken *et al.* 1996)and by the frequency of finding hyperhomocysteinaemia in patients with early CAD (Hopkins *et al.* 1995). This data suggests that hyperhomocysteinaemia can be a familial trait and this may contribute to cardiovascular disease (CVD).

Most cross-sectional and retrospective studies have found an association between tHcy and cardiovascular risk. A meta-analysis of 27 observational studies involving approximately 4000 subjects showed that hyperhomocysteinaemia was associated with an increased risk of fatal and non-fatal coronary heart disease (CHD)(Boushey *et al.* 1995). More recent studies have also reported an association between hyperhomocysteinaemia and CAD (Graham *et al.* 1997). The deleterious effects of Hcy include acute and chronic endothelial dysfunction, arterial intimal-mediated thickening through stimulation of vascular smooth muscle cell growth and collagen synthesis, wall stiffening, elevated plasma triglycerides, increased plasma peroxidation, oxidation of low-density-lipoproteins, decreased antioxidant activities, increased fibrinogen levels and a procoagulant activity by inhibiting several pathways of coagulation.

Prospective cohort studies have shown conflicting results. Twenty studies have reported statistically significant associations between hyperhomocysteinaemia and CVD. In the Physicians' Health Study, the adjusted relative risk of fatal and nonfatal myocardial infarction (MI) was 3.4 (95% CI 1.3-8.8, P=0.01) in subjects whose tHcy was in the highest 5% compared to those in the lowest 90% (Stampfer *et al.* 1992). A British study found an independent association between hyperhomocysteinaemia and the risk of stroke (Perry *et al.* 1995). Another British study found that the relative risk of fatal CHD was 2.9 (95%CI 2.0-4.1) among men whose tHcy levels were in the highest quartile compared to those in the lowest (Wald *et al.* 1998). Two studies in the Netherlands have reported a positive association

between Hcy levels and the risk of MI and stroke (Stehouwer *et al.* 1998;Bots *et al.* 1999). Nygard *et al* have reported a strong relation between tHcy levels and mortality in patients with angiographically proven CAD, with a mortality ratio of 4.5 (95%CI 1.2-17) for patients with the highest Hcy compared with the lowest (Nygard *et al.* 1997a). In a similar study, Hcy levels predicted mortality, independently of traditional risk factors, C-reactive protein and 5,10 MTHFR genotype (Anderson *et al.* 2000). Recently, in a case-control study of 30 CVD patients, levels of folate, tHcy, SAM, SAH, vitamin B_{12} and creatinine were measured. Plasma SAH appeared to be a much more sensitive indicator than tHcy in demonstrating the difference between the case and control groups (Kerins *et al.* 2001). Another aspect of CVD is the area of transient ischemic attacks and stroke (Bostom *et al.* 1999a;Fallon *et al.* 2003). The number of studies in this area is growing, the evidence pointing towards elevated tHcy being a strong predictor of increasing the risk of stroke or transient ischemic attacks. However, the inverse relationship between tHcy and risk of CVD has not been corroborated in some studies (Verhoef *et al.* 1994;Folsom *et al.* 1998;Fallon *et al.* 2001). Recently, the effect of folic acid grain fortification has been examined against the background of reduced homocysteine and raised folate status of the general population and its effect on CVD risk. The fortification has made an impact on supplementation trials as in one study patients were exposed to high doses of folic acid only resulted in very modest reductions in mean fasting tHcy (Bostom *et al.* 2002). Another study looked at the risk of stroke and transient ischemic attacks and low vitamin status, they found low vitamin B_6 but not tHcy was strongly associated with cerebrovascular disease in a post-fortification folate-replete sample of 320 case-control subjects (Kelly *et al.* 2003).

b. Cognition, Dementia and Alzheimer's Disease

A potential influence of hyperhomocysteinaemia on cognitive functioning and dementia has been postulated. Clarke and colleagues compared patients with histologically confirmed

Alzheimer disease (AD) with age-matched healthy control subjects. They found that individuals who had elevated serum Hcy concentrations >14μmol/L were 4.5 times as likely to have had AD (as confirmed by post-mortem histologic analysis) as those with low serum Hcy <11μmol/L (Clarke *et al.* 1998).Similar observations have been made in other studies (Nilsson *et al.* 1996;Joosten *et al.* 1997), including the recent finding that baseline Hcy concentrations predicted the risk of incident AD and dementia over an 8 year period in the Framingham Heart Study population (Seshadri *et al.* 2002). It remains unclear whether Hcy is a causative factor or simply a marker of coexisting vascular disease in AD patients (Miller *et al.* 2002). Significant correlations have been observed between Hcy concentrations and indices of cognitive function in case-control studies of patients with psychogeriatric conditions (Bell *et al.* 1992;McCaddon *et al.* 1998;Lehmann *et al.* 1999) and in cross-sectional, population-based studies of community dwelling older adults (Budge *et al.* 2000;Duthie *et al.* 2002;Miller *et al.* 2003;Ravaglia *et al.* 2003). Two studies, however, have found no significant associations between Hcy concentrations and cognitive function (Kalmijn *et al.* 1999;Ravaglia *et al.* 2000).

iii. MTHFR and Related Polymorphisms

SAM controls the commitment of one-carbon units to methylneogenesis or nucleotide synthesis through its effect on MTHFR activity. Under conditions of low SAM, MTHFR is stimulated to direct the pool of one-carbon units towards methylneogenesis. In contrast, elevations in SAM inhibit MTHFR activity, thus increasing the availability of one-carbon units for nucleotide synthesis. Dietary deficiency may also affect MTHFR directly (riboflavin bioavailability) or indirectly (folate, B_{12}, B_6, choline, methionine) by altering the cellular concentrations of the methioinine cycle intermediates.

Over recent years the enzyme methylenetetrahydrofolate reductase (MTHFR) has received much attention because of the presence of a common polymorphism within the

general population. This polymorphism is a thermo-labile enzyme variant that results in individuals who possess one or two T alleles have 30% and 65%, respectively, lower MTHFR activities in vitro than that of the CC genotype. It is inherited as an autosomal recessive trait and has been identified as a 677C→T mutation resulting in an alanine to valine substitution (Frosst *et al.* 1995a). The MTHFR enzyme, which is FAD dependent (riboflavin coenzyme), provides the 5-methyl THF necessary for the vitamin B_{12}-dependent remethylation of homocysteine to methionine. Recent studies have supported a role for riboflavin in the determination of MTHFR particularly in homozygotes (Hustad *et al.* 2000;McNulty *et al.* 2002;Jacques *et al.* 2002). Thus MTHFR as a critical enzyme it can direct the folate pool towards homocysteine remethylation at the expense of DNA and RNA biosynthesis. The prevalence of the MTHFR 677C→T variant is related to ethnicity and will vary within population groups. The frequency of homozygosity for the T allele (abnormal TT genotype) is approximately 10-15% in the UK (Schneider *et al.* 1998). Analysis of Caucasian and Asian populations typically show rates of ~12% for homozygosity and up to 50% for those who are heterozygotes (Brattstrom *et al.* 1998;Frosst *et al.* 1995a;Brattstrom *et al.* 1998). The allele frequency for sub-Saharan Africans is 0.07, 0.06 for Canadian Inuits, 0.24-0.54 in whites, Japanese and Chinese (Gudnason *et al.* 1998;Hegele *et al.* 1997;Pepe *et al.* 1998). There has also been a North-to-South increase in allele frequency seen in Europe (Botto and Yang 2000). An association between the 677T allele and 535G in intron 2, 31T in intron 6, 1298A in exon 7 and 262C in intron 10, this association between the 677T allele and the G-T-A-C haplotype may have stemmed from a founder effect, a strong selective advantage or both. Looking at population distributions, it has been hypothesized that MTHFR homozygosity conferred a survival advantage in populations with adequate folic acid consumption, which may explain the current variability in its prevalence in different populations (Rosenberg *et al.* 2002).

The concentrations of erythrocyte folate, used as an index of body stores, varies according to MTHFR 677C→T genotype. It also appears to be related to the type of folate assay used. This relationship was explained by the different response of the intracellular folate species to the various detection systems, there are inherent differences in response of the competitive binding of the radioassay and the microbiological assay (Molloy *et al.* 1998). It has been shown recently that formylated tetrafolate polyglutamates (~22% of erythrocyte folate) may accumulate in TT subjects at the expense of 5-methyl THF, the predominant form in CC and CT individuals. These formyl forms may cause re direction of carbon-one metabolism towards the more efficient maintenance of DNA repair under conditions of low folate status or intake, since they are associated with purine and pyrimidine synthesis than with methylation. However, it is clear that there is a lack of heterogeneity within TT individuals, as the percentage of formyl folate may vary from 0% to >60% (Bagley and Selhub 1998).

The effect of the MTHFR polymorphism on plasma Hcy and folates have been examined. In general, studies have found the TT genotype to have lower plasma folates then CT or CC individuals but at high plasma folate concentrations tHcy in TT's were as low as the other genotypes (DeBree *et al.* 2003;Harmon *et al.* 1996;Zittoun *et al.* 1998). In studies looking at how supplementation modulates MTHFR in relation to Hcy and folate response have been performed. The results of these suggest while all of the MTHFR genotypes respond to folic acid supplementation, the TT genotype may have a greater decrease in tHcy and may require higher amounts of supplementation to gain full benefit from the supplementation (Ashfield-Watt *et al.* 2002;Fohr *et al.* 2002;Guinotte *et al.* 2003).

Other "common" inherited enzyme polymorphisms intimately involved in deconjugation, transport and carbon-one metabolism include: a second MTHFR deficiency 1298A→C, methionine synthase deficiency 2756A→G, a vitamin B_2-dependent enzyme that regenerates methionine synthase (cystathionine β-synthase 844ins68), methionine synthase

reductase deficiency 66A→G, a vitamin B_{12}-dependent enzyme involved in the remethylation of Hcy to methionine (Silaste *et al.* 2001;Tsai *et al.* 2001;Wilson *et al.* 1999;Gaughan *et al.* 2001;Rady *et al.* 2003;Molloy 2002). There are also "common" polypmorphisms in the intestinal brush-border hydrolase folylpolyglutamate carboxypeptidase 1561C→T (Devlin *et al.* 2000) ,which resulted in a lowering effect of plasma folate and high plasma homocysteine, and the intestinal reduced folate carrier 80A→G (Whetstine *et al.* 2001), which predicted lower absorption. A single-nucleotide polymorphism recently identified to be associated with neural tude defect (NTD) incidence was the R653Q. This polymorphism is located on the trifunctional enzyme methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase, mothers of children affected by NTD were found to have an excess of the "Q" allele and QQ homozygotes were overrepresented in the NTD group compared with control individuals (Brody *et al.* 2002).

Although these polymorphisms have been implicated in the development of hyperhomocysteinaemia and some in the increase of risk of development of neural tube defects. It is not known to what exact extent they contribute to variance of folate levels and disease risk as studies in this area are thin on the ground and tend to have low numbers of participants. The linkage disequilibrium between C677T and A1298C polymorphisms in MTHFR has been examined in CRC to see if the combined heterozygosity conferred any effect on risk of developing CRC (Chen *et al.* 2002). The study concluded that the A1298C was a less substantial risk factor compared to C677T. Another study found the converse with the frequency of AA and AC genotypes was increased among hereditary CRC and no significance with C677T genotypes (Plaschke *et al.* 2003). The methionine synthase (MS) polymorphism was not associated with risk of developing colorectal adenomas (Chen *et al.* 1998a) in the Nurses Health Study. However, it was associated with lower risk of CRC in the Physicians Health Study, especially among those with low alcohol consumption, but the results should be

interpreted with caution as it had limited statistical power due to the low frequency of the MS variant genotype (Ma *et al.* 1999). Another study examined MS reductase A66G and found that the GG genotype was a risk factor for CRC in a small Japanese cohort (Matsuo *et al.* 2002).

iv. MTHFR and Disease

MTHFR C667T was found to be associated with increased plasma homocysteine and with possible increased risk of cardiovascular disease (Kang *et al.* 1991). A large body of evidence now exists to indicate that individuals with the TT genotype are prone to mildly elevated plasma tHcy levels compared with CT or CC individuals (Brattstrom *et al.* 1998;Gudnason *et al.* 1998;Harmon *et al.* 1999;Meleady *et al.* 2003b). A small increase in plasma Hcy concentration has been shown to be an independent risk factor for vascular disease (>~15μmol/L) and although the initial observation was that MTHFR could be an independent risk factor for vascular disease, the effect is now thought to be as a result of plasma Hcy and folate (Meleady *et al.* 2003a). The adverse effects of MTHFR related to elevated Hcy are thought only to be important in those with low folate or high alcohol intakes which creates an unstable environment that will have a more serious impact on the TT genotype (Jacques *et al.* 1996). A recent large meta-analysis of MTHFR C677T and CHD found homozygosity was associated with a 14% increase in CHD risk in European populations but not in North American populations (Klerk *et al.* 2002), this would support the argument that a good folate status (whether through diet alone, food fortification or multivitamin use) removes the impact of the TT genotype as a risk factor.

The incidence of NTDs have been shown to be dramatically responsive to supplemental folic acid taken during the periconceptual period (Scott *et al.* 1995a). The role of Hcy in the etiology of NTDs has been reviewed (Molloy *et al.* 1998;Isotalo *et al.* 2000a;Nelen

2001) but again the potential adverse role of MTHFR seems to be dependent on folate status (Shields *et al.* 1999;Isotalo *et al.* 2000b;Martinez, V *et al.* 2001;Volcik *et al.* 2003).

The interest in the possible role of MTHFR in cancer is a developing area of research. In the area of breast cancer, the reports are inconclusive and this could be down to a number of factors not been examined in the studies – the impact of dietary folate and alcohol intake, use of multivitamins, source of cancer data i.e. is it pre- or post-mandatory fortification in the US, cut-off timescales for the menopause and even in some studies the absence of plasma folate or tHcy analysis (Gershoni-Baruch *et al.* 2000b;Campbell *et al.* 2002;Langenlehner *et al.* 2003). The role of MTHFR in cervical cancer appears to be dependent on folate status as before, where those with low folate status and the TT genotype appear to be at increased risk of developing cervical dysplasia (Goodman *et al.* 2001b;Gershoni-Baruch *et al.* 2000a). A possible role of MTHFR in oesophageal and gastric cardia adenocarcinoma has been explored and a possible involvement speculated but studies to date have not been detailed enough (Miao *et al.* 2002). Rapidly replicating cells, such as haematopoietic cells, may be especially sensitive to alterations in intracellular folate; the possible involvement of MTHFR in acute leukaemia has been studied. Three case-control studies have evaluated the possible association (Skibola *et al.* 1999;Franco *et al.* 2001;Wiemels *et al.* 2001) and they suggest that the TT genotype is associated with a decreased risk of lymphoid leukaemias. However, no modification of risk was observed in myeloid leukaemia. As before, there were shortcomings in these studies as the sample size was small and folate status was neglected.

The main area of research regarding the association of MTHFR and cancer is in the area of CRC. Two large cohort studies, the US Physicians' Health Study (Ma *et al.* 1997) and the Health Professionals Follow-up Study (Chen *et al.* 1996;Chen *et al.* 1998a), and a large multi-centre control study (Slattery *et al.* 1999) all showed a 20-50% decrease in risk of colon cancer with homozygous status compared to either heterozygous or wild-type individuals.

However, those individuals who were TT but were folate deficient were at comparable risk to the other genotypes. The low folate status appears to negate the protective effect of the TT mutation. The suggested reason for this being that the reduced activity of the TT genotype could have a positive effect on nucleotide synthesis by increasing the availability of 5,10-MTHF required for normal DNA synthesis and cell division. The hypothesis is that cells from folate-replete individuals with TT genotype may be less prone to insufficiencies in the pools of nucleotide precursors available for DNA synthesis. Folate deficiency has been shown to depress thymidylate synthesis, which increases the misincorporation of uracil and increased the frequency of chromosome breaks in the human leucocyte DNA (Blount *et al.* 1997).

Two American studies of MTHFR genotype and colorectal adenoma (Ulrich *et al.* 1999a;Levine *et al.* 2000) showed similar baseline trends to the colon cancer studies. Although the Nurses Health Study (Giovannucci *et al.* 1998) showed a protective effect of dietary folate with colon cancer, they showed an increased risk of adenoma of about 30% in TT genotypes even when their folate status was high. A Norwegian study showed a high baseline risk of adenoma with homozygous status, a doubling of baseline risk of adenoma in those with low folate intake and a slightly protective effect of those with a high folate intake (Ulvik *et al.* 2001).A recent Japanese study supported previous findings (Marugame *et al.* 2000). A recent study examined the two polymorphisms of MTHFR and the alcohol dehydrogenase 3 polymorphism (ADH_3 (2-2) is associated with decreased alcohol catabolism) in relation to the risk of colorectal adenoma in the Health Professionals Follow-up Study (Giovannucci *et al.* 2003). The MTHFR genotypes were not significantly related to the risk of adenoma but there was a suggestive interaction (P=0.09) observed between the C677T genotype and alcohol intake; male TT's who consumed 30+g/day alcohol had an OR of 3.52 (95%CI 1.41-8.78) relative to drinkers of $\leq$5g/day with the CT/TT's. In addition, an increased risk of colorectal adenoma was observed in those with the ADH_3 (2-2) genotype and a high alcohol/low folate

intake compared to those with low alcohol/high folate intake and the ADH_3 (1-1) genotype (P=0.006). This indicates that alcohol interacts with MTHFR and that the interaction between alcohol and ADH_3 is stronger among those with low folate intakes, supporting the hypothesis that alcohol's carcinogenic influence is mediated through folate status.

A study looking at MTHFR in CRC examined the role of it in tumour development and the significance of allelic loss in tumour progression (Ryan *et al.* 2001). CT genotype was significantly associated with risk of developing CRC (OR 1.86, 95%CI 1.3-2.7, P<0.005). No increase was observed in TT homozygotes. The MTHFR "T" allele frequency was significantly higher in the cancer group (P<0.008). Loss of heterozygosity at the MTHFR locus was observed in 18% of informative cancers, with exclusive loss of the "T" allele in all cases. This suggests that functional MTHFR might play a role an important role in the survival and progression of a colonic neoplasm. This study follows onto another which looked at MTHFR and response of fluorouracil and other fluoropyrimidines in patients with metastatic CRC (Cohen *et al.* 2003). Of the 43 patients were examined, 5 were TT, 21 were CT and 17 were CC. When the relationship between MTHFR alleles and response rates to fluoropyrimidine chemotherapy was examined, there was a significant difference between responders versus non-responders who carried the "T" allele (OR 2.86, 95%CI 1.06-7.73, P=0.0351). This suggests that MTHFR genotyping may be of benefit in predicting response to chemotherapy in CRC.

5. FOLATE

i. Background

The link between folate and disease was made by Dr. Lucy Wills while studying macrocytic anaemia in pregnant, textile workers in Bombay. The anaemia was associated with poverty; she was the first to make the link between the poor quality of their diet (low in protein, fruit and vegetables) and their anaemia. The condition was reversed when she administered yeast or a yeast extract (Wills 1931). Folic acid was named in 1941 after it was isolated from spinach; folium is the Latin for leaf. In 1943, the pure crystalline form was synthesized which showed that it was composed of a pteridine ring, paraminobenzoic acid and glutamic acid so was called Pteroylglutamic acid (Angier *et al.* 1945). However, it soon became apparent that the structure of folic acid was different to that of naturally occurring folates; this was in time shown to be due to differences in the amount of glutamates present (polyglutamates), additional single carbon units (methyl groups) or reduction to tetra- or di-hydroforms of the pteridine ring. It was thus determined that folic acid refers to the synthetic, heat-stable, fully oxidised form of the Pteroylglutamic acid. The term folate is a generic term for the group of compounds that have a common vitamin activity; this includes the synthetic form and a wide variety of derivatives.

The active form of folate consists of a substituted pteridine ring linked to *p*-aminobenzoic acid (pABA) (together forming pteroic acid) and glutamic acid. The term folic acid refers to pteroylglutamate, which is the monoglutamyl form. Reductions and substitutions in the pteridine ring constitute 5-methyl-H_4 folate, the circulating form of the vitamin. The addition of up to seven glutamates in the γ–carboxyl linkage enhances folate-dependent reactions by producing polyglutamyl folate, the major form in which both dietary and intracellular folates occur. The principle function of the folate co-enzymes is the transfer of single-carbon units in reactions essential to the metabolism of several amino acids and to

nucleic acid synthesis (Shane and Stokstad 1985). The principle interaction with vitamin B_{12} is in the action of methionine synthetase. H_4 folate is essential in turn for polyglutamation and for the synthesis of 5,10-methylenetetrahydrofolate (5,10-MTHF), a co-factor of thymidylate synthetase that converts deoxyuridylate into thymidylate in the process of DNA synthesis. Lack of either folate or vitamin B_{12} decreases the supply of tetrahydrofolate, thus limiting the production of both polyglutamyl folate and DNA.

ii. Dietary Sources

Folate was originally identified in green leafy vegetables and high levels have been identified in asparagus, Brussels sprouts, spinach and kale (>100µl/serving). Other fruit and vegetables that contain lower but significant amounts are broccoli, cabbage, cauliflower, iceberg lettuce, parsnips, spring greens and oranges (50-100µg/serving). Folate is found in a concentrated form in liver, as it is the major site of animal storage. It is also found in yeast, yeast extract and beer. However, in the diet, it is potatoes that provide the richest vegetable source; fortified bread and breakfast cereals supply around 30% of the average daily intake and dairy products are another important source.

The bioavailability of folate has been a topic of active research over the years. Much of the interest in this area originated from reports that showed a wide range of bioavailability of endogenous folate in a wide variety of common foods (Tamura and Stokstad 1973;Babu and Srikantia 1976). Interest increased when a series of studies were published on the apparent incomplete bioavailability of folic acid added to native cereal foods in South Africa (Coleman 1982). Bioavailability has been defined for the application to dietary component analysis and it was as the fraction of an ingested nutrient, which has the potential to meet functional demands in target tissues. The definition implies that bioavailability refers to post-absorptive processes, namely bioefficacy and biodelivery, as well as absorption (Strain and West 1999). An early

study looked at the bioavailability of naturally occurring food folates and found that it was about 50% lower than that of folic acid (Sauberlich *et al.* 1987). This study looked at the bioavailability of folate in a typical mixed diet compared to a formula diet; however, in view of the small number of subjects and the variability of the indices used, the value of $\leq$50% was quite imprecise. It has been reported that natural folates result in a significantly lower increase in red-cell folate concentrations compared with folic acid supplements or foods fortified with folic acid (Cuskelly *et al.* 1996), conducted on free-living subjects and whereas not a quantitative study of bioavailability, this study showed lower aggregate bioavailability for the high folate foods used. It should be recognised that not all food sources exhibit poor bioavailability, for example Brouwer and colleagues found an assortment of fruits and vegetables to exhibit 60-90% bioavailability relative to folic acid (Brouwer *et al.* 1999). The bioavailability of folic acid when consumed in the form of fortified foods is though to be less than that of folic acid alone. An estimate of 85% bioavailability from fortified foods was used to calculate the dietary folate equivalents in the USA. It has been further calculated that folic acid or fortified foods is 1.7 times more bioavailable than food folates. However, recent studies using a dual-isotope methodology have suggested that some cereal-based vehicles of fortification (particularly bran) may inhibit folate absorption to below the expected 85% bioavailability (Gregory 2001;Finglas *et al.* 2002). Three-quarters of dietary folate occurs in the polyglutamyl forms and the remaining quarter as monglutamyl. Food preparation, particularly boiling, is known to destroy dietary folates (Herbert 1987b). The availability of folate from different food sources is also limited by the presence of endogenous inhibitors of folate hydrolase and other factors. Thus, in a study of changes in urinary folate excretion (an index of absorption) following intakes of different foods of known folate content, the availability of folate from lettuce, eggs, oranges and wheatgerm was found to be about half that from lima beans, liver, yeast and bananas (Tamura and Stokstad 1973). It is assumed, for the

purposes of estimating requirements, that about 50% of polyglutamyl dietary folate is absorbed. Although recently, a much higher absorption rate was found in patients with an ileostomy for spinach than expected – 79% when compared against a folic acid supplement. The study calculated these results by having the folate content of the foods to be ingested and the folate content of the ileostomy effluents (Konings *et al.* 2002b). However, these results could be queried as patients with established ileostomies have a greater absorptive capacity due to the decreased length of the gut and possibly a different mechanism. Therefore, it may be then argued that the mechanism of absorption in these patients may not reflect the normal *in vitro* situation.

It has been proposed that there are three main factors involved in the determination of the bioavailability and which account for the variance seen between publications. Firstly, it is likely that foods such as orange juice have partially incomplete bioavailability of polyglutamyl folates due to the presence of natural inhibitors. Secondly, in vitro evidence strongly suggests that some chemical instability of reduced folate in the digestive system before absorption also accounts for some variation seen and thirdly, it appears that the cellular structure of plant materials can partially inhibit the absorption of folates by entrapment (Gregory 2001).

iii. Absorption and Metabolism

Folate exhibits complex whole-body physiology and metabolism (Shane 1995). Before absorption across the intestinal mucosa can take place, the polyglutamates must be hydrolysed to the monoglutamate form in the gut lumen by brush-border γ-glutamyl hydrolase or conjugase (folylpolyglutamate carboxypepidase) (Halsted 1980). Human brush-border deconjugase has been shown to catalyse the stepwise hydrolysis of the polyglutamates and be a zinc-dependent exopeptidase (Reisenauer and Krumdieck 1977). The mucosal cells of the proximal small intestine (jejunum) then take up the monoglutamates by primarily a saturatable

active energy-dependent carrier-mediated process at physiological concentrations with little selectivity among various folates, and by passive diffusion at higher concentrations (Selhub *et al.* 1984). Many foods contain inhibitors of absorption through inhibition of brush-border enzyme activity and/or the folate transport system (Tamura and Stokstad 1973). The efficiency of deconjugation and absorption in the gut varies considerably with the degree of glutamation, the presence of other dietary constituents and various physiological factors. Absorption does not decline with age (Bailey *et al.* 1984) but pH will markedly influence folate absorption. 6.3pH has been shown to allow maximal absorption, while at a higher pH there was a sharp decrease (Russell *et al.* 1979). Dairy products contain folate-binding proteins and this may decrease the bioavailability of the folates present (Wagner 1985).

Once the monoglutamate folates are absorbed, they are converted into 5-methyltetrahydrofolic acid (5-methyl THF) while moving through the intestinal mucosa. The 5-methyl THF are then transported in the hepatic portal vein, where they can then travel to the liver. In man, folate is mainly stored in the liver (Hopner and Lampi 1980), where it has been assumed that approximately 50% of total body folates (5-20mg) are stored. Folate undergoes substantial enterohepatic recirculation, as much as 100μg undergoing daily bilary re-excretion (Herbert and Das 1993). It has been shown that most of this was reabsorbed in the small intestine (Weir *et al.* 1985), the efficiency of this system may be influenced by dietary composition if digestion was taking place concurrently.

5-methyl THF via the liver is transported into the plasma of the systemic circulation. The liver has been thought to initially remove a proportion of the absorbed dose; this has been called the "first pass effect". The liver is the major site of one-carbon metabolism and has a larger mass of folate and higher folate concentration than other tissues (Herbert 1987b). Retention of folates in hepatic and other cells is aided by conversion to poly-γ–glutamyl derivatives and by association with specific folate-binding proteins, most of which are enzymes

involved in folate metabolism. Because folate is secreted in bile and reabsorbed, enterohepatic circulation is an important aspect of folate physiology. Bilary folate concentration varies considerably with folate intake. A calculation based on a mean bilary folate concentration of ~90nmol/L and bile flow of 600-700ml/day suggests about 60nmol/day enters enterohepatic circulation (Gregory *et al.* 1998). This suggests that at least 10% of daily folate intake would enter enterohepatic circulation, which is mainly 5-methylTHF because it is a poor substrate for hepatic folylpolyglutamate synthase (Yong-Hae *et al.* 1999). Thus, newly absorbed 5-methylTHF, and that formed from folic acid, is more extensively exported through biliary secretion than are other folates entering the liver. The intestine has been shown to be capable of both reduction and one-carbon substitution (methylation) of physiological doses of folic acid. However, the process was shown to be saturable but there was also the additional capacity of passive diffusion, as a result if a dose >280μg has been ingested in one dose, unaltered folic acid appeared in the circulation (Kelly *et al.* 1997). The appearance of 5-methylTHF in plasma is related to the folic acid dose by a Michealis-Menten function (Lucock *et al.* 1989). With this as the exception, 5-methyl THF is generally the folate form found in circulation and subsequently transported into the cells via folate transport systems. The cellular retention of 5-methylTHF occurs mainly in proportion to the extent of its conversion to other folates as mediated by methionine synthase. By this process, folate entering a cell as 5-methylTHF is converted to forms of folate that are more suitable for cellular retention through polyglutamation, specific protein-binding and additional participation in one-carbon metabolism. For the 5-methyl THF to be stored intracellularly, it must be converted to tetrahydrofolate through the B_{12}-dependent enzyme methionine synthase and then on to a polyglutamate. Circulating excess folic acid can be taken up by cells and become functional after reduction via dihydrofolate to tetrahydrofolate. The routes of folate turnover have been well documented; however, disagreement exists regarding their relative quantitative

contributions. The existence of faecal folate has been attributed to microbial synthesis of the vitamin, although unabsorbed dietary folate as well as folates from bile, other gastrointestinal secretions and sloughed off cells contribute to faecal losses of folate. Because synthesis and breakdown of folate by the colonic microflora, identification of the origin of faecal folates and catabolic products is challenging. Evidence from tracer studies indicates that faecal excretion is a significant route of folate turnover. Urinary excretion of folate constitutes a small, intake-dependent loss of body folate (Gregory *et al.* 1998). The kidney serves an important role in folate metabolism through its carrier-mediated reabsorption of folate from the glomerular filtrate (Selhub *et al.* 1987) and secretion of folate from renal tubular cells into urine also occurs (Morshed *et al.* 1997). Folate catabolism constitutes another important in vivo mechanism for the loss of folates (Murphy *et al.* 1976). The predominant catabolism of the folate molecule occurs by cleavage to yield a pterin and *p*-aminobenzoylglutamate. Typically at least 80% of the *p*-aminobenzoylglutamate is excreted as the acetamido derivative, which appears to be the product of a specific acrylamine acetyltransferase (Minchin 1995). Some folate "catabolism" probably occurs by non-specific oxidation. Recent studies suggest that specific mechanisms of folate cleavage also exist (Suh *et al.* 2001).

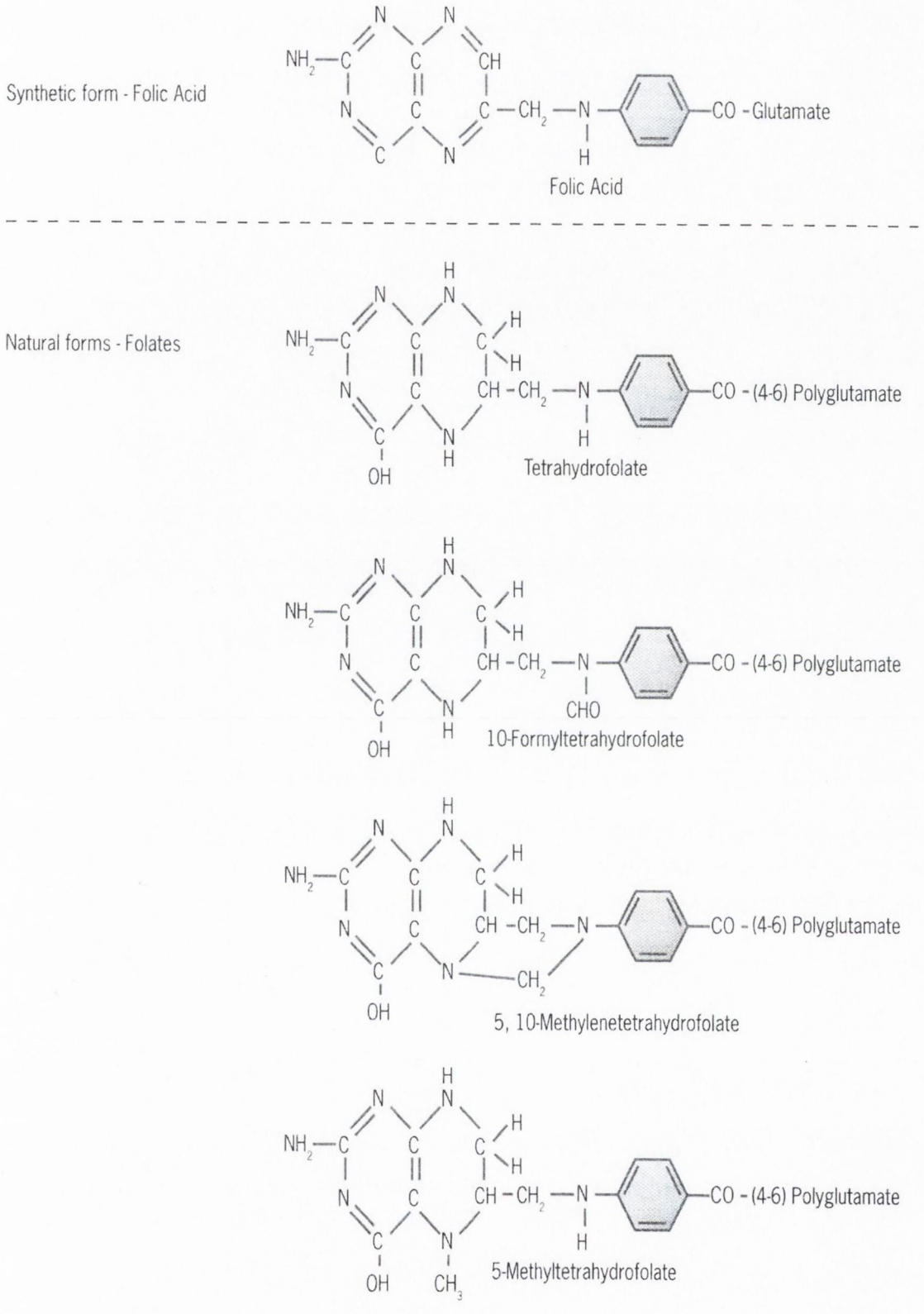


Figure 1.xii Structural formula of folic acid and tetrahydrofolic acid

(Department of Health 2000)

One-carbon metabolism is a network of interrelated biochemical reactions in which a one-carbon unit from a donor compound is transferred to tetrahydrofolate (THF) for subsequent reduction or oxidation and/or transfer to other compounds. Folate co-enzymes in mammalian tissue thereby act as acceptors or donors of one-carbon units in a variety of reactions involved in amino acid and nucleotide metabolism.

The major source of cellular one-carbon groups is the conversion of serine to glycine with transfer of the serine C_3 to THF, yielding 5,10-methyleneTHF (Pasternack *et al.* 1994). Subsequent glycine metabolism to CO_2 and NH_4^+ yields a further molecule of 5,10-MTHF. Formate contributes 25% of one-carbon groups for purine synthesis even when the cell is serine deplete. The donation of the methyl group of unconjugated imported 5-methyl THF to methionine synthase contributes little to the cellular pool. Three formyl derivatives of THF exist in equilibrium: 10-formylTHF, 5-formylTHF and 5,10-MTHF (Stover and Schirich 1992). The trimeric enzyme methyleneTHF dehydrogenase/methylTHF cyclohydrolase/formylTHF synthase provides a conduit for the flux of folate groups between formate and methyleneTHF. Thus, the latter co-enzyme stands at the crossroads of cellular transmethylation reactions and nucleotide synthesis. Increased levels of methyleneTHF result in preferential fluxing of this metabolite towards nucleotide synthesis rather than cellular methylation reactions (Green *et al.* 1988).

Folate is required for de-novo synthesis of purines.10-formylTHF serves as the major folate species in the process, donating two carbons (C_2 and C_8) through the actions of the enzymes glycinamide ribonucleotide (GAR) transformylase and 5-amino-imidazole-4-carboxamide ribonucleotide (AICAR) transformylase respectively. The activity of the enzymes varies with tissue requirements, being several fold greater in the marrow than in the liver (Smith *et al.* 1982).

Deoxynucleoside triphosphates are the immediate pre-cursors of DNA. The de novo synthesis of thymidylate (dTTP) is mediated by the dimeric thymidylate synthase (5,10-methyleneTHF: dUMP C-methyltransferase). This enzyme forms a ternary complex with dUMP and methyleneTHF, with the transfer of the methylene group to the 5' position of the pyrimidine ring followed by the hydride transfer. Polyglutamation of the folate co-enzyme is structurally important in this reaction (Dev *et al.* 1994). Following thymidylate synthesis, the dihydrofolate unit is reduced to THF. Cell levels of thymidylate synthase are autoregulated by feed-back inhibition of the translation of its own mRNA (Chu *et al.* 1991). Thymidylate is functionally compartmentalised into several pools: de novo, salvage and replication pre-cursor. The latter pool is small (<5%), sited close to the replication fork and draws on the other two pools, though preferentially on the salvage pool (Taheri *et al.* 1981).

Methionine is necessary for the growth and development of mammals. This essential role derives from its utilisation in the synthesis both of proteins and S-adenosylmethionine (SAM), the cornerstone of cellular methylation reactions. The methyl group of methionine is derived through the activity of two enzymes, MTHFR and MS. Methionine metabolism presents a central methionine-SAM-homocysteine cycle with four outlets (i) bi-directional flow between methionine and protein (ii) utilisation of decarboxylated SAM in polyamine formation (iii) transfer of the SAM methyl group to a variety of methyl acceptors (iv) transsulphuration of homocysteine to cysteine.

MTHFR catalyses the reduction of methyleneTHF to methylTHF. This enzyme is a flavoprotein specific for FAD that utilises NADPH. NADPH recruits active forms of the enzyme, whereas SAM recruits inactive forms, inducing conformational change that greatly reduces the affinity for NADPH (Matthews *et al.* 1986).

Mammals re-methylate homocysteine to methionine in addition to utilising dietary sources. Mammalian MS is a cobalamin dependent enzyme that catalyses the transfer of

methyl groups from 5-methylTHF through methylcobalamin to homocysteine. The transfer of methyl groups from 5-methylTHF requires their activation. It has been postulated (Matthews *et al.* 1986) that iron functions to activate the methyl group. MS is regulated predominantly through substrate/product influence (Krebs *et al.* 1976). Methionine is required for aminopropylation reactions and the transmethylation of lipids, proteins and nucleic acids. SAM, formed by the ATP activation of methionine, serves as the methyl donor group in these reactions. Transmethylation reactions account for up to 80% of SAM metabolism.

SAM serves as the precursor of polyamines in addition to its role as a substrate for transmethylation reactions. The product of transmethylation reactions is S-adenosylhomocysteine (SAH), an inhibitor of many of the SAM dependent methyltransferases. The ratio of SAM to SAH is important in the regulation of transmethylation reactions (Schalinske and Steele 1992). SAH is hydrolysed to Hcy, which may cycle back through MS, completing a cycle with a high degree of conservation of the Hcy moiety. Alternatively, Hcy undergoes transsulphuration to cysteine. In the transsulphuration pathway, Hcy condenses with serine to form cystathionine in an irreversible reaction catalysed by the pyridoxal-5-phosphate containing cystathionine-β-synthase (Selhub and Miller 1992).

In parallel with vitamin B_{12} dependent methyltransferase described, exists an alternative choline-betaine pathway for MS. The enzyme betaine methyltransferase uses preformed methyl groups derived from dietary choline and is independent of cobalamin. In humans, the importance of this pathway may be limited as it is only found in liver and kidney tissue (McKeever *et al.* 1991).

Cell methionine homeostasis is maintained through several processes depending on the degree of repletion of the individual. When methionine levels are high, resulting in a surplus of carbon-one units, shunting of folate away from MS occurs. Excess methionine and SAM are converted to metabolites including sarcosine. Conservation of methionine is achieved through

a shift away from methylation reactions towards protein synthesis and increased remethylation of homocysteine. Cystine can replace approximately 70% of the dietary requirements for methionine through a redistribution of homocysteine between remethylation and transsulphuration reactions (Finkelstein and Martin 1984).

Intracellular folates are restricted in activity by several forms of compartmentation, primarily to the cytosol (50-80%) and the mitochondria (20-50%). Transmembrane passage of serine, glycine and formate enable communication between these pools (Pasternack *et al.* 1994).

iv. Folate Deficiency

Folate is the second most common vitamin deficiency worldwide (Hoffbrand 1987). Although inadequate dietary intake is the single greatest contributor, secondary deficiency can result from other causes. Low folate blood levels may accompany coeliac disease (Hjelt and Kransilnikoff 1990), atrophic gastritis (Russell *et al.* 1986), impaired pancreatic function (Benn and Cooke 1971), post-enteritis enteropathy (Paerregaard *et al.* 1990), scleroderma (Hendel *et al.* 1987), small bowel sarcoidosis (MacRury *et al.* 1992), HIV (Dowling *et al.* 1993)and inflammatory bowel disease (IBD) (Steger *et al.* 1994). Other causes not related to the gastrointestinal tract are haemodialysis (Ono and Hisasue 1992), leukaemia (Rose 1966), critical illness (Hehir and Phelan 1992) and psoriasis (Iwama *et al.* 1992). A broad spectrum of pharmacological agents influence folate metabolism. These effects may arise due to the direct consequences of enzyme inhibition or another mechanism. Therapy with phenobarbitione, phenytonin, primidone, carbamezapine and valproic acid result in reduced serum, red cell or cerebro-spinal fluid folate levels. Polytherapy gives rise to greater depletion of folate than monotherapy (Edeh and Toone 1985). During pregnancy such deficiency has been cited as a mechanism for teratogenesis (Bower 1994). This deficiency may be due to inhibition of γ-

glutamyl carboxy-peptidase cleaving dietary polyglutamate forms. Additionally, defective absorption of the monoglutamates can also be caused by an increase in the luminal pH of the intestine by barbiturates.

Macrocytic anaemia is commonly encountered with chronic alcohol abuse. Although nutritional deficiency may account for some degree of this, a direct toxic effect on folate metabolism has been shown. Alcohol influences the uptake of folate by isolated hepatocytes, prevents the utilisation of 5-methylTHF and inhibits body retention of folate (Hidiroglou *et al.* 1994). Ethanol and other alcohols inhibit the intestinal transport of folates to a degree related to alcohol concentration and chain length (Said and Strum 1986).

Blood folate deficiency is associated with the use of steroids and sulphonamide containing oral contraceptives and the long-term use of triamterene (Steeger-Theunissen *et al.* 1993;Mason *et al.* 1991). Triamterene and probenicid may affect folate levels through influencing ion channels. The drugs that are used in the treatment of IBD, in particular those containing sulphonamide, cause folate depletion. This may arise through inhibition of dihydrofolate reductase. Sulphasalazine (sulphapyridine/5-amino salicylic acid) impairs the absorption of folic acid in patients with IBD (Franklin and Rosenberg 1973). This effect was due to inhibition of intestinal transport. Sulphasalzine can inhibit dihydrofolate reductase, 5-methylTHF and serine transhydroxymethylase (Selhub and Rosenberg 1978). The therapeutic benefit of methotrexate is mediated through inhibition of dihydrofolate reductase, thus reducing levels of suitable substrates for polyglutamation and thymidylate synthesis. The low folate serum levels seen with methotrexate are due to enhanced urinary loss and impaired intestinal absorption (Zimmerman 1992). Cholestryamine reduces the bioavailability of gut lumen folate (Hopner and Lampner 1991).

The major clinical feature of folate deficiency is megaloblastic anaemia in association with variable degrees of pancytopaenia. Though a similar picture is seen with vitamin B_{12}

deficiency, the course is considerably more protracted (Hoffbrand 1987). The overt clinical sequelae of folate deficiency arise from the haematological disturbances, these include: organomegaly, congestive cardiac failure and easy bruising. Folate deficiency may also impair function of the immune system (Dhur *et al.* 1991).

v. Analysis Methodologies

The laboratory investigation of folate status is largely based on quantitative tests, estimates of serum and red cell folate being the mainstay of clinical practise. The use of functional tests such as the deoxyuridine suppression and FIGLU are reserved for specific research purposes.

a. Quantitative Tests

The early stages of folate depletion can be measured using serum folate levels, as these tend to reflect short-term balance during the preceding 1-2days. The measurement of serum folate nevertheless has substantial predictive value in defining the risk of megaloblastic anaemia as well as other indicators of systemic folate status, such as Hcy levels (Selhub *et al.* 1993). In contrast, the folate that is packaged into a developing blood cell (which is primarily a function of folate availability at the time of erythropoiesis) is no longer metabolised after maturation of that red cell occurs. Red cell folate concentration therefore represents an integration of dietary folate intake during the preceding 120days, the half-life of the red cell. It is therefore not surprising the red blood cell (RBC) folate tends to be a more accurate reflection of tissue folate status (Bailey 1990). Full blood count and blood film are poor indicators of folate status but can serve to identify those with anaemia. The interpretation of serum and red cell folate tests has been reviewed by Herbert and he grouped the manifestations of folate deficiency into three broad stages: (i) persistently low serum folate (<3ng/ml), at three weeks this will result in a slight increase in the size of average marrow normoblasts (ii) folate

deficiency erythropoiesis (sub-clinical deficiency) ~17 weeks depletion (iii) folate depletion anaemia, taking ~19 weeks (Herbert 1987a). Total body stores of folate are in the range of 7.25±2.5mg, the single largest reserve being the liver. Care must be exercised in interpreting normal serum and red cell folate results in patients with severe iron deficiency anaemia, as such values may be erroneous. Conversely, it should be recognised that 50% of those with folate deficiency will have significant iron deficiency (Herbert 1985). Venous blood yields higher folate levels than found with arterial samples and blood transfusions have little influence on measurements taken within fourteen days (Ho 1992).

Although value ranges vary somewhat from laboratory to laboratory, two standard deviations below the mean for serum and RBC folate concentrations are typically 3ng/mL and 160ng/mL, respectively, and values less than that are associated with a markedly increased risk of developing megaloblastic anaemia; serum folate concentrations of 3-5ng/mL are considered to be marginally deficient. Serum and RBC folate continue to be important tools in the clinical evaluation of folate status although it is worth remembering that what we presently consider to be normal ranges may need to be modified in view of the growing consensus that subtle degrees of folate depletion appear to have pathologic consequences.

b. Methods

Since the 1930's, microbiological assays have been used to measure folate status. Assays have been become simplified with time. The use of low concentrations of ascorbate to stabilise reduced folate forms has permitted the long-term storage of samples prior to assay and the introduction of conjugase into protocols has permitted assays to be carried out in cells found outside the circulation. The use of differential microbiological assay has allowed specific folate species to be identified, although this has been superceded by the use of high performance liquid chromatography (HPLC). The coupling of microtitre plate techniques and

automation with the use of chloramphenicol resistant organisms has permitted large numbers of samples to be processed under non-sterile conditions.

Other assay techniques have been described including radioassay and ELISA. Radioassay kits are based on the competition of a radiolabelled folate co-enzyme with serum/red cell folate for sites on a folate binding protein such as milk powder or β-lactoglobulin. These kits compare favourably though not uniformly with the microbiological assay in respect of accuracy and ease of performance (Arnaud *et al.* 1992). The main problem with the use of the radioassay kits is the choice of the pH because PGA (the normal calibrant used in the kit) and 5-methylTHF exhibit different pH-dependent affinity binding curves with the milk folate binding protein (Givas and Gutcho 1975). Equal affinity between both forms is found at pH 9.3 and therefore this is the pH of choice in most kits. However, small differences in assay pH may strongly affect the results. Typical values obtained from recent European intercomparision studies for folate determinations in serum and red blood cell using the microbiological and radioassay gave between laboratory variation of about 30 to 40%, respectively. The variation for red blood cell only for the microbiological assay was lower at about 25% (Wright *et al.* 1998). Similar results have also been reported for serum and red cell folate using the microbiological assay, radioassay and HPLC methods (Gunter *et al.* 1996).The authors found two- to nine-fold differences in folate concentrations between methods, with the greatest variation occurring at the critical low concentrations.

Some concern has been expressed that the current extraction procedures for red blood cell need re-evaluation. Published literature suggests that increased deoxyhaemoglobin (which can bind folate electrostatically) yields more assayable folate by microbiological assay, and increased oxyhaemoglobin (which cannot bind red cell folate) yields less assayable folate (Wright *et al.* 1998). In addition, there has been some debate as to the most appropriate procedure for the determination of red cell folate. Significant differences in folate values have

been obtained in individuals who are homozygotes for MTHFR using the microbiological and radioassay (Molloy *et al.* 1998). It has been further postulated that the folate form in these individuals may have an increased response in the radioassay compared with the assay calibrant, giving erroneous higher values.

HPLC procedures for folate analysis in blood use both fluorometric and electrochemical detection (Lucock *et al.* 1993). More recently, a stable isotope dilution gas chromatography-mass spectrometry procedure was reported for the quantitation of red blood cell folate using bacterial synthesised $^{13}C_6$-labelled folate as an internal standard (Santhosh-Kumar *et al.* 1995). This approach gives high sensitivity, specificity and accuracy/precision and may, in the future, be suitable as a reference procedure.

c. Functional Tests

Though serum methionine levels remain unaffected, those of homocysteine are elevated by folate depletion and provide a sensitive measure of functional deficiency(Cravo and Camilo 2000;Jacob *et al.* 1994). The differential effects of folate and vitamin B_{12} on serum levels of Hcy and methyl malonic acid (MMA) provide a means for differentiating between functional deficiencies of these two vitamins, although MMA is rarely measured. The appearance of raised levels of formimino glutamic acid (FIGLU) in the urine implies an interference with folate metabolism. Following a standard dose of L-histidine, at least two factors determine the amount of FIGLU excreted. Firstly, the catabolism of FIGLU is limited by the availability of folate groups to accept the imino group. Secondly, there is impaired metabolism of FIGLU from urocanic acid. The test provided poor results in pregnancy and has been superceded by the assay of Hcy levels.

The competition between the de novo pathway (methylation of deoxyuridine monophosphate dUMP) and the salvage pathway (phosphorylation of preformed thymidine by thymidine kinase) of thymidylate synthesis provides a means to quantify the functional effect

of both folate and vitamin B_{12} deficiency. The deoxyuridine suppression test is based on the presumption that under conditions of normal folate and cobalamin supply, the addition of non-radioactive deoxyuridine to short-term cultures of bone marrow, whole blood, lymphocytes or rat colonic epithelium promotes the de novo synthesis of thymidylate by thymidylate synthase. There is concomitant suppression of radioactive thymidine incorporation into DNA by the salvage pathway. When folate and cobalamin are deficient, the degree of suppression is less pronounced because of impaired de novo synthesis of thymidylate and greater utilisation of the salvage pathway (Cravo *et al.* 1991).

vi. Requirements

Folate requirements historically have been defined as the quantity of intake necessary to prevent a severe deficiency with clinical symptoms (Food and Nutrition Board and National Academy of Sciences 1989). More recently, the focus has shifted to identifying intakes associated with maintenance of normal one-carbon transfer reactions using functional indicators of folate status to identify sub-optimal intakes. A great deal of current research is involved in identifying the optimal intake associated with risk reduction for chronic disease and developmental defects.

In the UK, a value for the Recommended Daily Amount (RDA) was first set for folate back in 1979 at 300μg for adults (Department of Health and Social Security 1979). This value was subsequently withdrawn over scientific uncertainties, particularly in the area of the biochemical assay of the active forms of folate. In 1991, new values were declared based on balance studies, which sought to titrate dietary intakes against markers of folate status (red cell and serum values) and the concentration found in liver at post-mortem examination. The values for infants took account of the folate content of breast milk, while the values for children were interpolated between the values for infants and those for adults, as there was lack of scientific

data in this area. Increments were added for pregnancy and lactation to account for the known increase in folate requirements in late pregnancy and the need to replace folate lost through breast milk (Department of Health 1991). The Dietary Reference Values (DRVs) set define an estimated range of dietary requirements in different groups of individuals. They take into account normal biological variation. The DRVs are comprised of 3 levels of intake: the Estimated Average Requirement, the Reference Nutrient Intake, which is sufficient to cover the needs of 97.5% of the population group and the Lower Reference Nutrient Intake, sufficient only for those with the lowest requirements and unlikely to meet the needs of the general population. The European Union Scientific Committee for Food set reference nutrient values and energy intakes for adults in the EU but they were very similar to those from the UK (Commision of the European Communities 1993). The US National Academy of Sciences has since published (Institute of Medicine 1998) a revision of their RDA and the development of recommended dietary intakes. The revised RDA looked at plasma Hcy as well as the common systemic markers. Folate and folic acid bioavailability were also accounted for and were defined as Dietary Folate Equivalents (DFE). DFE takes the folate value of the foodstuff and factors in the fact that folic acid in food is 1.7 times more available than naturally occurring food folates. The RDA set was 400μg DFE/day for adults, increasing to 600μg DFE/day for pregnancy and 500μgDFE/day during lactation. The Irish RDA for dietary folate is higher than the EU Population Reference Intake values, the US RDA and the UK Reference Nutrient Intake values. However, it was the opinion of the expert panel that women should take 400μg of folic acid in tablet form or through supplementation in the peri-conceptual period to help prevent neural tube defects (NTDs). The Irish RDA for females (11years+) is 300μg/day. This increases to 500μg for the second half of pregnancy and to help prevent megaloblastosis. The working group advised that the 1983 RDA be retained on consideration of the high incidence of NTDs in Ireland (Food Safety Authority 1999).

The addition of folic acid to all enriched cereal grain foods was mandated by the Food and Drug Administration in January 1998. Although this program was designed such that typical folate intakes would be increased by ~100μg/day and the risk of intakes >1000μg/day (the FDA's safe upper limit of daily intake) would be minimal. Linear regression analysis of data published has been examined and it was found that the increase in circulating folate levels was linear related to folic acid intake over the range of 100-1000μg/day ($r=0.984$, $P<0.0001$). Predicted increases in folic acid intake from fortified food ranged from 215 to 240μg/d. Typical intakes of folic acid from fortified foods are more than twice the level originally predicted. The effect of this much higher fortification must be carefully assessed i.e. the metabolic effects, health benefits and risks, before any changes to the current fortification program are made (Kirby 2000).

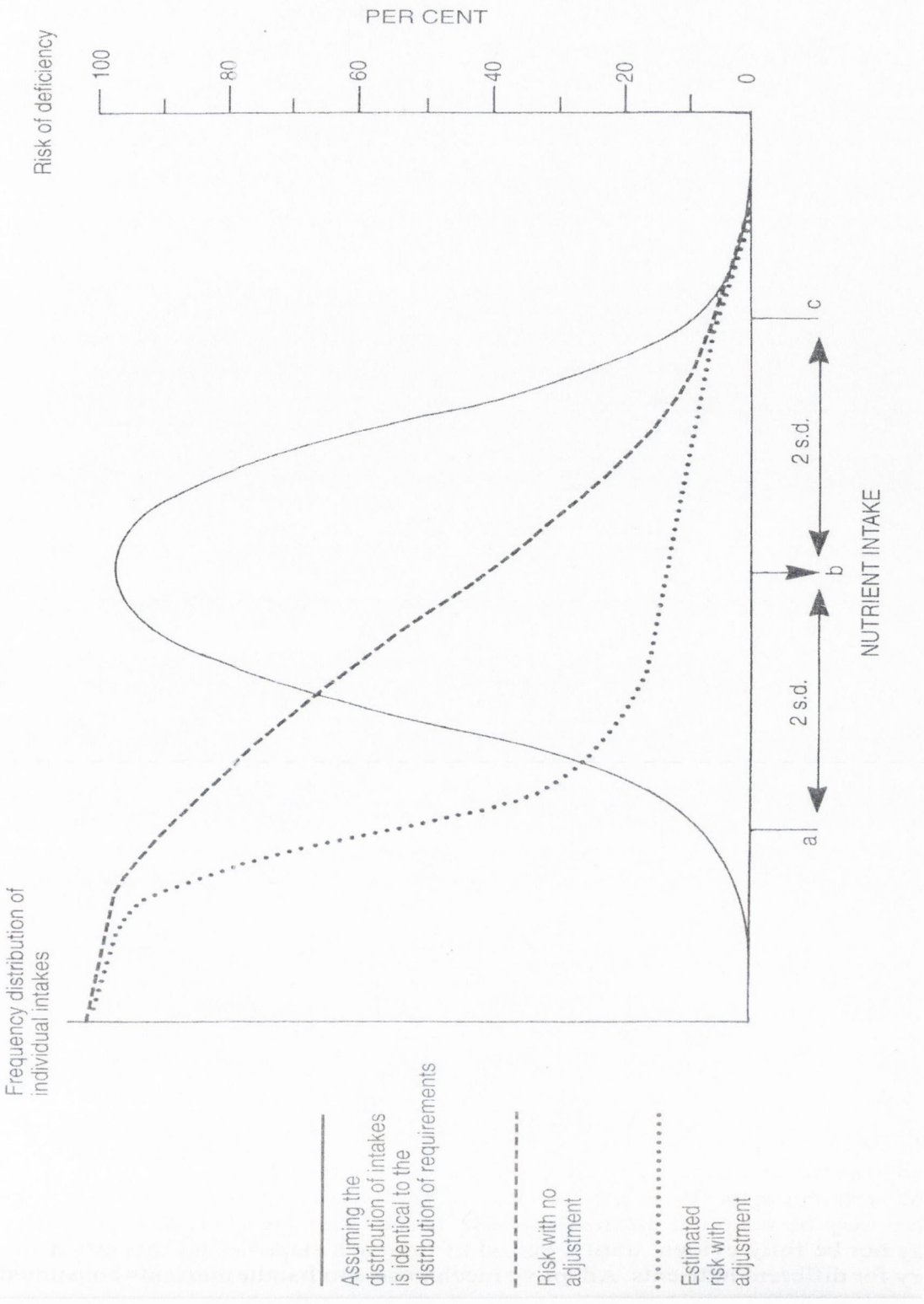


Figure 1.xiii Dietary Intakes and risk of deficiency; *a* represents LRNI, *b* represents EAR and *c* represents RNI. (Department of Health 2000)

Table 1.v **Dietary Reference Values for Folate (μg/d) in the UK**

Age	Lower Reference Nutrient Intake	Estimated Average Requirement	Reference Nutrient Intake
0-12 mth	30	40	50
1-3 yr	35	50	70
4-6 yr	50	75	100
7-10 yr	75	110	150
11 yr onwards	100	150	200
Pregnancy			+100
Lactation			+ 60

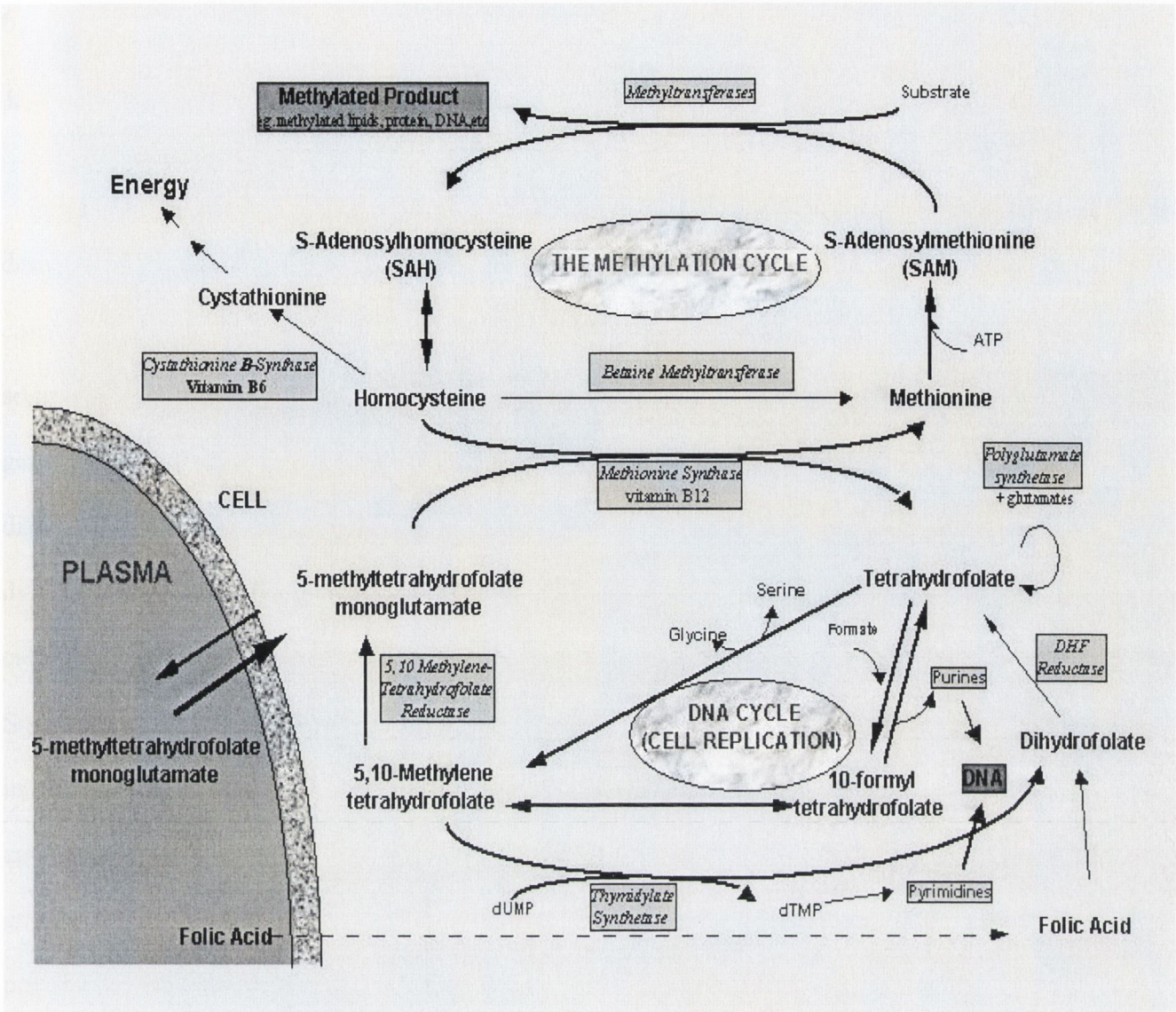


Figure 1.xiv **The Folate Cycle (Courtesy of Scott,J.M.)**

6. COLORECTAL CANCER – GENETIC BASIS AND PATHWAYS

i. Adenoma to Carcinoma Sequence

The adenoma-carcinoma sequence in the histological genesis of CRC was first demonstrated by Morson (Morson 1974). This sequence was later renamed the dysplasia-carcinoma sequence(Morson *et al.* 1983) to allow for extrapolation to other cancer. The sequence of adenoma to carcinoma generally commences with a field defect (presence of a generalised abnormality in the colonic epithelium) such as altered proliferation, altered differentiation, resistance to apoptosis and altered methylation (figure 1.xv). Within this, dysplastic aberrant crypt foci can develop. Aberrant crypt foci are characterised by groups of crypts with enlarged diameter, thickened hypercellular epithelium and altered mucin pattern. Some of these dysplastic foci progress to adenomas, which are initially low risk of developing into adenocarcinomas. These adenomas are characterised by small size, tubular architecture and low-grade dysplasia. A small subset of adenomas will continue to progress, generally growing in size, and develop villous architecture and high-grade dysplasia. A further small percentage develops adenocarcinoma. Neoplastic transformation is also increasingly understood in terms of evolutionary mechanisms, and it is now understood that cancer development is a multistep process involving natural selection of somatic mutations (Vogelstein and Kinzler 1993;Klein 1998). In close agreement with this view is a growing amount of research indicating that tumour-specific genetic alterations are not random but closely related to different tissues and specific classes of carcinogens (Levine *et al.* 1995). A number of reports also reveal distinct patterns of genetic alterations in different tumours, thus implying that the carcinogenic process may proceed through alternative genetic pathways (Shackney and Shankey 1997).

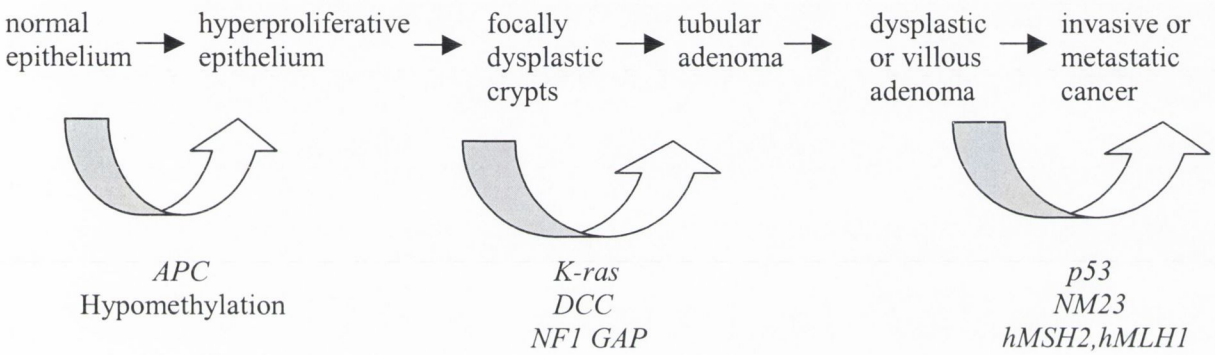


Figure 1.xv The Adenoma to Carcinoma Sequence (Zingg and Jones 1997)

ii. Microsatellite Instability

Microsatellite Instability (MSI) is recognized as genome-wide alterations in repetitive DNA sequences (Lothe 1997). Such mutation patterns have been found in almost all analysed malignant tumours from patients with HNPCC and in about 15% of sporadic cases. MSI can be caused by defects in the nucleotide mismatch repair (MMR) machinery, which in addition to correcting mismatched nucleotides also repairs incision/deletion mutations in repeated DNA sequences. Germ-line mutations in MMR genes have been identified in HNPCC patients and somatic MMR alterations in tumours from hereditary and sporadic tumours with MSI. One gene, BAX, has a central role in the promotion of the apoptosis process, and frameshift mutations have been identified in this gene have been identified in more than 50% of tumours expressing MSI (Rampino *et al.* 1997). MMR deficiency generates frameshift mutations in numerous encoding sequences, resulting in potentially immunogenic proteins. Induction of apoptosis is a central immunologic mechanism and immunorecognition may be a mechanism by which MMR deficiency promotes apoptosis. In fact, MSI tumours display a profound lymphocyte infiltration that may be related to this immunological phenomenon (Kim *et al.* 1994). MMR deficiency and MSI have been related to cellular agents N-methyl-N-nitrosourea and N-methyl-N'-nitro-N-nitrosoguanine. Different studies have suggested that such methylation tolerance arises through the acquisition of defects in the MMR pathway (Karran and Hampson 1996;Kawate *et al.* 1998). The two methylating agents named above induce methylation preferentially in the O^6 position in guanine residues in the DNA molecule. The enzyme O^6-methyguanine-DNA methyltransferase, which transfers the methyl group to cysteine residue within its own structure, reverses such DNA damage (Pieper 1997). However, this enzyme is susceptible to epigenetic silencing through hypermethylation (Qian and Brent 1997) and expression in the colorectal mucosa is low compared to other tissues. The resulting low levels thus promote accumulation of O^6-methylguanine residues in the colorectum.

Several studies implicate bile acids in CRC (Nagengast *et al.* 1995) and epidemiologically this association is most evident in the proximal colon. About 90% of sporadic and 70% familial CRC expressing the MSI phenotype are localized to this area (Lothe *et al* 1993, Breivik *et al* 1997). Bile acid conjugates taurocholic acid and glycocholic acid can undergo N-nitrosation to form compounds which are analogous to methylating agents (Busby, Jr. *et al.* 1985). Considering the data, that link MMR deficiency and methylating agents, this suggests that bile acid conjugates constitute the selection pressure that favours MSI in the proximal colon. In contrast to MSI tumours, where mutations are predominantly localized to repetitive DNA sequences, the majority of colorectal tumours have numerous alterations at the chromosome level. These alterations can vary from chromosomal rearrangement, loss or gain of fragments of the genome and abnormal numbers of chromosomes or shifts in the total nuclear DNA content (Thompson and Thompson 1998). Tumours expressing such features are classified as aneuploid, in contrast to the diploid properties of normal cells and MSI tumours. Carcinogens can be divided into methylating or bulky-adduct-forming (BAF) agents depending on the lesions and repair mechanisms that they induce (Mitra *et al.* 1989). BAF carcinogens include UV radiation, free oxygen species and some chemicals; these induce relatively large alterations in the three-dimensional structure of the DNA, such lesions induce growth arrest and are exclusively repaired by nucleotide excision repair (NER) (Sancar 1995). NER is the most important of the DNA repair pathways and appears to function as a back up for other repair mechanisms. The NER process involves removal and resynthesis of large fragments of DNA. BAF carcinogens may inhibit cellular growth by activating the mitotic checkpoint and constitute a selection pressure that favours breakdown of this control mechanism.

iii. Anatomical Site Dependence

In a study of 282 different patients with CRC, different genetic alterations were found to be dependant on age and the gender of the patient (Breivik *et al.* 1997). It has been clear for 20 years that the epidemiology of cancer of the proximal colon differs from that of the distal colon and of the rectum (De Jong *et al.* 1972;Jensen 1984;Slattery *et al.* 1996). Cancers of the proximal colon have a higher excess of female cases, are more likely to be associated with previous gastric or gallbladder surgery, are more likely to follow the microsatellite-instability genetic pathway and are less likely to have a demonstrable adenoma precursor. After the age of 70, proximal tumours predominate over distal ones (Slattery *et al.* 1996). The determination of site-specific differences in tumour development is important because right- and left-sided CRC appear to have different aetiologies and thus different strategies may be required to protect against cancer at different sites (Bufill 1990). Colon cancer incidence in Japan has increased, coincident with a progressive Westernisation of the diet (Honda *et al.* 1999). Importantly, only the distal cancer incidence has increased and not the proximal, this would suggest that proximal cancer incidence may not be directly related to diet (Hong *et al.* 2001). Although cancers in the proximal colon are associated with intrinsic factors (bile acids, sex hormones), distal cancers are more closely related to environmental factors such as diet, cigarette smoking and alcohol consumption (Giovannucci and Martinez 1996). Aneuploidy and *p53* alterations have consistently been related to distal tumour location (Breivik *et al.* 1997). Distally occurring CRC tends to be more infiltrating, recur more often and are associated with a poorer clinical outcome compared to proximal lesions (Tang *et al.* 1995). Genetic alterations such as aneuploidy, *p53* alterations and transversions have all been related to an unfavourable prognosis (Borresen-Dale *et al.* 1998). Conversely, proximal colon tumours tend to grow as polypoid fungating masses and be associated with a more favourable prognosis. Furthermore,

MSI has been associated with a better prognosis and are found almost exclusively in proximal tumours.

There has been a well documented shift in the site incidence of CRC, the shift being towards a higher incidence in the proximal colon (Beart *et al.* 1983;Kee *et al.* 1992;Slattery *et al.* 1996;Hayne *et al.* 2001). This shift is thought to be down to the advancing age of the Western populations and improved methods of diagnosing CRC in the upper colon. Another possibility is the differences in the epidemiology of proximal and distal CRCs could result from the presence of different procarinogenic factors in the ascending versus descending colon, acting on cells with similar or distinct biological characteristics. In a study examining the pattern of gene expression, found more than 1000 genes were differentially expressed in adult ascending versus descending colon. 165 genes showing > 2-fold and 49 genes showing > 3-fold differences in expression were found but there were only 87 genes that were differentially expressed in ascending versus descending foetal colon. This would indicate that although anatomically relevant differences are already established in embryonic colon, additional changes occur post-natally which may be due to environmental exposure (Glebov *et al.* 2003).

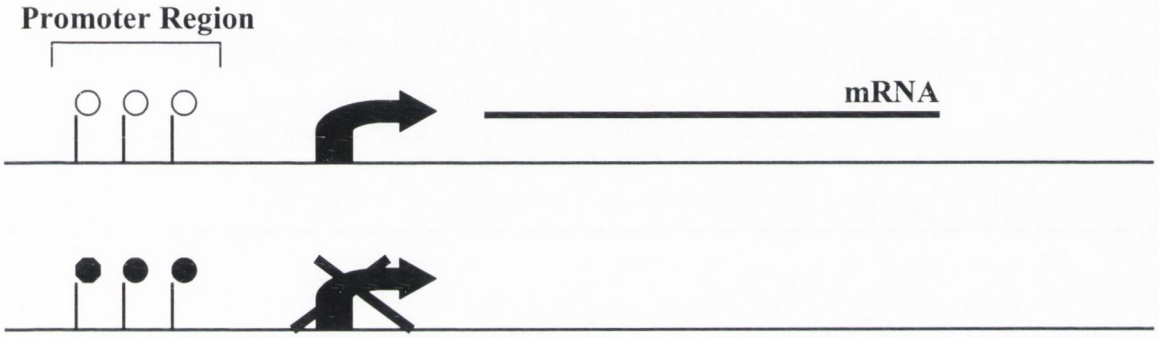


Figure 1.xvi Methylation of CpG islands in the promoter regions of genes causes gene silencing. (○ unmethylated CpG; (● methylated CpG. (Mathers 2003)

iv. DNA methylation

Methylation of cytosine residues at CpG sites is found throughout the human genome, characteristically clustered in "CpG islands" in the gene promoter regions. Methylation of cytosine residues confers structural stability. The presence of 5-methylcytosine in the AT rich areas of a gene has a local effect on the unwinding of DNA for the initiation of both replication and transcription (Hodges-Garcia and Hagerman 1995). Approximately 4% of all cytosines in mammalian DNA are methylated. Most of these 5-methyldeoxycidine residues are found in the palindromic sequence, cytosine guanine dinucleotide (CpG) (Riggs and Jones 1983) Hypo- and hypermethylation of these regions are related to activation and inhibition of transcription, respectively. DNA methylation patterns are maintained during cell division by 5-cytosine DNA methyltransferase (Ramsahoye *et al.* 1996). This enzyme preferentially methylates hemimethylated CpG sites, thus copying established methylation patterns to the newly synthesized DNA strands. This epigenetic replication process ensures that information related to gene expression and chromatin structure is maintained through cell division. DNA methylation has an important role in determining chromosome structure, spontaneous mutation rates, the direction of mismatch repair, the programmed destruction of chromosomes (aging), DNA/RNA synthesis rates and the regulation of transcription initiation or termination (Kim 1999). Different tissues and stages of development are related to specific methylation patterns and a growing amount of data has related cancer development to epigenetic alterations (Baylin *et al.* 1998). Furthermore, DNA methylation has been shown to be susceptible to different biological agents; this shows that DNA methylation despite a relatively high-fidelity maintenance, can respond to endogenous as well as exogenous factors (Slattery *et al.* 1997b). Different studies have implicated DNA methylation as a key factor in the aging process, increasing age has been related to a general hypermethylation of CpG islands in different tissues (Issa *et al.* 1996;Issa 2002) and appears to be a normal process in the aging process

(hypermethylation increases towards the distal segments). S-Adenosylmethionine (SAM) is the substrate for DNA methyltranferase, essential to CpG methylation and is the prime methyl donor for the body. The dietary factors choline, methionine, folate and vitamin B12 are key components of SAM synthesis and an insufficient supply may promote hypomethylation (Jhaveri *et al.* 2001;Friso *et al.* 2002). Vitamin B_{12} and methylcobalamin significantly enhance DNA methylation in the presence of SAM. It has been suggested that methylcobalamin can act as a methyl donor independent of SAM (Pfohl-Leszkowicz *et al.* 1991). A direct relationship has been shown between dietary folate deficiency, decreased SAM/SAH ratio and global hypomethylation of DNA (Balaghi and Wagner 1993). It has been also shown that supplementation with folic acid increases the degree of methylation present in DNA (Cravo *et al.* 1994). Hypomethylation of DNA does not occur in the presence of vitamin B_{12} deficiency, though hypomethylation of choline occurs; suggesting that methylation of DNA is conserved but other less vital structures will be sacrificed.

Conversely, ethanol and a number of experimental and environmental carcinogens will inhibit SAM synthesis and promote hypomethylation. Different chemotherapeutic agents also interfere with the DNA methylation process. The nucleotide analog, 5-azacytidine, is the most studied inhibitor of DNA methyltransferase; similarly, methotrexate interferes with methionine metabolism and affects the methylation process. Cisplatinum and 5-fluorouracil promote hypermethylation (Zingg and Jones 1997).

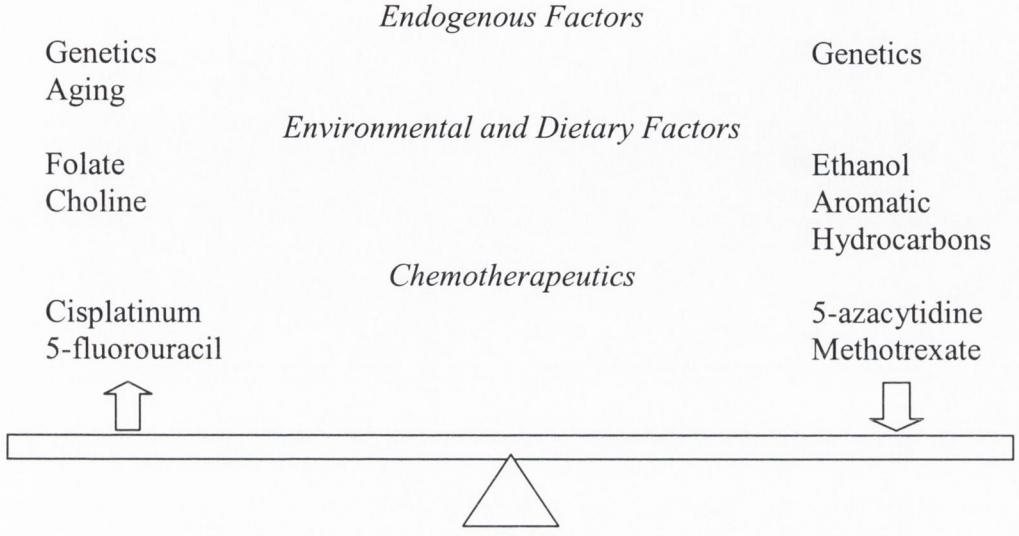


Figure 1.xvii **Factors affecting methylation status (Breivik and Laudernack 1999)**

Mismatch repair (MMR) is a post-replicative DNA repair system recognising mispaired bases that have eluded the exonuclease function of the DNA polymerase. To ensure that a misincorporated base is removed, discrimination of newly synthesised DNA from parental strands is necessary. DNA methylation permits such differentiation as the specific methylation of CG couplets is delayed for several hours after strand synthesis. Additionally, methylated cytosine residues influence the selection of DNA cleavage sites by restriction /mismatch repair enzymes. The presence of 5-methylcytosine in DNA may lead to spontaneous mutations due to deamination to thymine leaving a mismatched GT pair. Methylation compensates for such errors by permitting strand discrimination for correction (Griffin *et al.* 1994).

DNA methylation occurs in a specific fashion and influences gene expression. In general, the presence of a methyl group is suppressive and altered patterns of methylation have been found in several clinical syndromes such as Prader-Willi and Fragile-X (Reis *et al.* 1994). The phenotypic expression of changes to DNA methylation is dependent on site (promoter/inhibitor region) and gene type (suppressor/activator). The influence of such methylation patterns may be mediated through altered binding of regulatory proteins (Verdine 1994). The control of gene expression by DNA methylation is conserved across all evolutionary groups. However, the relationship between DNA methylation and gene expression is not as consistent as might be implied as genes may be unmethylated yet remain un-expressed.

Changes in the pattern of DNA methylation are a consistent finding in cancer cells. It has been proposed that damage to DNA, with subsequent repair, just before or after DNA replication can lead to the loss of methyl groups from specific DNA sequences adjacent to structural genes. Altered genomic methylation (hypo- or hypermethylation) is thought to cause more than one-third of all transitional mutations responsible for human cancer (Tornaletti and Pfeifer 1995) and has been reported in a wide spectrum of cancer types: colon, ovary, lung, kidney, brain, breast, leukocytes and liver (Kim 1999). Age-related genomic methyl group

changes may also predispose individuals to sporadic tumourigenesis (Issa *et al.* 1996). Many cancers display global hypomethylation compared to normal tissue and this has been proposed to represent the central phenomenon in the neoplastic process (Counts and Goodman 1995a). Demethylation has been related to overexpression of known oncogenes and 5-azacytidine promotes demethylation and cancer in animal models (Wainfan and Poirier 1992). In both animals and humans methyl-deficient diets are related to increased risk of CRC (Giovannucci *et al.* 1993) and feeding methyl-deprived animals with SAM leads to remethylation of DNA and reversal of the tumourigenic state (Christman *et al.* 1993). Conversely, many tumours may display regional hypermethylation associated with the inactivation of tumour suppressor genes and increased mutation rate at CpG sites (Zingg and Jones 1997). Furthermore, mice models deficient in DNA methyltransferase exhibit resistance to colorectal tumourigenesis initiated by mutation of the *APC* tumour suppressor gene and further demethylation by 5-azacytidine treatment enhances this resistance (Laird *et al.* 1995). Alterations in DNA methylation occur very early on in the neoplastic process.

In human studies using lymphocytic DNA, a consistent relationship between folate status and genomic DNA methylation has been observed. Marginal folate depletion has been shown to diminish genomic DNA methylation (Jacob *et al.* 1998;Rampersaud *et al.* 2000). The induction of genomic hypomethylation under these circumstances is probably due to the associated rise in Hcy, which in turn increases tissue concentrations of SAH, an inhibitor of methylation reactions (De Cabo *et al.* 1995). It has been for this reason that SAH and not SAM concentrations have been found to be most predictive of DNA methylation (Yi *et al.* 2000). Also in a recent study, a lower "methylation" ratio of SAM:SAH was observed in pathological adenocarcinoma tissue from resected colon compared to normal tissue, mainly due to much higher SAH concentrations (Varela-Moreiras *et al.* 2003). Cell proliferation is affected by DNA methylation. Specific reduction of cellular DNA methyltransferases in human cancer

cells result in inhibition of cell proliferation (Akoglu *et al.* 2001). DNA hypomethylation at critical loci might enhance expression of proto-oncogenes, such as *ras* and *c-myc*, or other genes related to carcinogenesis, resulting in excess cell proliferation (Ryan and Weir 2001).

iv. Gene-Specific Methylation Status

Hypomethylation at critical loci is probably a more important factor than genomic hypomethylation from the perspective of carcinogenesis. *p53* is the single gene most frequently altered in human malignancies and in about 70% CRC (Goh *et al.* 1994). The protein has a key role in the cellular defense against DNA damage and appears to function as a "guardian of the genome" (Lane 1992). *p53* is stabilized by bulky DNA adducts and single-stranded DNA (Lee *et al.* 1995) and the resulting accumulation promotes cell cycle arrest or apoptosis (Gotz and Montenarh 1996). Both BAF carcinogens and NER-induced strand breaks will thus promote *p53*-dependent growth arrest or apoptosis. This will lead to a selection pressure that favours the inactivation of p53. *p53* inactivation appears to be a very early event in some cancers but in CRC, alterations are very rare at this stage. The inactivation of *p53* appears to be involved in the malignant conversion of advanced adenomas (Fearon and Vogelstein 1990). Observations in folate-deficient rats have confirmed that dietary deprivation of folate is sufficient to produce hypomethylation within the "hypermutable region" of the *p53* gene (Kim *et al.* 1997). Subsequent studies have demonstrated that this depletion also decreases steady-state concentrations of *p53* mRNA (Kim *et al.* 2000). Consistent with the concept that these changes are involved in carcinogenesis are observations, which indicate that hypomethylation within exon 8 of the colonic *p53* gene, was also observed in animal models of carcinogenesis. More recently, folate depletion produced gene-specific, rather than genomic, DNA methylation in human nasopharyngeal carcinoma KB cells (Jhaveri *et al.* 2001). Cell culture studies indicate that these foci of aberrant methylation within the coding region may serve as initiators of

mutation (Shen *et al.* 1992) and may induce susceptibility to breakage of the DNA backbone at the site of hypomethylation (Pogribny *et al.* 1995) by increased uracil insertion and strand breaks (Blount *et al.* 1997). Hypomethylation of the coding regions of critical genes, which have been observed in experimental models of folate depletion, can lead to instability of the region either because it becomes more susceptible to endogenous nucleases or because the site of hypomethylation is more likely to undergo deamination to uracil (Blount *et al.* 1997). The latter situation is particularly prone to occur under conditions in which intracellular SAM concentrations are low (Zingg and Jones 1997). Alterations in methylation status within promoter regions is a very important epigenetic mechanism in gene control (Baylin *et al.* 2000). Because methylation of the promoter generally suppresses transcription, promoter hypomethylation can activate protooncogenes and promoter hypermethylation can inactivate tumour suppressor genes. Experimental deficiency of methyl nutrients in animals may instead cause a paradoxical hypermethylation of certain loci (Pogribny *et al.* 1997a), which is of interest because the promoter regions of several tumour suppressor genes such as *p16*, *p53* and *APC*, are frequently found to be hypermethylated in cancers (Schroeder and Mass 1997;Hiltunen *et al.* 1997). This hypermethylation may suppress the expression of the gene and is, therefore, hypothesized to contribute to the evolution of the cancer.

v. Misincorporation of Uracil

Folate deficiency induces breaks in chromosomes and these breaks are associated with an increased risk of cancer in humans. DNA strand breaks, that occur in the phosphodiesterase backbone of DNA, seem to be the molecular basis for this disruption in chromosomal integrity. Folate deficiency in cell culture and animal experiments have been shown to create an excess of strand breaks in DNA (Pogribny *et al.* 1995). In rat models, dietary folate deficiency induced gene-specific DNA strand breaks in the hypermutable region of *p53* in the colon and

decreased steady-state *p53* in that organ (Kim *et al.* 1997;Kim *et al.* 2000). This has also been seen in human studies (Lathrop-Stern *et al.* 2000). The most likely mechanisms by which folate deficiency may create such breaks are uracil misincorporation (Duthie *et al.* 2000a) and impaired DNA repair (Choi *et al.* 1998) . Folate deficiency reduces deoxythymidylate synthesis from deoxyuridylate, and the ensuing nucleotide imbalance increases the incorporation of uracil bases into DNA. Uracil in DNA is excised by the repair enzyme, uracil glycosylase, and in the process, a transient single-strand break develops in the DNA. Simultaneous removal and repair of 2 adjacent uracil residues on opposite strands can then result in a double-strand break, which is difficult to repair and further exacerbates DNA instability. Unrepaired double-strand breaks enhance cellular transformation in culture and increase cancer risk. Similarly, an in vitro study using immortalised human colon epithelial cells demonstrated that folate deficiency induced uracil misincorporation and increased DNA strand breaks (Duthie and Hawdon 1998). Excessive DNA uracil content has been observed in folate deficient humans also and both defects are easily reversed by folate supplementation (Blount *et al.* 1997). DNA excision repair, in a folate-deficient rat model, was impaired in colonic mucosal cells when compared to normal folate conditions (Choi *et al.* 1998). Folate supplementation above the RDA lessened chromosome breakage below concentrations observed in healthy, folate-replete persons (Fenech *et al.* 1998).

vi. Inherited Genetic Disorders

a. Familial Adenomatous Polyposis (FAP)

FAP is an autosomal dominant disorder in which multiple adenomatous polyps develop in the colon during the second and third decades of life. These polyps are histologically identical to those preceding sporadic CRC. Although individually each polyp has little risk of malignant transformation, the sheer numbers (reaching thousands in some patients) mean that the risk of developing CRC by the age of 40 years is very high. Linkage studies have shown

the gene involved, *APC*, is on the long arm of chromosome 5 (between q21 and q22). In FAP, multiple polyps are found throughout the gastrointestinal tract, the colon and the duodenum being the most noted. Constant endoscopic surveillance is necessary in all patients, as all will go on to develop cancer if followed for long enough. An attempt should be made to remove all polyps but this is often impossible. Owing to the high risk of cancer in this group and in any relatives found to have polyps (relatives must be screened after 12 years of age), a colectomy with an ileorectal anastamosis is performed with long-term surveillance of the anal stump. The Non-Steroidal Anti-Iinflammatory Drug sulindac has been shown to decrease the number and size of polyps. Congenital Hypertrophy of the retinal pigment epithelium can be seen in two-thirds of FAP patients and is useful in the screening of younger patients. Gardner's syndrome is a variant of this condition in which patients will also develop mesodermal tumours and pigmented occular fundal lesions is almost 100% (Caukwell and Quirke 1996).

The gene that causes FAP, the adenomatous polyposis coli (*APC*) gene has been cloned and sequenced. This means that advanced RNA and DNA assays have been developed that can detect mutations in symptom free family members with a sensitivity of 100%. Clinical studies have been done of phenotypes of specific mutations. Families that do not show congenital hypertrophy of the retinal pigment as a clinical marker of FAP generally have a mutation 5' to exon 9 of the *APC* gene (Olschwang *et al.* 1993). Less severe forms of FAP (fewer polyps and more patchy distribution) are associated with mutations at the extreme ends of the gene (Scott *et al.* 1995b). By contrast, profuse FAP has been localized to exon 15, codons 1255-1467 (Nagase *et al.* 1992).

The non-mutated *APC* gene encodes a 300 kDa cytoplasmic protein, and most of the mutations seen result in a loss of expression or truncation of the protein. Only one affected allele is required, which suggests a dominant negative effect of the mutant product in prevention of effective action of the wild-type protein. Although the precise function of the

wild-type *APC* gene product has yet to be characterized, it does bind to cellular transcription factors and β-catenin (Morin *et al.* 1997), which in turn binds cadherins and suggests a role in cell-to-cell interactions, adhesions and metastasis (Kemler 1993). . The *APC* protein is best known as a tumour suppressor in the human colon, this is due to the key role it plays in down regulating β-catenin in the *wnt* signalling pathway. *APC* mutations occur in the majority of colorectal neoplastic lesions, with no difference in the frequency of mutations from the smallest benign adenoma to carcinomas, which suggests it has a major role to play in the early development of CRC (Powell *et al.* 1992). Loss of function of *APC* can occur through gene silencing as result of hypermethylation of the promoter region of the gene. Hypermethylation occurs in approximatly 18% of sporadic CRC and in both adenomas and carcinomas. It appears to be an early event in tumourigenesis(Esteller *et al.* 2000). Somatic mutations are found mainly in the proximal half of the gene, with a preponderance in the so-called mutation cluster region ranging from codon 1250-1550 (Fearnhead *et al.* 2001). Mutations in this region are believed to be tumourigenic because they prevent β-catenin down regulation and result in the expression of oncogenes: such as *c-myc* (He *et al.* 1998), which give the initiating cell a selective advantage, cyclin D1 (the multidrug resistance 1 gene), which may initiate tumourigenesis by suppressing cell death pathways in colonocytes (Yamada *et al.* 2000), the dominant negative helix-loop-helix regulator Id2 (Rockman *et al.* 2001). In summary, *APC* is the gatekeeper gene for the colonic epithelium (Fodde *et al.* 1999) and loss of function of both *APC* alleles underlies both initiation and promotion of CRC (Fodde *et al.* 2001).

b. Hereditary Non-Polyposis Colorectal Cancer (HNPCC)

HNPCC describes an autosomal dominant predisposition to CRC that lacks the excess polyposis seen in FAP. The disorder is thought to account for up to 6% of CRC cases. HPNCC is classified into two Lynch syndromes, and an international group has defined a set of diagnostic criteria – the Amsterdam Criteria (Lynch *et al.* 1993;Vasen *et al.* 1991). Clinical

features of HNPCC include an association with other tumours (endometrium, ovary, hepatobiliary tract, skin), a predilection for the right side of the colon and an increased frequency of poorly differentiated mucinous tumours, which have a high propensity for invasion. The incidence of associated tumours can be strikingly high. The prognosis for the colon cancers arising in HNPCC is better than that for sporadic colon cancer. Nevertheless, prophylactic subtotal colectomy is appropriate. Screening is also an important part of surveillance in this group – risk of developing CRC can be reduced by 50% and mortality by up to 65% with 3 yearly screening colonoscopies (Jarvinen *et al.* 2000).

Molecular research in HNPCC was initially hampered by the absence of a gross haplotype abnormality. In 1993, linkage to a locus on the short arm of chromosome 2 was established for HNPCC patients in Canada and New Zealand. The gene responsible for this group was cloned, sequenced and named human Mut S Homolog (*h MSH2*) because of an observed homology to bacterial genes necessary for repair of mismatched DNA (Fishel *et al.* 1993). This accounts for 60% of HNPCC cases. Since then, three other involved genes have been identified – *h MLH1* (3p), *h PMS1* (2q) and *h PMS2* (7p). They account for 30%, 5% and 5% of cases respectively (Bronner *et al.* 1994;Nicolaides *et al.* 1994). All of these genes encode enzymes involved in DNA surveillance; if any are defective, mismatch bases in replication will not be repaired and genome-wide mutations will follow. Mutated mismatch genes produce an identifiable genetic marker referred to as microsatellite instability. Microsatellites are short-repeat DNA sequences that exhibit length polymorphisms and are especially susceptible to mispairing during replication, which creates DNA loops. In normal people, these loops are repaired. With dysfunctional repair genes, however, the mismatched loop is undetected and new alleles occur. Such alleles can be identified in HNPCC patients by amplification of the regions of DNA that contain microsatellites. Evidence suggests that the mismatch repair deficits cause cancer by inhibition of growth of colonic epithelium by the

tumour growth factor β receptor (*RII*) acting as a tumour-suppressor gene in colon cells. Inactivation of *RII* mutations are present in more than 90% of patients with microsatellite instability (Grady *et al.* 1997). Furthermore, 85% of cancers in HNPCC patients carry alterations in the *RII* gene at a short polyA repeat sequence (Akiyama *et al.* 1997). The frequency of the mutation in adenomas of HNPCC patients is lower (57%), which suggests that alterations of the tumour growth factor β *RII* gene might correlate with adenoma to carcinoma progression. Recently, *h MLH3* was suggested to be causative in HNPCC but most of the mutations were found in low risk patients, suggesting that it was a low risk gene which caused the hypothesis that the mutations work together in an additive manner to elevate risk in susceptible individuals (Liu *et al.* 2003b).

7. FOLATE AND CANCER

i. Introduction

Folate was identified as a possible important factor in the development of cancer through its involvement in the transfer of one-carbon groups, which are subsequently involved in the biosynthesis of purines and thymidylates. Consequently, folate deficiency in tissues with rapidly replicating cells results in ineffective DNA synthesis, resulting in reduced cell proliferation, impaired cellular physiology and abnormal cytologic morphology. This biological function has been applied to chemotherapy utilizing a number of anti-folate agents such as methotrexate and 5-fluorouracil to inhibit tumour growth. Conversely, it has been hypothesised that an imbalance in folate status may predispose epithelial tissues towards the adenoma-to-carcinoma sequence initiation. The possible role of folate in various cancers has been examined:

ii. Lung

Lower serum and RBC folate concentrations have been identified in smokers (Piyathilake *et al.* 1994;Voorrips *et al.* 2000) and they have also been observed to have significantly lower folate concentrations in the buccal mucosa than non-smokers. This observation suggests the repeated exposure to cigarette smoke might result in folate depletion of the respiratory epithelium making cells more at risk from the carcinogens in cigarette smoke (Piyathilake *et al.* 1992). Chemical compounds in cigarette smoke appear to convert some forms of folate into biologically inactive compounds (Khaled *et al.* 1986). However, the epidemiological studies that have been conducted to date have not yielded consistent results (Le Marchand *et al.* 1989;Bandera *et al.* 1997;Voorrips *et al.* 2000). The earlier case-control study found no significant association between dietary folate intake and the risk of lung cancer, while the more recent studies did find the most significant protective effect of dietary folate

was on the development of squamous cell carcinoma in the lungs of heavy smokers (Bandera *et al.* 1997;Voorrips *et al.* 2000). A recent study examined dietary folate intake in former smokers and its association with lung cancer. They found a significant dose-response relationship between increasing dietary folate and decreasing risk of lung cancer (P for trend <0.001). A more pronounced inverse association was observed in subjects who drank alcohol, had smoked more, who did not take supplemental folate and who had a family history of lung cancer (Shen *et al.* 2003). The randomised intervention studies published have found a significant improvement in the regression of bronchial metaplasia in those supplemented with 10-20mg folic acid and 500-750μg vitamin B_{12} compared to the control groups even though the numbers were low (73 and 38 smokers respectively)(Bailey and Krumdieck 1988;Saito *et al.* 1994). More evidence is needed from intervention trials using large numbers and epidemiological studies to confirm this relationship.

iii. Breast

The primary mechanism of oestrogen carcinogenesis is thought to be related to oestrogen receptor-mediated proliferation associated with spontaneous DNA replication errors (Henderson *et al.* 1988). However, catechol metabolites of oestrogen (catechol oestrogens) can also contribute to the carcinogenic process through their involvement in redox cycling and their metabolism to quinines, which can directly adduct DNA. As with catecholamines, the methylation of catechol oestrogens catalysed by catechol-O-methyltransferases (*COMT*) prevents quinone formation and redox cycling (Miller *et al.* 1996). The *COMT* genotype is polymorphic and a val to met coding polymorphism is present in up to 75% of Caucasians and results in a 3-4 fold decrease in the enzyme's activity. The low activity *COMT* allele has been found to be associated with breast cancer (Lavigne *et al.* 1997;Yim *et al.* 2001).SAM is the necessary methyl donor for the *COMT* catalysed reactions. Upon *COM*T substrate methylation,

SAM is converted to SAH, which is a known *COMT* inhibitor thus representing a negative feedback loop and it is through this function that folate is suspected to act as a chemopreventative agent in breast cancer. A recent study looked at data from two nested case-control studies and examined the relationship between *COMT* genotype, serum folate, vitamin B_{12}, pyridoxal-5'-phosphate (PLP) or active vitamin B_6, cysteine and Hcy (Goodman *et al.* 2001a). This study was the first to be able to show that low activity *COMT* and high Hcy or low levels of circulating folate were associated with breast cancer risk. However, the study number was relatively low and obtaining the SAM:SAH ratio would have been an advantage to further explore the *COMT* genotype's impact on the folate cycle. Further work is required to elucidate folate's role in breast cancer, also examining pre- and post-menopausal status may be of benefit owing to the different results obtained by studies using varying age groups (Lavigne *et al.* 1997;Yim *et al.* 2001).

iv. Cervical

Folate deficiency was first suggested as a risk factor for cervical cancer when several cytological similarities were noted between epithelial cells of folate deficient women and those from dysplastic cells from the same area, the cervix (Van Niekerk 1966). Subsequently, decreased blood serum concentrations were demonstrated in women using oral contraceptives (Streiff 1970), which are a known risk factor for cervical cancer (Stern 1977;Vessey *et al.* 1983). Examining case-control studies, the risk of developing cervical dysplasia is reduced by 60-70% in those with the highest intake of dietary folate compared to those with the lowest (McPherson 1989;VanEenwyk *et al.* 1992). Human Papilloma Virus-16 infection and persistence have been identified as the cause of most, if not all, cervical cancers. Women consistently positive for the oncogenic type HPV infections are at greater risk of developing cervical dysplasia, compared with those transiently infected. Folate status has been evaluated

in conjunction with HPV status but the results have been confounding (Butterworth *et al.* 1992a;Sedjo *et al.* 2003). The intervention studies completed to date in this area have proven also inconclusive (Butterworth *et al.* 1982;Butterworth *et al.* 1992b;Childers *et al.* 1995), this may be due to short duration of trials, small numbers of participants and some poorly controlled i.e. the selection criteria, but to-date the picture on cervical cancer and folate remains unclear.

v. Upper Gastrointestinal Tract

Although oesophageal cytology studies have been reported as being very similar morphologically to those associated with folate deficiency (Jaskiewicz 1989), further study into this area revealed that oesophageal cancer was more common in regions where overall nutritional status was poor and that it was this that predisposed individuals rather than folate uniquely. However, a recent case-control study, conducted in Switzerland in a well-nourished population, included 749 patients with incident cancer of the oral cavity and pharynx, and 1772 hospital controls with acute, non-neoplastic conditions. They used a validated food frequency questionnaire. The ORs were 0.68 (95% CI 0.52-0.88) for the intermediate tertile and 0.53 (95% CI 0.40-0.69) for the highest tertile of dietary folate intake, compared with the lowest tertile. No heterogeneity was found in strata of gender, age, methionine intake or alcohol consumption. The combined OR for low-folate and high-alcohol intake versus high-folate and low-alcohol intake was 22.3 (95% CI 13.1-38). This study supported a protective role of folate against oral and pharyngeal carcinogenesis. Compared with low folate intake, a consistent reduction in risk was already observed from intermediate levels of intake, suggesting that cancer risk may be related to relative folate deficiency.

The data relating folate and gastric cancer points more towards a similar picture, again it seems to be folate alongside other micronutrients that positively influence the health of the

tissue (La Vecchia *et al.* 1994;Nikias *et al.* 1995;Nomura *et al.* 2003). There have been few case-control studies on pancreatic cancer and the association of dietary factors. One such study looked at 104 cases and 253 controls and found that folate intake was inversely correlated with the risk of pancreatic cancer after correction for total energy, alcohol, tobacco use and other nutrients. The risk of developing pancreatic cancer was reduced by 64% in the individuals with the highest intake of folate (OR=0.36; 95%CI 0.18-0.74) compared to those with the lowest (Baghurst *et al.* 1991). More recently, it has been shown that dietary folate intake was inversely associated with pancreatic cancer and the risk was greater with heavy smoking (Stolzenburg-Solomon *et al.* 1999a;Stolzenburg-Solomon *et al.* 2001). It has also been shown that serum Hcy and erythrocyte SAH and SAM levels are raised and lowered respectively in patients with diabetes mellitus and renal failure. This is likely to be reflecting disturbed methylation patterns, which may contribute to the pathogenesis of several pancreatic lesions (Longnecker 2002). The possible involvement of folate in hepatic cancer has been explored but to date the extent of this research has been in animal models and cell lines. A recent study looked at methyl-CpG-binding protein (*MeCP2*) that binds specifically to methylated DNA and induces chromatin remodelling and gene silencing. Dietary folate deficiency has been shown to decrease tissue SAM and cause global DNA hypomethylation, hepatic steatosis, cirrhosis and ultimately hepatic tumourigenesis in rodents (Rogers 1993). The study investigated the effects of a diet deficient in folate, choline and methionine on the expression of *MeCP2* during the preneoplastic transformation of rat liver (Esfandiari *et al.* 2003). The level of *MeCP2* mRNA was increased in the deplete models after 9 weeks and the *MeCP2* protein level was reduced almost two-fold in the deplete rats after 9 and 36 weeks. A known target gene of *MeCP2*, the tumour suppressor gene metallothionen-I, was overexpressed in the deficient rats at nine and 36 weeks; this suggests that the reduction in *MeCP2* has functional consequences. Methyl deficiency also caused an increase in the ratio of long to short variants of *MeCP2* transcripts,

suggesting reduced *MeCP2* protein level is as a result of reduced rate of translation. Reduction of *MeCP2* protein expression may influence the initiation and/or progression of hepatic cancer induced by methyl deficiency.

In summary, there has been some research done in the area of folate and upper gastrointestinal cancer but to date the results do not allow any definite conclusions to be drawn, as there is insufficient data available.

vi. Colorectal

The role of folate deficiency and supplementation in CRC has been extensively studied and diminished folate status (assessed through systemic markers of folate status) has been found to be associated with increased risk of CRC in most, but not all, epidemiological studies.

a. Ulcerative Colitis

Lashner and colleagues were the first to investigate the role of folate in CRC (Lashner *et al.* 1989). The study used a chronic ulcerative colitis (UC) population group as this group have a 10- to 40-fold increased risk of developing colorectal dysplasia and CRC compared to the general population and the frequent use of sulphasalazine alongside frequent intestinal losses due to inflammation (Longstreth and Green 1983). Sulphasalazine is thought to impair folate absorption through competitive inhibition of both folate transport enzymes and enzymes involved with folate metabolism, the presence of luminal sulphasalazine can decrease folate absorption by more than 30% (Selhub and Rosenberg 1978). In the 1989 retrospective cohort study, 99 patients with longstanding UC were included. 35 patients with neoplasia were compared with 64 patients without abnormalities, there was information on folate supplementation for 88 of these with daily doses ranging from 0.4 to 1mg. Folate use was associated with a decreased risk of finding cancer or dysplasia during the surveillance period (OR 0.38, 95%CI 0.12-1.2). Furthermore, chronic administration of sulphasalazine was

associated with a 50% increase in the risk of dysplasia (OR 1.5, 95% CI 0.43-5.19); unfortunately, the association was not statistically significant for both observations. Interpretation of the results is limited by the lack of information regarding the surveillance and biopsy practises at colonoscopy, the duration of folate supplementation and lack of measurements of systemic folate status. A subsequent study by Lashner and co-workers, looked at a cohort of 98 patients with chronic UC who had intact colons and in whom no dysplasia or cancer had developed within 2 years of referral were included (Lashner *et al.* 1997). This time, folic acid supplementation (0.4 or 1mg/day) was analysed as a dichotomous variable, which use for at least 6 months compared to less than 6 months or no use. The adjusted relative risk of development of dysplasia and cancer for folic acid use of at least 6 months was 0.72 (95%CI 0.28-1.83). An insignificant trend towards a dose response effect was seen. In another case-control study involving 67 patients with UC (6 cases with neoplasia and 61 controls), Lashner found that RBC folate was 13% lower in cases (453.7±123ng/ml vs. 519.8±297.7ng/ml). Following construction of a logistic regression model, the risk of dysplasia or cancer was significantly decreased by 18% for each 10ng/ml increase in RBC folate (OR 0.82, 95%CI 0.68-0.99). However, other case-control studies have found no effect or sulphasalazine being protective rather than increasing the risk of developing colonic neoplasia (Fiedler *et al.* 1993;Pinczowski *et al.* 1994).

b. Animal Models

Animal models have been used to explore the pathways through which folate might modulate CRC risk. An early study investigated experimental folate deficiency to see if it increased the likelihood of developing neoplasia in rats treated with the carcinogen dimethylhydrazine. Weanling male Sprague-Dawley rats were fed with an amino acid-defined diet containing either 8 or 0 mg/kg folic acid. After 5 weeks of defined diet, weekly s.c. injections of dimethylhydrazine (DMH) (20 mg/kg) were administered to both groups. Serum,

whole blood, liver, and colonic folate concentrations at the time of sacrifice were significantly lower in folate-depleted animals ($P < 0.001$). There were significant differences in the incidence of colonic neoplasia between the two groups after 20 weeks of dimethylhydrazine exposure: folate-deficient rats had a greater incidence of dysplasia (6 of 7 versus 2 of 7 animals; $P < 0.05$) and carcinoma (6 of 7 versus 1 of 7 animals; $P < 0.01$). Furthermore, a significantly greater proportion of folate-replete rats than folate-deficient rats were free of neoplastic lesions (5 of 7 versus 0 of 7 animals; $P < 0.05$). These results suggest that, in that animal model, folate deficiency increases the risk of malignancy when there was an underlying predisposition to colorectal cancer (Cravo *et al.* 1992). Other points to note from this study, 100% of rats fed the folate-depleted diet developed microscopic neoplasms after 20 weeks compared to 29% of rats on the control diet, while the incidence of macroscopic neoplasms were 86% and 43% in the folate-depleted and control groups respectively ($P=0.09$). This supports the effect of folate on early CRC development. In a further study using the same DMH rat model of CRC, the rats were fed diets containing 0, 2 (daily dietary requirement), 8 or 40 mg folate/kg diet for 20 weeks. After five weeks of diet, rats were injected with DMH (44 mg/kg) weekly for 15 weeks. Fifteen weeks after the first injection of DMH, all rats were killed. Folate status was determined, and the entire colorectum from each rat was analysed for macroscopic and microscopic neoplasms. Plasma and colonic folate concentrations correlated directly with dietary folate levels ($p < 0.005$). The incidence of microscopic neoplasms was similar among the four groups. However, the incidence and the average number of macroscopic tumours per rat decreased progressively with increasing dietary folate levels up to 8 mg/kg diet ($p < 0.05$). These data indicate that in this rat model increasing dietary folate up to four times the basal requirement leads to a progressive reduction in the evolution of macroscopic neoplasms from microscopic foci; and that folate supplementation beyond four times the requirement does not convey further benefit (Kim *et al.* 1996). A study looking at

uracil misincorporation found that a significant increase in the level of uracil in the liver was seen as early as 3 weeks after initiation of a folate/methyl deficient diet and that it was accompanied by parallel increases in DNA strand breakage and premutagenic apyrimidine sites. Uracil was also detected in the *p53* gene sequence which implies that the capacity for uracil base excision repair was exceeded with chronic folate/methyl deficiency (Pogribny *et al.* 1997b). An interesting study in *Apc+/- Msh2-/-* mice, which carry a heterozygous mutation in the *Apc* gene and a null mutation in the *Msh2* gene (*Apc+/-Msh2-/-*), were used to determine the effect of dietary folate on intestinal tumourigenesis. *Apc+/- Msh2-/-* mice were randomized to receive either 0 or 8 mg of folate/kg diet starting at either 3 or 6 weeks of age. The 3- and 6-week diet starts represent intervention before and after the establishment of neoplastic foci, respectively. At 11 weeks of age, the mice were killed, and the small intestines and colons were analyzed for adenomas and aberrant crypt foci (ACF). Serum folate concentrations accurately reflected dietary folate levels ($P < 0.005$). Folate supplementation, started before the establishment of neoplastic foci, significantly decreased the number of small intestinal adenomas by 2.7-fold ($P = 0.004$) and colonic ACF by 2.8-fold ($P = 0.028$) and colonic adenomas by 2.8-fold ($P = 0.1$) compared with a moderate degree of folate deficiency. In contrast, a moderately folate-deficient diet, started after the establishment of neoplastic foci, significantly reduced the number of small intestinal adenomas by 4.2-fold ($P = 0.001$) but had no effect on colonic ACF and adenomas compared with folate supplementation. Genomic DNA methylation and microsatellite instability do not seem to play a major role in folate-modulated intestinal and colonic tumourigenesis in this model. In this murine model, dietary folate supplementation significantly protected against small intestinal and colorectal tumourigenesis if it was provided before the establishment of neoplastic foci. However, if it was provided after the establishment of neoplastic foci, dietary folate seemed to have an

opposite effect. These data suggest that the timing of folate intervention is critical in providing an effective and safe chemopreventive effect on intestinal tumourigenesis.

Acetaldehyde has been postulated as a possible factor responsible for the ethanol-associated CRC risk. High levels of acetaldehyde accumulate in the large intestine via the microbial oxidation of alcohol. Acetaldehyde degrades folate in vitro. Thus, it is possible that high intracolonic acetaldehyde levels break down folate in the colon. A study tested the effect of high alcohol and acetaldehyde concentrations in the gut on systemic and local intestinal folate levels in rats. Twenty rats received 3 g/kg of ethanol twice a day for 2 weeks with or without concomitant ciprofloxacin administration. Twenty control rats received saline with or without ciprofloxacin. All rats were fed a diet with normal folate content. Alcohol treatment led to very high intracolonic acetaldehyde levels (387 +/- 185 μM), which were markedly decreased by concomitant ciprofloxacin treatment (an antibiotic that reduces the aerobic and facultative anaerobic flora of the gut, which significantly reduces the microbiological production of acetaldehyde from ethanol) (21 +/- 4 μM). Erythrocyte, serum and small intestinal folate levels were unaffected by alcohol treatment. Alcohol administration decreased significantly colonic mucosal folate levels by 48%, and this effect was prevented by ciprofloxacin. It was concluded that alcohol administration for 2 weeks leads to local folate deficiency of colonic mucosa in rats, most probably via the degradation of folate by the high levels of acetaldehyde microbially produced from ethanol (Homann *et al.* 2000).

In a recent study, a "Western-Style" diet (increased fat content and reduced calcium, vitamin D, fibre) was fed to normal C57/B16 mice that also had reduced methyl-donor nutrients (folic acid, methionine and choline) to nutrient-density levels approximating to those consumed by humans. A second diet was also constructed which was as above except it contained increased amounts of calcium and vitamin D, a control group was also used. The results of feeding this diet for 12-18 months, without exposure to carcinogens or use of

targeted mutation murine models, were that the increased level of calcium and vitamin D in the second western-style diet were insufficient to prevent the development of tumours in the colon, highlighting the importance of dietary folate and methyl-donors in prevention of tumourigenesis (Newmark *et al.* 2001). Another study examined the levels of SAM and SAH and of global DNA methylation in the pre-neoplastic small intestine of Min (multiple intestinal neoplasia) mice, the effect of folate/choline deficiency on these parameters and on tumor multiplicity was also examined. In folate-adequate Min mice, a positive linear correlation between SAM and SAH and tumor numbers ($R^2 = 0.38$, $P < 0.005$; $R^2 = 0.26$, $P = 0.025$, respectively). A positive correlation between global DNA hypomethylation and tumor multiplicity was also observed ($R^2 = 0.29$, $P = 0.014$). SAM, SAH and DNA hypomethylation may, therefore, serve as early markers of cell transformation. Folate/choline deficiency, however, did not produce a consistent effect on tumor numbers in three separate experiments. As an increase in tumor numbers was observed only in folate- and choline-deficient mice with low levels of SAM and DNA hypomethylation, the modulatory role of folate may be dependent on the transformation state of the cell (Sibani *et al.* 2002). The issue of age and its relationship with folate and CRC risk was examined in weanling and 1-year-old Sprague-Dawley rats using 0,4.5 or 18μmol/kg folic acid (deplete, replete and supplemented groups respectively). Folate-deplete and replete older rats had 30-45% lower colonic folate concentrations than the weanlings. 5-MTHF was uniformly depleted in colons of the older, folate-deplete rats, whereas this depletion occurred only in a minority of weanlings. At the final stage of the study, the older rats had 50% more uracil incorporated into their colonic DNA than the weanlings ($P<0.05$). In the older rats, this misincorporation was incremental across the diet groups (P-test for trend <0.05), whereas there was no excess misincorporation seen in the young rats. This showed that the colonic mucosa of older rats was more susceptible to folate depletion, and its consequences, than young rats. However, folate supplementation at four times the basal

requirement was just as effective at repairing depletion in the older rats as the younger ones (Choi *et al.* 2003).

c. Dietary Folate and Colorectal Cancer

Several case-control and prospective studies have investigated the relationship between dietary folate intake and the risk of colorectal adenoma development, which is a well-established precursor of CRC (Fearon and Vogelstein 1990). These studies have examined dietary folate intake through various food frequency questionnaires. In the case-control studies (Benito *et al.* 1993;Bird *et al.* 1995;Tseng *et al.* 1996;Boutron-Ruault *et al.* 1996) an average reduction in risk of 35% was found in individuals with the highest dietary folate compared to the lowest. Most showed a statistically significant inverse relationship between the risk of developing colorectal adenomas and dietary folate intake in a dose-dependent fashion. The protective effect was more evident in women than men in some studies (Tseng *et al.* 1996;Boutron-Ruault *et al.* 1996). The most convincing epidemiological evidence comes from the Nurses' Health Study and the Health Professionals Follow-up Study (Giovannucci *et al.* 1993). A semi-quantitative food frequency questionnaire was used to determine the intake of certain nutrients. Histopathologically confirmed cases of adenomatous polyps of the distal colon and rectum (N=895) were included in the final analysis. Dietary folate was inversely associated with risk of colorectal adenoma in a dose-dependent manner. Folate intake from foods had a weak inverse association but when alcohol intake was considered, those with moderate to high intakes had a significantly increased risk of developing colorectal adenomas. The level of folate defined as the lowest quintile was <166μg/d for women and <241μg/d for men, which is the RDA for the U.S. population. This suggests that only a modest decrease in folate intake was associated with an increased risk of developing colorectal adenomas. Another prospective study (Baron *et al.* 1998), showed that after adjustment for energy intake, dietary folate had a significant protective association with the risk of recurrent colorectal adenomas

(P=0.04). The relationship was removed when adjusted for fibre and fat (P=0.69). The use of folate supplementation was not associated with reduced risk but it must be noted that the follow-up period was only for 4years. Moderate-to-high alcohol was again associated with increased risk (OR = 2.04; 95% CI 1.28-3.26).

The relationship of dietary folate and the risk of CRC have been examined in several case-control and prospective studies. The case-control studies have, in general, found a 35% reduction in the risk of developing CRC in those with the highest dietary folate intake compared with those with the lowest (Benito *et al.* 1991;Ferraroni *et al.* 1994;Glynn *et al.* 1996;La Vecchia *et al.* 2002;Freudenheim *et al.* 1991;Meyer and White 1993;La Vecchia *et al.* 1997;White *et al.* 1997). Some of the studies showed a gender or site-specificity, while a dose-dependent inverse relationship between dietary folate and CRC, risk was found in the majority of studies. One study that took a different slant on the role of folate in CRC risk looked at thymidylate synthase, which is a key enzyme in folate metabolism. Thymidylate synthase repeat polymorphisms of the promoter enhancer region (2repeat versus 3repeat of 28bp) are associated with decreased expression and a 6bp deletion in the 3' untranslated region may affect RNA stability. These polymorphisms were investigated in a case-control study of adenomatous polyps (510 cases and 604 controls). There was a significant gene-nutrient interaction seen between the thymidylate synthase enhancer region polymorphism and folate intake: among 3repeat/3repeat individuals (greater expression), folate intake >440μg/day (highest tertile) against ≤440μg/day was associated with a two-fold decreased risk (OR 0.5, 95%CI 0.5-0.9). However, among 2repeat/2repeat individuals, a high folate intake was associated with a 1.5 fold increase in risk (OR reference group 0.6, 95%CI 0.4-0.9 versus OR 0.9, 95%CI 0.5-1.5, P=0.03) (Ulrich *et al.* 2002), similar results were found by Chen and co-workers (Chen *et al.* 2003). This may have implications for folate supplementation and

chemoprevention as well as for chemotherapy treatment as 5-fluorouracil targets thymidylate synthase primarily.

Prospective studies have examined both dietary folate sources (Giovannucci *et al.* 1995b;Su and Arab 2001;Konings *et al.* 2002a) and supplement use (Giovannucci *et al.* 1998;Fuchs *et al.* 2002;Jacobs *et al.* 2003) and risk of developing CRC. The results from the dietary folate analysis studies have yielded support for the inverse relationship between dietary folate and CRC risk. The Health Professionals Follow-up Study showed a non-significant 15% reduction in risk in those with the highest folate intake compared to the lowest as well as a non-significant inverse dose-response relationship (Giovannucci *et al.* 1995b). However, when those individuals with low alcohol, high folate and methionine diets were compared with those with high alcohol, low folate and methionine, the risk reduction was 70% (RR=0.30, 95%CI 0.14-0.63) with a predominant protective effect on the distal colon (RR=0.13, 95%CI 0.03-0.58). The NHANES I Epidemiological Follow-up Study consisted of almost 14,500 subjects with a 20year follow-up (Su and Arab 2001). In this study a marginally significant association was observed between folate intake and reduced CRC risk but when gender and alcohol consumption were examined, dietary folate was significantly inversely associated with colon cancer in men (RR=0.40, 95%CI 0.18-0.88) who consumed more than 249μg/day of folate and there was a significant dose-response relationship (p=0.03). The association did not reach statistical significance in women. Using a composite dietary profile, a significant increase in risk was found for men who consumed a high alcohol, low folate and methionine diets when compared with male, non-drinkers with a high folate and methionine diets (RR=2.67, 95%CI 1.16-6.16). The Netherlands Cohort Study comprised of 120,852 men and women aged 55-69 and after 7.3 years follow-up the occurrence of CRC was examined (Konings *et al.* 2002a). An inverse association was found between dietary folate and CRC risk for the highest total dietary intake against the lowest, an inverse association was also found for male rectal carcinoma risk.

Of the studies that looked at folate supplementation, Giovannucci and co-workers evaluated this alongside overall energy adjusted dietary folate intake. 88,792 women took part in the Nurses' Health Study at the initial time point in 1980 and they were reassessed in 1994 (Giovannucci *et al.* 1998). Higher folate intake in 1980 was related to a lower risk of CRC (RR=0.69, 95%CI 0.52-0.93) for intakes of >400μg/day compared with intakes of ≤200μg/day after controlling for other impacting factors. Women who used multivitamins for 15 years or longer showed a markedly lower risk of developing CRC (RR=0.25, 95%CI 0.13-0.51). This represented 15 new cases of colon cancer per 10,000 instead of 68 in women aged 55 to 69 years of age. Folate from dietary sources alone was related to a modest reduction in risk for CRC and the benefits of multivitamin use were present across all levels of dietary intakes. Further analysis of this study group was carried out to examine the impact of familial risk on the above (Fuchs *et al.* 2002). The inverse association of folic acid with colon cancer risk was greater in women with a family history. Compared with women who consumed 200 μg or less of folic acid/day as part of a multivitamin or as a supplement, the age-adjusted RR of colon cancer for those who consumed >400 μg/day was 0.81 (95% CI 0.62-1.07) in women without a family history of colorectal cancer and 0.48 (95%CI 0.28-0.83) in women with a family history (P = 0.02). The influence of family history was markedly diminished by use of multivitamins containing folic acid (P = 0.04). High levels of dietary methionine also reduced the effect of family history (P = 0.05), whereas moderate to heavy alcohol consumption increased the risk associated with family history (P = 0.004). Recently, the Cancer Prevention Study II Nutrition Cohort was examined to see if regular multivitamin use reduced CRC incidence among 145,260 men and women for a 10 year period (Jacobs *et al.* 2003). The results supported the previous studies in finding regular use of multivitamins was associated with reduced risk if used for > 10years (RR=0.71, 95%CI 0.87-1.23).

d. Folate Status and Colorectal Cancer Risk

The relationship between systemic markers of folate status and the risk of colorectal adenomas is still under debate. Prospective clinical studies have produced varying results, in some the serum folate was not significantly different between cases and controls (Paspatis *et al.* 1995;Meenan *et al.* 1996;Meenan *et al.* 1997;Kim *et al.* 1998) while in others significance was found (Kim *et al.* 2001b;Kim *et al.* 1998). The same results have been shown in RBC folate in the same papers; although Paspatis' group were unable to show significance, the RBC folate in the case group was 28% lower than the controls even though it was still within the normal range (536ng/mL) suggesting that a modest reduction in status can increase risk. Sample size seems to be the most common fault of all studies so finding no difference in systemic measures may be because of a type II error. Studies looking at higher numbers of cases (Bird *et al.* 1995;Kato *et al.* 1999) and controls have supported folates role in CRC. The Bird study group looked at 332 subjects and 350 controls and found a protective effect of RBC folate concentration against the development of colorectal polyps, at least in men (OR=0.53, 95%CI 0.32-0.87). The New Yorker University Women's Health Study Cohort (Kato *et al.* 1999) examined 105 cases and 523 matched controls and found the risk of CRC was almost twice as high in cases with below-median serum folate and above-median total alcohol intake compared with those with above-median serum folate and below-median alcohol consumption (OR=1.99, 95%CI 0.92-4.29). There have been few studies in the area of local colonic folate concentrations and CRC risk. All completed studies to date have suffered from small sample size as to obtain the colonic tissue folate concentration, either the patient must undergo a full colonoscopy or sigmoidoscopy and this has, in itself, has ethical considerations as it is an invasive procedure. Another problem comes from the different methods of analysis of the biopsy tissue. The methodology used in the U.S. looked at whole biopsy tissue folate while the Meenan method examined epithelial cell folate. Both methods have had criticism levelled at

them. For the whole biopsy method, it can be argued that the entire biopsy will be composed of a variety of cell types - the average punch biopsy will contain epithelium, lamina propria, lymphatic nodules, smooth muscle fibres, muscularis mucosa, some submucosa containing lymphatic vessels, venules and arterioles. The evidence for CRC and folate's involvement is derived from the epithelial layer being involved in the adenoma-to-carcinoma sequence and not from any other cell types so it can be argued that whole biopsy tissue folate may not be reflecting the true involvement of folate in CRC at tissue level. For the epithelial cell isolation method, it has been argued that the processing of the biopsy to isolate the epithelial cells may allow cellular folate to equilibrate from the time of biopsy to when the epithelial cells are isolated. The studies that examined whole biopsy folate have found, in general, correlations between tissue folate and systemic folate status (Kim *et al.* 1998;Kim *et al.* 2001b), which homocysteine being the most significant. However, in the latter study where supraphysiological folate supplementation was given for a year, these correlations were not seen and the correlations shown for the systemic markers against colonic tissue folate appear to be driven by the supplementation group. In contrast, the studies carried out by Meenan and co-workers were unable to show a relationship between serum or RBC folate and colonic epithelial cell folates (Meenan *et al.* 1996) It may also be worth noting that plasma Hcy was not measured in the Meenan studies. Another study showed that folate depletion occurred in the neoplastic but not the adjacent normal tissue ($p<0.02$). While adenomas showed lower levels of epithelial cell folates, this did not reach statistical significance ($p<0.06$) (Meenan *et al.* 1997). The effect of folate on the colorectal mucosa may not be entirely derived from the blood because the colorectal mucosa is exposed to luminal folate synthesized by intestinal microflora or that has escaped small intestine absorption (Camilo *et al.* 1996). Therefore, the absolute relevance of the systemic markers has been queried, particularly in light of studies which have shown a modest reduction in folate status resulted in increased risk.

Recent studies have been looking at the possible pathways through which folate status may be involved. Pufulete and colleagues have examined the influence of folate status, DNA methylation, and polymorphisms of methylenetetrahydrofolate reductase (MTHFR 677C-->T and 1298A-->C), methionine synthase (MS 2756A-->G), and cystathionine-beta-synthase (CBS 844ins68) on risk for developing colorectal neoplasia (Pufulete *et al.* 2003b). Thirty-five patients with adenoma, 28 patients with cancer, and 76 controls were recruited for a case control study. Blood samples were obtained for determination of blood folates, vitamin B_{12}, Hcy, DNA methylation, and genotypes. Tissue biopsy samples were obtained at colonoscopy for determination of DNA methylation in colonic mucosa. Constructing a score from estimates of dietary intake and serum and erythrocyte folate assessed folate status. Cancer patients had 26% lower folate status (95% CI 6%- 44%, P = 0.01) and 21% lower serum vitamin B_{12} concentration (95% CI -38% -1%, P = 0.06) compared with controls. [^{3}H] methyl incorporation into colonic DNA was 26% higher in patients with adenoma (95% CI 8%-56%, P = 0.009) and 30% higher in patients with cancer (95% CI -3%-48%, P = 0.08) compared with controls. High folate status was associated with decreased risk for cancer (P = 0.01 for trend). Colonic and leukocyte DNA hypomethylation were associated with increased risk for adenoma (P = 0.02 and P = 0.01 for trend, respectively) and a non-significantly increased risk for cancer (P = 0.09 and P = 0.08 for trend, respectively). Friso and co-workers were able to show genomic DNA directly correlated with folate status and inversely with plasma Hcy levels (P<0.01). TT genotypes had a diminished level of DNA methylation compared to those with CC genotype (P<0.0001). Moreover, in TT individuals DNA methylation status was correlated with the methylated proportion of RBC folate and was inversely correlated with formylated proportion of the red blood cells folate (P<0.03) that is known to be solely represented in these individuals. These results indicate that the C677T polymorphism influences DNA methylation status through an interaction with folate status (Friso *et al.* 2002). Reduced DNA repair

capacity and folate deficiency have been implicated in increased cancer risk. In a recent study (Wei *et al.* 2003) found that calorie-adjusted total folate intake was an independent predictor of DNA repair capacity ($P<0.001$). Additional stratification analysis indicated that this association was more pronounced in those who did not use folate supplements compared to those who did.

e. Intervention Studies

Several intervention studies have been carried out but again to date these have all had small numbers of participants. Cravo and colleagues have shown that folic acid supplementation at 10mg/d for 6 months in patients with either colonic adenoma or cancer (n=22) followed by removal of these lesions can significantly reverse genomic hypomethylation in normal rectal mucosa. During the washout period, it was shown that DNA methylation values moved toward the initial values in most cases (Cravo *et al.* 1994). In another study, 60 subjects with colorectal adenoma were randomised to receive either 1mg/day folic acid or placebo for 2 years after removal of the adenoma. Although adenoma recurrence was reduced by 40% to 46% in the folic acid supplementation group compared to the placebo group at year 1 and 2 colonoscopic follow-up, respectively, the results fell short of statistical significance (Paspatis and Karamanolis 1994). An intervention study in Portugal looked at the effect of a 3-month supplementation of 5mg/day in 20 subjects post-adenoma removal. The subjects with only one adenoma responded to the folic acid supplementation with a 40% increase in genomic DNA methylation ($p=0.05$), whereas those with greater than one adenoma showed no increase (Cravo *et al.* 1998). Another study ran an intervention for 1 year using 5mg/day in a placebo-controlled manner in colonic adenoma patients. The folate supplementation group and placebo showed increased genomic DNA methylation and decreased strand breaks in exons 5 through 8 of the *p53* tumour suppressor gene ($p<0.01$) (Kim *et al.* 2001a). Suggesting that factors other than folate supplementation were responsible for the

parallel increase in methylation of DNA and increased stability of the *p53* gene. An Australian group investigated the effect of the combination of 700μg folic acid and 7μg vitamin B_{12} per day over a 12 week period and then 2mg folic acid and 20μg vitamin B_{12} over an additional 12 weeks in relation to DNA methylation and damage as measured by micronucleated cell frequency in healthy volunteers (18-32 years of age) (Fenech *et al.* 1998). The micronucleated cell frequency was significantly reduced by 25.4% in those individuals who were initially in the high 50th percentile ($p<0.01$) but overall the genomic DNA methylation status was not altered in the supplementation group. It has been shown in a trial of patients with colonic polyps that a 3 month supplementation of 5mg folic acid /day resulted in a 35% decrease in abnormally high ornithine decarboxylase activity in polyps that was accompanied by a 43% increase in SAM content in polyps (Bukin *et al.* 2001). An Italian study was able to show that a dose of 15mg/day folic acid significantly reduced cell proliferation in the upper 40% of the colonic crypts (Biasco *et al.* 1997) of UC patients; an expansion of the proliferative compartment from the lower 60% into the upper 40% of the crypts has been observed in patients with CRC (Bostick *et al.* 1997). A further study examined the same end point using a supplement dose of 2mg folic acid for 3 months duration in patients with recurrent adenomatous polyps (n=6) and a control group receiving placebo (n=5). Rectal biopsies were taken at commencement, 4, 12 and 18 weeks. The biopsies at each time point were immediately incubated in culture medium enriched with BrdUrd. The S phase cells, which incorporated the BrdUrd into their DNA, were identified following immunohistochemical staining. BrdUrd labelling indices (LIs) were calculated for the entire crypt and for each of five equal compartments running consequently from the base to the luminal surface, 25 orientated crypts were identified for each time point and the number and position of BrdUrd positive and negative cells were counted. The LIs of the treatment group were lowered after 12 weeks of supplementation (9.1 (6.7-12.3) v 7.4 (5.3-9.6)). The LIs for compartments within the crypt

showed that the most significant drop in number of proliferating cells was in the upper most regions of the crypt. This study indicated that folate supplementation decreased the colonic mucosal cell proliferation and that the most significant reduction was in the luminal aspect of the crypt (Khosraviani *et al.* 2002).

8. VITAMIN B12

i. Introduction

Vitamin B_{12} consists of a cobalt-substituted porphyrin ring and is linked to ribose and phosphoric acid. The general name used is cobalamin. The metabolically important cobalamins differ from each other in having either a methyl group or a 5'-deoxyadenosyl group attached to the cobalt. Cyanocobalamin, the commercial and therapeutic product, is present in minimal amounts in the diet and tissue. The principal natural and dietary forms are 5-deoxyadenosylcobalamin, methylcobalamin and hydrocobalamin. Cobalamin is a cofactor for only two known human enzymes, methionine synthase (MS) and methyl-malonyl-CoA mutase (Cooper and Rosenblatt 1987). In the methionine synthase reaction, the methyl group of 5-MTHF is transferred to cobalamin to form methylcobalamin, which then donates the methyl group to Hcy. The end products of the reaction are methionine, cobalamin and hydrofolate. Recently, the molecular mechanism for the regulation of Hcy metabolism by cobalamin supplementation was investigated. Cobalamin supplementation did not alter mRNA or protein turnover rates but induced translational up-regulation of MS by shifting the mRNA from the ribonucloprotein to the polysome pool. The cobalamin-responsive element was localised to a 70bp region located at the 3' end of the 5' untranslated region of the MS mRNA. The cellular consequences of the cobalamin response is a 2- to 3.5-fold increase in flux of Hcy through the MS-dependent transmethylation pathway of the cell lines examined (Oltean and Banerjee 2003). It suggests that cobalamin-induced up-regulation of MS may have evolved as an adaptive strategy for rapidly sequestering an essential and rare nutrient whose availability may have been limited. Methylmalonyl coenzyme A mutase reaction occurs in the mitochondria, using deoxyadenosylcobalamin as cofactor, and results in the conversion of methylmalonyl-CoA to succinyl-CoA, into the Krebs cycle. The reaction is essential for the degradation of propionate and odd-chain fatty acids, especially in the nervous system. In most

mammals, propionate arises from the degradation of certain amino acids and after β-oxidation of odd-chain fatty acids. The essentiality of cobalamin for this reaction may account for the accumulation of methylmalonic acid, with acidosis and high serum glycine and glucose concentrations in cobalamin deficiency.

ii. Absorption and Transport

At physiological levels of intake, the overall efficacy of vitamin B_{12} absorption is about 70%, decreasing to less than 10% at intakes greater than five times the RDA (Herbert 1987c). Dietary cobalamin is liberated from its protein binders by gastric acid and pepsin, then immediately bound avidly to R (rapid electrophoretic mobility) proteins in the stomach. The R proteins are secreted in saliva, gastric juice and bile. In the alkaline environment of the duodenum, pancreatic proteases, particularly trypsin, release the cobalamin from the R factor, but it is immediately bound to intrinsic factor (IF) (a glycoprotein secreted by the parietal cells of the stomach). The cobalamin-IF complex binds to a specific receptor on the brush-border membrane of the terminal ileum, where it is taken up and split within the enterocyte (Kapadia *et al.* 1983). The cobalamin is then transferred to another protein (transcobalamin II or TC-2) at the basolateral border, and transported to the liver (Chanarin *et al.* 1978). The entire absorptive process, from ingestion to its appearance in the portal vein, takes 8-12 hours. The TC-2 bound vitamin is taken up into tissues by specific receptors (Nexo and Hollenberg 1980). R factors, TC-1, TC-3 and haptocorrin are names for the glycosylated binding proteins found in plasma and in most secretions. In addition to their role in cobalamin absorption, R proteins are thought to play a role in the transport of cobalamin analogues and their disposal in the bile (Kanazawa and Herbert 1983). Recently, a polymorphism of TC has been identified, P259R and its distribution and effects have been studied in Caucasians (Namour *et al.* 2001). The blood apo-TC concentration of the 259-P homozygotes were significantly higher

(P<0.0001) than those found to be heterozygotic (P<0.0006). The heterozygotes 259-R/P had significantly higher Hcy concentrations than the homozygotes 259-R and 259-P (P=0.02 and P=0.01 respectively). This suggests that the R259P polymorphism affects TC plasma concentration and may interfere with cobalamin cellular availability and Hcy metabolism. More than 95% of intracellular cobalamin is bound to either cytoplasmic MS or to mitochondrial methyl-malonyl-CoA mutase.

iii. Deficiency

The body pool of vitamin B_{12} is 2-3mg, with a daily excretion of 1.2-1.3μg divided equally between urine and faeces. About 80% of the body store is in the liver, and turnover is extremely slow (0.05-0.2% of the body pool per day). Four to ten years are required to develop cobalamin deficiency when intestinal absorption is disrupted by IF deficiency. Cobalamin deficiency will develop much more rapidly if the enterohepatic circulation is interrupted by ileal disease or surgery. Conversely, efficient conservation of cobalamin in the enterohepatic circulation may preserve stores for longer than 10 years if the deficiency originates from the diet (Herbert 1987c).

Cobalamin deficiency usually occurs as the result of acquired gastrointestinal disease and rarely because of inadequate diet or congenital disorders of absorption and transport. Because dietary cobalamin is exclusively of animal origin, strict vegetarians and vegans are at risk of deficiency after prolonged periods following this diet. Cobalamin is a large molecule and unlike other water-soluable vitamins, less than 3% is absorbed by diffusion so the main cause of deficiency is through malabsorption. In practise, deficiency is rare because vegetarians in the western world take multivitamins containing cobalamin and in the developing world there is sufficient bacterial contamination of the diet to obtain bacterially synthesized cobalamin.

a. Pernicious Anaemia

Pernicious anaemia (PA) is associated with gastric atrophy and deficient IF secretion. A survey of 16 million people registered with general practitioners in the UK showed a prevalence of PA of 127 per 100,000 population (Scott 1960). It is a disease of later life as only 11% of cases occur before the age 40. In North West England, 3.7% of people aged over 75 were diagnosed with PA (Chanarin 1990). A small study in the US looked at 729 people over 60 years and over found that 14 people without PA had the antibodies to IF and only 9 of these had subnormal serum cobalamin levels (Carmel 1996). If these results are similar to incidence in the UK then around 150,000 people aged 60 and over with undiagnosed cobalamin deficiency are at risk. About half the cases are hereditary and mostly of Scandinavian descent; other cases result from the progressively acquired atrophy of ageing (Herbert and Coleman 1988). Circulating antibodies to IF are found only in patients with hereditary PA. The autoimmune nature of the hereditary form of the disease is suggested by the frequent finding of antibodies to IF in other tissues. In both types of PA, gastric atrophy is associated with destruction of the gastric parietal cell and an absolute lack of gastric acid, even in response to stimulation. Gastric atrophy predisposes to gastric cancer, which may occur in as many as 10% of patients with PA. IF deficiency also occurs in all patients who have had surgical total gastrectomy and in about 20% of patients who have had a partial gastrectomy for peptic ulcer disease (Herbert and Coleman 1988). Between 30 and 50% of cases with a gastric bypass for the treatment of severe obesity will become deficient after 5 years (Flickinger *et al.* 1987). Cobalamin deficiency is a consequence of the bacterial stasis syndrome, which occurs in individuals with small intestinal diverticulosis, strictures or fistulas, and in scleroderma with abnormal intestinal motility (Toskes and Donaldson 1983). In these syndromes, contaminating bacteria are presumed to take up and utilise cobalamin as it passes through the intestinal lumen. Bacteria that overgrow the small intestine are also capable of the efficient conversion

of cobalamin to metabolically inactive analogues. The fish tapeworm *Diphyllobothrium latum*, which is endemic in Finland, also utilises cobalamin thus producing deficiency in the host.

b. Malabsorption

Cobalamin deficiency can also result from malabsorption in the ileum, as the Cobalamin-IF receptor is only present on the ileal surface. The malabsorption can be due to disease processes or surgical removal. Vitamin B_{12} malabsorption is nearly universal in tropical sprue but rare in Coeliac Disease, because the latter affects mainly the jejenum. Patients with Crohns disease or radiation enteritis are at risk of malabsorption if the ileum is affected. Cobalamin deficiency can be predicted in patients who have had more than 50cm of terminal ileum removed surgically (Trier 1983).

c. Drug-Nutrient Interactions

Several drugs (Colchicine, neomycin, ethanol and p-aminosalicylic acid) can produce cobalamin deficiency by interacting with the Cobalamin-IF receptor. Medications used in the treatment of peptic ulcer disease that inhibit the secretion of acid by pariental cells have the potential to interfere with cobalamin absorption. Megaloblastic anaemia results from repeated exposure to nitrous oxide, an anaesthetic, as a result of the oxidative conversion of cobalamin to a form incapable of binding to MS. Nitrous oxide exposure has also caused subacute combined degeneration of the spinal cord in humans and several experimental animals. In chronic pancreatitis, R protein is not destroyed by pancreatic enzymes, which upsets the balance between R protein and IF avidity to bind to cobalamin.

d. Homocysteine

In addition to its role in preventing anaemia and neurological damage, cobalamin has been shown to influence Hcy levels. The Hcy-lowering potential of a folic acid supplement was compared with that of supplements containing various doses of cobalamin in addition to folic acid. In this study, 150 women of childbearing age were given a placebo initially for 4

weeks followed by a 4-week treatment period of either 400μg of folic acid or 400μg folic acid plus 6μg cobalamin or 400μg of folic acid plus 400μg cobalamin. Significant reductions in plasma Hcy levels were observed in all groups but the reduction with supplements containing cobalamin was larger than the folic acid alone group (-11%). Folic acid in combination with 6μg cobalamin reduced Hcy levels by 15% while 18% was observed in the larger cobalamin dose group (Brönstrup *et al.* 1998). Following on from this association, the role of MTHFR polymorphisms (C677T and A1298C) have been investigated in relation to differences in folate and cobalamin status on tHcy status. 186 females aged 20-30 years were examined, plasma Hcy was negatively associated with plasma cobalamin concentration (P=0.015) and serum folate (P=0.049), with the degree of correlation between plasma cobalamin and Hcy dependent on MTHFR genotype. The plasma Hcy decreased as cobalamin concentration increased (P=0.0005) in those individuals who were heterozygous for both C677T and A1298C variants and non-significant trends were found for the homozygotes. In contrast, the wild-type genotypes, Hcy was not associated with changes in plasma cobalamin concentrations. The data suggesting that enhancing cobalamin status may significantly decrease Hcy even when cobalamin concentrations are within the normal range (Bailey *et al.* 2002).

e. Clinical Sequelae

Vitamin B_{12} deficiency can result in abnormalities in bone marrow, small intestine and nervous system. Cobalamin deficiency produces two very distinct clinical sequelae – megaloblastic anaemia identical to that seen in folate deficiency and sub-acute combined degeneration. The typical patient with untreated PA develops weakness, glossitis (sore, inflamed tongue) and diarrhoea. These symptoms result from the defect in DNA synthesis that causes cell enlargement (megaloblastosis) of the red cell precursors in the bone marrow and of the absorbing enterocytes in the intestinal lining. Megaloblastosis leads to anaemia, whereas

the defect in the small intestine may cause generalized intestinal malabsorption. The most widely accepted explanation of cobalamin's role in megaloblastic anaemia is called the "Methyl-trap hypothesis". It suggests that 5-MTHF formed in cobalamin-deficient cells becomes metabolically trapped in this form and therefore unavailable to participate in purine and pyrmidine biosynthesis. As folate deficiency can cause the same clinical megaloblastic anaemia, the phenomenon of cobalamin deficiency in conjunction with normal folate status could mask the cobalamin deficiency and allow the potentially irreversible neurological complications to progress unchecked. This was a major concern when mandatory fortification with folic acid occurred in the US in 1998. However, a recent study has examined this problem and has found that there was no evidence that an increase in low cobalamin concentrations without anaemia has occurred since fortification (Mills *et al.* 2003). Another aspect that food fortification has uncovered is the possible role of cobalamin in disease risk reduction. Low maternal plasma cobalamin has already been shown to be an independent risk factor for neural tube defects (Kirke *et al.* 1993). Recently, cobalamin has been uncovered as the main determinant of plasma Hcy once folate status has been normalise through fortification (Quinlivan *et al.* 2002). This discovery could have implications on risk of developing vascular disease as plasma Hcy has been identified as an independent risk factor. The results of this study also point to the benefit a dual fortification policy could have on the reduction of both NTD's and CVD.

Neurological symptoms are less common and consist of paresthesiae of hands and feet, abnormal vibratory and proprioceptive sensation with loss of postural sense and spastic ataxia. These symptoms result from subacute combined degeneration, with myelin degeneration in the dorsal and lateral tracts of the spinal cord. Cobalamin deficiency results in a deficiency of SAM and that will cause a rise in SAH levels, since Hcy cannot be re-methylated when MS is deficient. The result is that the SAM:SAH or methylation ratio is reduced, resulting in

inhibition of methylation reactions. This will cause a state of hypomethylation in the brain, which in turn causes subacute combined degeneration. Cobalamin neuropathy is more frequent in males with an incidence ratio of 10 male to 7 female, although PA is more common in females (1.6:1)(Chanarin 1990). Lindenbaum and co-workers described a group of patients with neuropsychiatric disorders, including paresthesiae, weakness, ataxia, dementia and psychosis (Lindenbaum *et al.* 1988). The patient group were not anaemic but had biochemical evidence of cobalamin deficiency with subnormal levels of serum cobalamin and elevated Hcy and MMA. Therapy with cobalamin corrected the biochemical abnormalities in every case and improved the neuropsychiatric symptoms in most cases. The degree of response has been shown to vary with the severity, duration of the disorder and the age of the patient. The older the patient, the lesser the potential of recovery.

Early diagnosis and treatment are essential at all ages, especially in older people. Improvement can be seen within days and can continue for a year or more (Healton *et al.* 1991). Low serum cobalamin without anaemia has been detected in up to 5% of psychiatric admissions and up to 10% or more of psychogeriatric or elderly populations. Recent studies, involving additional techniques (cerebrospinal fluid folate, plasma Hcy and MMA) have confirmed these deficiency states in psychiatric and elderly populations and suggest that they may contribute to the cognitive decline in older people (Bottiglieri *et al.* 1995;Clarke *et al.* 1998). Another study examined the use of cut-offs for Hcy (15.0μmol/L) and MMA (0.35μmol/L) to identify persons who are at risk of deficiency. The study examined 1562 people $\geq$ 65 years and found that ~10% of those 65-74 years were at high risk and of those $\geq$75 years around 20% were at high risk. About 10% and 20%, respectively, were also at high risk of folate deficiency and about 10% of those with folate deficiency also had cobalamin deficiency (Clarke *et al.* 2003). A similar study looked at the prevalence of hyperhomocysteinaemia and vitamin B_{12} deficiency in 103 poorly nourished, elderly people

after food fortification in the US (Johnson *et al.* 2003). The study was able to show cobalamin deficiency was prevalent in 23% of subjects and that it was associated with poor cognition, anaemia and hyperhomocysteinaemia. The high level of cobalamin deficiency detected was most likely due to advanced age of the subjects (76.4±8.1) and the probable long-term poor nutrition of the group.

iv. Dietary Sources and Requirements

All natural cobalamin is synthesized by bacteria, fungi or algae. It reaches the human diet after incorporation into animal protein following intestinal bacterial synthesis. Human intestinal bacteria may also synthesize small amounts but the availability is uncertain. The amount of cobalamin consumed varies from 3 to 15μg/day (Herbert 1987c). The forms of cobalamin in the diet include mainly 5-deoxyadenosyl- and hydroxocobalamin, with lesser amounts of methylcobalamin in dairy products and little or no cyanocobalamin. Dietary B_{12} may be partly destroyed by heating in an alkaline medium.

The average requirement to prevent or cure the megaloblastic anaemia of cobalamin deficiency in adults appear to be less than 1μg/day and this is the intake now recommended by FAO (Food and Agriculture Organisation 1988). The Lower Reference Nutrient Intake was set at 1μg/day, Estimated Average Requirements was set at 1.25μg/day and the Reference Nutrient Intake at 1.5μg/day, which would not only reduce the risk of megaloblastic anaemia but also allow for the provision of adequate stores to withstand periods of zero intake. An increase of 0.5μg/day was allowed for lactation as there is 0.2-1.0μg/L cobalamin contained in breast milk. Patients with PA are treated with intramuscular injections of 100-1000μg cyanocobalamin monthly (Herbert and Coleman 1988). In a recent study where cobalamin deficiency was examined in 315 older adults alongside dietary sources, deficiency was found in 13% of patients. 46% of patients reported taking synthetic cobalamin in the form of a

supplement and these patients were significantly less likely to be cobalamin deficient than those not taking supplements (8% vs. 17%, P=0.02) (Rajan *et al.* 2002).

v. Clinical Assessment

The serum cobalamin assay remains the conventional means of assessing vitamin B_{12} status. Two standard deviations below the mean are typically found to be 150pg/mL, and levels below that are clearly associated with a high risk of developing clinical manifestations of deficiency (Weir and Scott 1998). Some individuals develop the clinical manifestations of deficiency even though their serum levels are in the low-to-normal range (150-400pg/mL) (Lindenbaum *et al.* 1988). It has been estimated that up to 5-15% of the elderly whose serum B_{12} fall into the low-to-normal range are functionally deficient (Stabler *et al.* 1997). The measurement of serum methylmalonic acid (MMA) and Hcy were shown to be more effective means of detecting subtle deficiencies although MMA is more specific because it does not rise in folate deficiency (Savage *et al.* 1994). Nevertheless, confounding factors that may artefactually alter MMA levels include renal insufficiency or hypovolaemia (Stabler *et al.* 1988) and concurrent use of antibiotics (Lindenbaum *et al.* 1990). Measuring levels of the serum transport protein, transcobalamin II bound to cobalamin, has been suggested as a sensitive means of detecting B_{12} depletion (Herbert 1994) although it still has to be tested in large clinical trials. A recent study examined cobalamin deficiency using various clinical markers – MMA, Hcy, plasma cobalamin and holotranscobalamin II (Rogers *et al.* 2003). Holotranscobalamin II was shown in this study to be a less sensitive indicator than MMA in 533 children, of whom 11% had low plasma cobalamin and 22% had marginal concentrations. This study can be contrasted with another recent study looking at cobalamin status in vegetarians (Herrman *et al.* 2003).This study found that the assessment of holotranscobalamin II, accompanied by that of Hcy and MMA, offered sensitivity and reliability in diagnosing

early cobalamin deficiency. However, both studies used varying cut-off points for deficiency in each metabolite studied and this could have influenced the end results found.

CHAPTER 2

METHODOLOGY DEVELOPMENT

1. METHODS

i. Epithelial Cell Isolation Technique

a. Introduction

Previous work has been done on colonic epithelial cell isolation based on various methodologies. Enzymatic methods have been successfully used to isolate epithelial cells. Several cocktails have been used, early studies used trypsin, hyaluronidase and DNAses but these resulted in low yields and purity. Gibson and co-workers used (Gibson *et al.* 1995) dispase type I with high grade collagenase type IV to isolate colonic crypts. This method yielded high viability but high erythrocyte contamination (30%). Another method used a solution of 0.03% collagenase IV and 0.1% pronase (Benya *et al.* 1991) to isolate cells from rabbit distal colon. Purification of the cells was required over a percol gradient.

Another method is the use of perfusion supported mechanical disaggregation, which separated the underlying layers of the colonic biopsy from the epithelium. The epithelial layer was then minced and incubated with Hanks' Balanced Salt Solution (HBSS) supplemented with 2mg/ml proteinase K and 1mg/ml collagenase P for 60 min in a shaking water bath at 37^0C. The cells were then washed and pelleted for use (Schaeferhenrich *et al.* 2003b). However, contamination with erythrocytes was a problem and required a subsequent purification step.

The final type of dissagregation technique was ion-chelation. This method was based on the fact that divalent cations, in particular calcium, weaken the inter-cellular tight junctions. This renders the bond between the epithelial cell baso-lateral membrane and the basement membrane vulnerable to light mechanical disruption (Madrigal *et al.* 1993). Dithiothreitol (DTT) was required as an adjunct in human tissues because of the presence of mucous but at high concentrations, this can decrease viability. The method devised by Meenan and co-workers was developed to optimise the isolation of epithelial cells from small colonic biopsies.

This method resulted in good cell yield, purity and viability (Meenan *et al.* 1997). The only drawback of the ion-chelation method devised by Meenan was the long incubation periods in each solution.

b. Method Comparison

Different isolation methods were examined for their suitability in the Comet assay and folate analysis. The methods examined were an enzymatic method devised by Everett to look at gastric epithelial cells (Everett *et al.* 2000) and the Meenan method used on colonic biopsies.

The Meenan method involved placing the biopsy tissue in HBSS (without calcium or magnesium) (Sigma, Dorset, UK) supplemented with 0.75mM DTT (Sigma, Dorset, UK) for a period of 2h to breakdown any mucous present. The biopsies were then transferred into 2mM EDTA (Sigma, Dorset, UK) HBSS solution at 37^0C for 1h in a rotating basket at a 45-degree angle. This allowed for the ion-chelation and a gentle shear force to be applied. The result of this was an epithelial cell suspension, which was then centrifuged at 980*g* for 10min. The supernatent was then discarded and the resulting cell pellet used for subsequent assay work.

The Everett group method involved suspending biopsies in Dulbecco's modified Eagle Medium (Sigma, Dorset, UK) supplemented with 0.1% pronase and 0.03% collagenase (Sigma, Dorset, UK). These were then incubated at 37^0c for 45min with gentle shaking. The incubation was limited to 45min to avoid complete digestion of the biopsies. At the end of the incubation, the biopsy remnants were removed and the cell suspension was centrifuged at 2400*g* for 7sec and the supernatent was discarded.

Nine patients were recruited from the Endoscopy Clinic of St. James Hospital, Dublin. Fully informed consent was obtained from each patient prior to the procedure. Four descending colon biopsies were obtained and placed into fresh HBSS. The biopsies were then split into 2 sets of two biopsies and analysed using the two different methods.

c. Results of Method Comparison

The non-enzymatic / Meenan method proved repeatedly to be more robust and superior to the Everett / Enzymatic one, in terms of suitability for the comet assay. Although the non-enzymatic method takes longer to perform, it was more consistent in terms of cell yield and viability. Furthermore, a lower level of DNA damage was observed in the resulting Comets following non-enzymatic biopsy disaggregation (Figure 2.i). On this basis, the non-enzymatic / Meenan method was chosen.

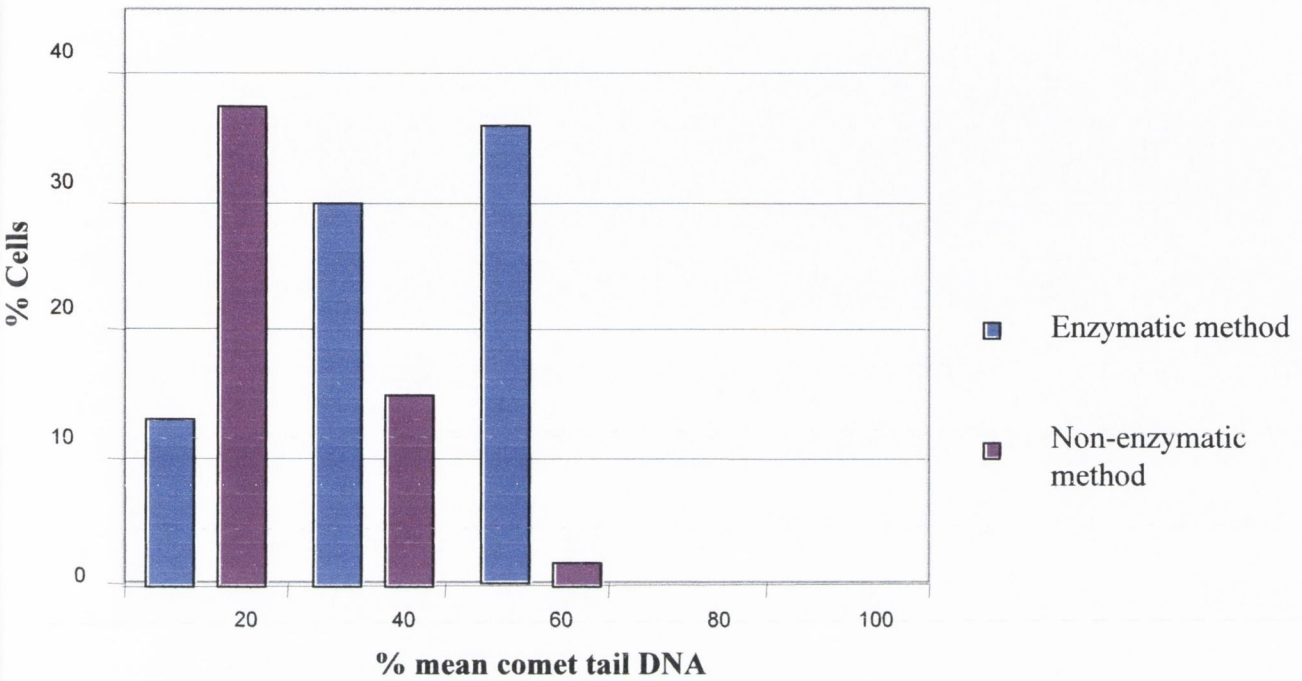


Figure 2.i **Biopsies disaggregated using enzymatic and non-enzymatic methods yield different levels of DNA damage from the same site.**

ii. Transport

Owing to the conjoint nature of the study, the slides containing the freshly isolated colonic epithelial cells needed to be transported in such a manner that they remained intact and viable in transit, without any artefactual damage to the DNA. A variety of methods was attempted.

a. Microbeads

Initial work was done incorporating the cells once isolated into molten liquid paraffin where they form microbeads. The cells became encapsulated inside the beads and were then transported in suspension. Upon arrival in Coleraine, they were mixed with LMP agarose to form the top layer of the Comet slide. This method was disregarded due to the loss of cells in the preparation and processing of the beads and poor electrophoresis across the interface of the bead, which meant that the cell comets were not forming properly.

b. Slide-Based

Slide-based transportation was the next option examined. A variety of slides types were used: fully frosted (Richardson Supply Company, UK), diamond pen etched and electrostatically-charged (polysine) slides (Sigma, Dorset, UK). Descending colon cell populations from 3 patients with a normal appearing mucosa at endoscopy were divided in half and resuspended in 0.75% LMP agarose (Sigma, Dorset, UK). The cell suspension was then pipetted on top of a 1% NMP set agarose layer (Sigma, Dorset, UK) and spread with a coverslip. This was performed in duplicate for each slide type. The slides were then sent to Coleraine overnight via courier in neutral lysis solution (2.5M Lithium Chloride, 0.3M EDTA, 10mM Tris, 0.1% Lithium Dodecyl Sulphate, pH 8.0)(Sigma, Dorset, UK) containing proteinase K to a final concentration 0.03mg/ml, to enable efficient de-proteinisation of the cells and inhibit any DNA-degrading enzymes which could have caused artefactual damage.

Only the fully-frosted slides resulted in gel survival and subsequent comet analysis so these were chosen as the slide to transport the cells.

c. Transportation Container

Different transportation methods were attempted with the aim of decreasing gel loss in transit. Four descending colon biopsies were taken from 13 patients with normal appearing mucosa. The sets of biopsies were divided equally into 2 groups. Both groups were processed as per the disaggregation protocol with two sets of two comet slides being produced. One group were sent in a rectangular slide holder containing 20ml neutral lysis supplemented with 0.03mg/ml proteinase K. The other group of slides were placed on PBS-dampened tissue in a flat slide holder. All sets were transported using the same overnight courier service and on the same day. Increased levels of DNA damage were detected in the damp tissue group so this method of transport was abandoned.

d. Proteinase K

The pre-incubation of the slides in the lysing solution for 1 hour at 37°C was investigated. Six biopsies from an endoscopically normal descending colon were taken and disaggregated according to the protocol. The final cell volume was then split equally into 3 pellets. Each pellet was then made into 3 comet slides. One set of slides had 0.03mg/ml proteinase K added to the neutral lysis solution and was pre-incubated for 1h at 37^0C prior to delivery to Coleraine via courier overnight. Another set was pre-incubated for 1h at 37^0C but with 0.06mg/ml proteinase K and the final set received the standard amount of proteinase K but no incubation. The slides that received the double dose of proteinase K were equivalent in DNA damage level to those with the standard amount so 0.03mg/ml dose was chosen. The slides that lacked the pre-incubation step showed an increased degree of DNA damage observed, probably due to activity of DNA degrading enzymes. This proved that the pre-incubation was essential to activate the proteinase K.

iii. Biopsy Collection and Storage

a. Timing of Patient Recruitment

Patient recruitment was dependent on the endoscopy clinic times and to this end, it was important to ensure that the assay was robust enough to allow flexibility around these times. For patients recruited in the morning, the above protocol could be followed, as the courier was able to collect the processed slides that afternoon.

b. Overnight Storage

For patients recruited in the afternoon, it was essential that a method be devised to allow for next day processing. Firstly, we examined the possibility of storing the slides after preparing them as per the protocol above. Four sets of four descending colon biopsies were obtained from four separate patients with endoscopically normal colons. Two biopsies were processed and sent on to Coleraine on the day of collection. The other two biopsies were processed on the same day but the slides were stored overnight at 0-4^{0}C in neutral lysis and sent the following day. No significant difference was seen between the slides sent the same day and those sent the following but the quality of the gels did suffer from the lengthened storage in neutral lysis. Storage of the whole biopsy overnight was also examined.

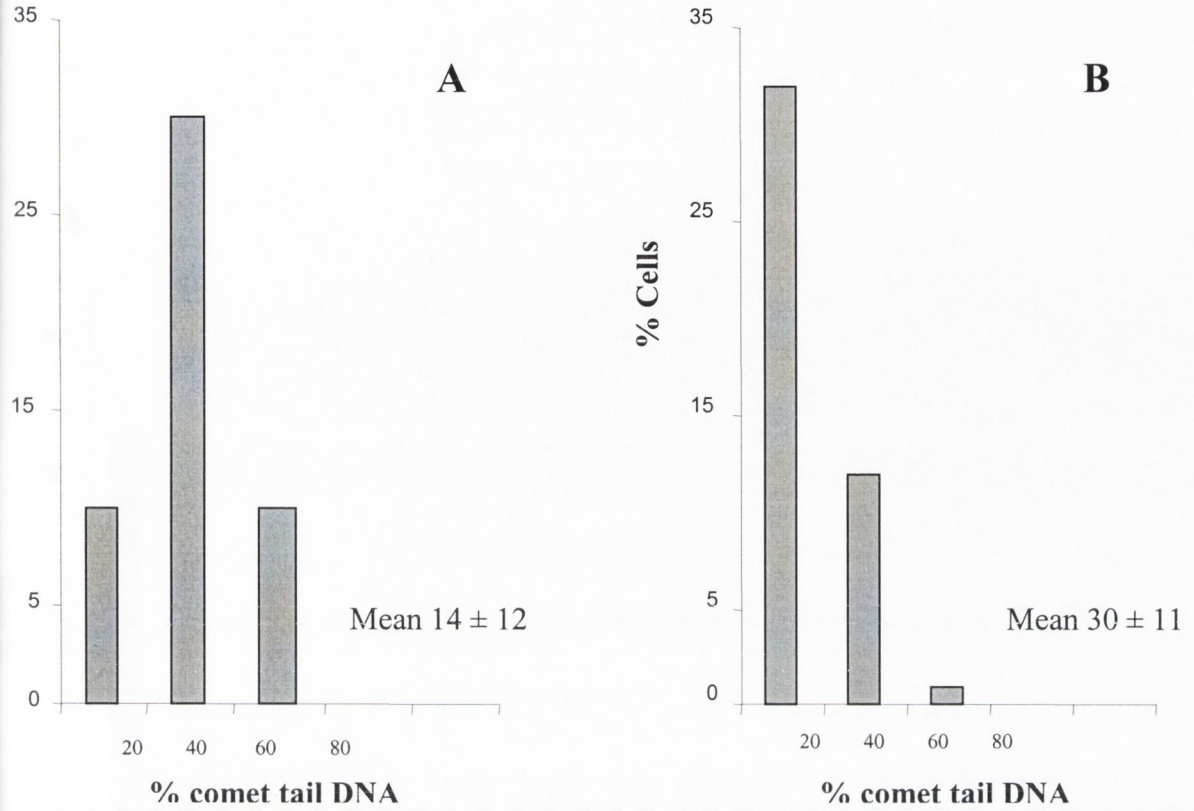


Figure 2.ii Pre-incubation with Proteinase K reduces baseline DNA damage.

A. Comet slides without pre-incubation at 37°C before courier transport

B. Comet slides pre-incubated at 37°C in neutral lysis buffer and proteinase K for 60min before courier transport

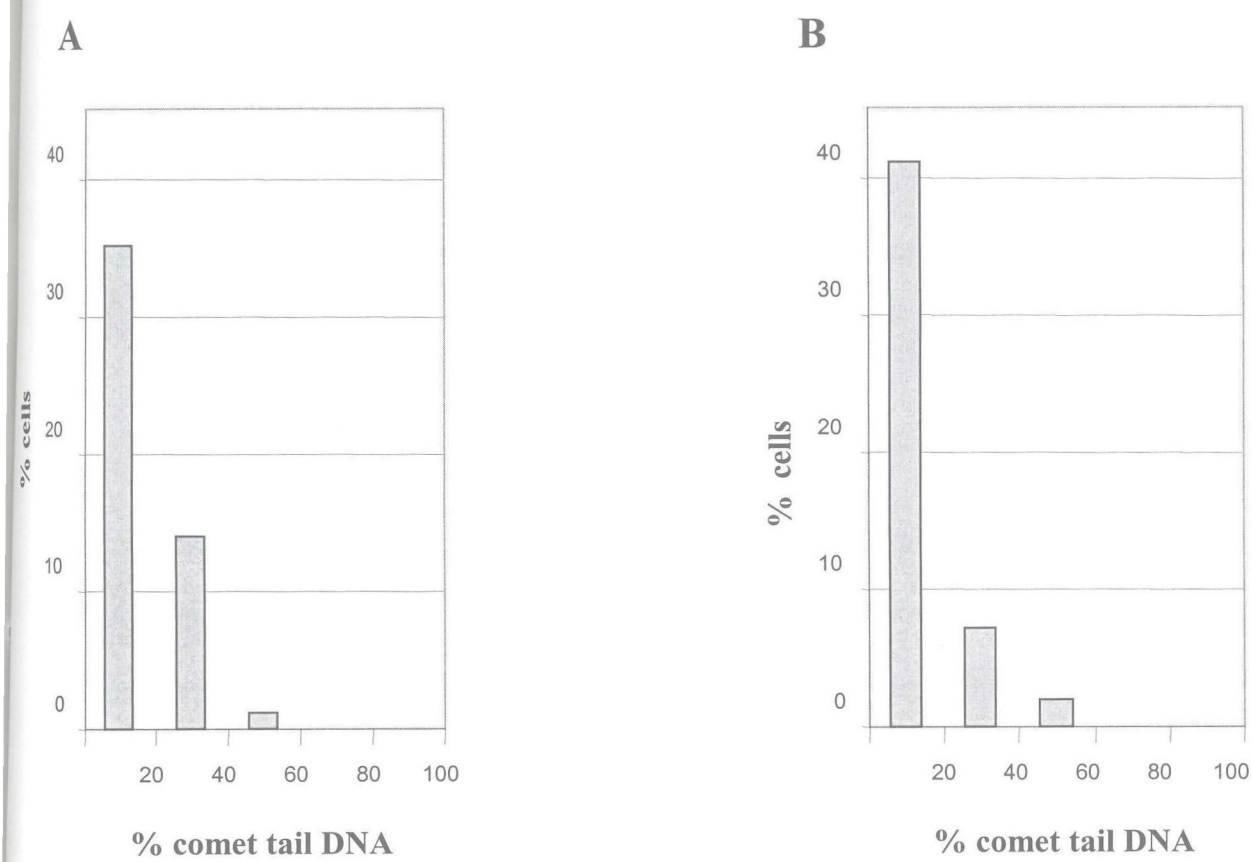


Figure 2.iii Overnight storage of biopsies prior to comet analysis.

A. Biopsy disaggregated and Comet slides transported on day of collection

B. Biopsy stored overnight at 0-4^{0}C before next day disaggregation and transport

c. Storage for >24hours

To allow for biopsy collection on Friday and storage over the weekend, we investigated storage of biopsy material for 48h (n = 5) and 72h (n =3) . These experiments found no significant difference between those processed the same day, 48h later or those processed 72h later. It was further established that the biopsy specimens are not adversely affected in the event of courier delays in delivery to Coleraine. To this end, it was demonstrated that extended storage in neutral lysis (for slides delayed in transport overnight) or in PBS at 4°C (for slides that may arrive late) does not result in any increase in DNA damage as determined by Comet assay parameters.

iii. Comet Assay

The Comet assay was used to detect single strand breaks and alkali-labile sites in the DNA of individual cells. Following arrival at the Coleraine laboratory, the slides are immersed in freshly prepared alkaline lysis buffer (2.5M Sodium Chloride, 100mM EDTA, 10mM Tris, pH 10.0) (Sigma, Dorset, UK) with 1% Triton X-100 added just before use. The slides are immersed for 1h at 0-4^{0}C, but before which a methylation enzyme digestion may be conducted by adding the required enzyme in reaction buffer at a concentration of 1 unit per slide and incubating at 37^{0}C for 1h followed by washing in PBS (calcium and magnesium free) (Sigma, Dorest, UK).

The slides were removed from the lysing solution, drained. Uracil Glycosylase enzyme digestion may be conducted at this stage as above. Slides are then placed in a horizontal electrophoresis tank side by side with the gaps filled with blank slides. The tank was filled with fresh electrophoresis buffer (0.3M Sodium Hydroxide, 1mM EDTA) (Sigma, Dorset, UK) till just covering the slides and left for 20min to allow unwinding of the DNA before

electrophoresis. Electrophoresis was carried out for 20min at 25V adjusting to 300mA by rising or lowering the buffer level in the tank.

After electrophoresis, the slides were drained, placed on a tray and flooded with three changes of neutralization buffer (0.4M Tris, Sigma, Dorset, UK) for 5min each time. This removes the alkalis and detergents that would interfere with the ethidium bromide staining. Slides were drained before staining with 50µl of 2µg/ml ethidium bromide and covered with a 22x50 coverslip (Sparks, Dublin). Slides can be stored for 1-2 days on damp tissue in a box shielded from the light. The slides were viewed using an epifluorescence microscope equipped with a Chroma filter at excitation 540nm and emission 635nm. Analysis of the slides was done using an image analysis system and Komet 4.0 software.

iv. Folate Microbiological Assay

Systemic and localized cell folates were analyzed using the previously determined method (Molloy and Scott 1997). Folate buffer was prepared using 0.5% (w/v) Sodium Ascorbate (Sigma, Dorset, UK) in double-distilled, deionised water. The folic acid standard was prepared to a final working standard concentration of 500pg/ml using pre-prepared stock standard solution and folate buffer. The growth medium was prepared with 7g folic acid broth (DIFCO,), 3mg chloramphemicol (Sigma, Dorset, UK) added to 100ml double-distilled, deionised water (dH_2O). The solution was heated till just before the boiling point and 30µl Tween-80 (Merck, Germany) was added and the solution was brought to the boil for 1min, and then cooled on ice. Prior to use, 75mg ascorbic acid (Sigma, Dorset, UK) was added and dissolved in the medium. *Lactobacillus Rhamnosus* (formerly known as *Lactobacillus Casei*) (NCIB 10463; Torrey Research Station, Scotland) was cryopreserved at -80^0C as a glycerol suspension and thawed just prior to use in the growth medium, where it was added to give a final concentration of 200µl/100ml medium. The medium was then mixed for 10min using a

magnetic stirring plate. Samples for analysis were diluted using folate buffer to the desired concentration and pipetted in the wells of the 96-well microtitre plate. A standard plate was prepared using folate buffer and the working standard. Additions of this standard were made to the wells of the microtitre plate to give a range of concentrations from 0-50pg/well (0-100µl standard). All well volumes were made up with folate buffer. All the 96-well microtitre plates were given a 42h incubation at 37^0c and then read at 590nm spectrophotometrically (Labsystems Multiskan Plus). The data from the spectrophotometer was then extrapolated using Genesis Lite version 3.03 software (Medical Supply Company, Ireland).

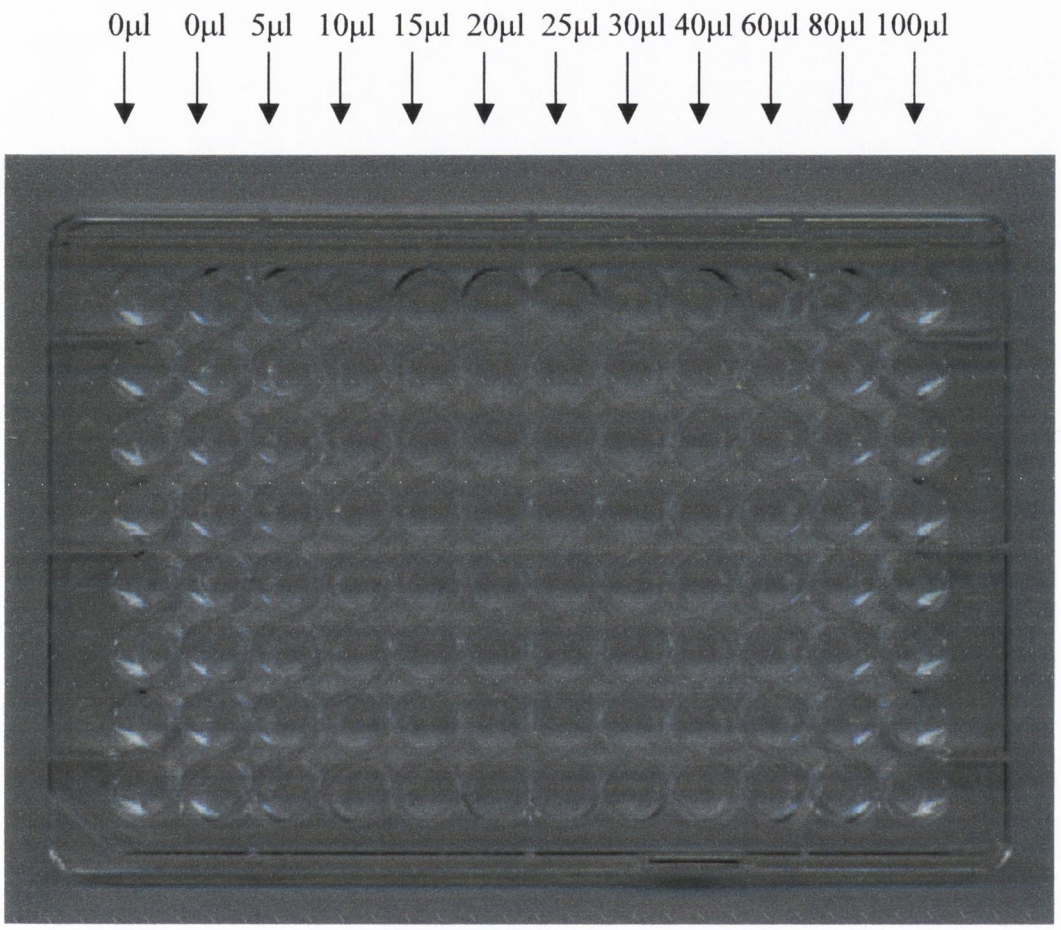


Figure 2.iv Folate standard microtitre plate, amounts in figure indicate the volume of standard that was added to each row and the remainder of the 100µl volume was made up using folate buffer.

v. Homocysteine Assay

The Imx Hcy assay (Abbott, Germany) was based on the Fluorescence Polarisation Immuno Assay (FPIA). Using the method, the concentration of tHcy was determined using 2 technologies: competitive protein binding and fluorescence polarization. Each Hcy reagent kit contained 4 different reagents: DTT (reduces the Hcy and protein bound forms of Hcy to free-Hcy), SAH-hydrolase and adenosine (which converts free-Hcy to S-adenosyl-L-Hcy (SAH)), monoclonal antibody molecules (SAH and fluorescein tracers compete for sites on the antibody). 55µl of each sample was reverse pipetted into a new sample cartridge placed in an FPIA carousel alongside new glass cuvettes around the central section. Internal and external controls should be included in each run. Once the cartridges are filled, the carousel should be locked into position inside the Imx machine and the reagents placed in position. The lid should then be closed and the 1h run commenced. All controls should be within the desired ranges before the run is accepted.

vi. B_{12} Assay

Vitamin B_{12} was assayed microbiologically using *Lactobacillus Leichmannii* (NCIB 12519; Torrey Research Station, Scotland). B_{12} extraction buffer was prepared from an acetate and a 0.1% (w/v) sodium cyanide (NaCn) solution. The acetate solution was prepared with 8g sodium hydroxide dissolved in 200µl dH_2O. To 160ml of that solution, 500ml dH_2O was added as well as 22.8ml 100% glacial acetic acid (pH 4.5). This solution was then made up to 1L with dH_2O and mixed well. The working B_{12} buffer was freshly prepared from the acetate and NaCn stocks using 54ml acetate solution and 24ml NaCn made up to 1L with dH_2O. B_{12} medium was prepared using 6.2g medium powder (Difco, Becton-Dickinson microbiology systems, USA). To this 100ml dH_2O were added, alongside 11mg colistin sulphate. The solution was heated till just before the boiling point and 150µl Tween-80 (Sigma, Dorset, UK) was added and the solution was brought to the boil for 1min, and then cooled on ice. *Lactobacillus Leichmannii*

was cryopreserved at –80^{0}C as a glycerol suspension and thawed just prior to use in the growth medium, where it was added to give a final concentration of 200µl/100ml medium. The medium was then mixed for 10min using a magnetic stirring plate. B_{12} Standard was prepared from a stock standard prepared from 1mg/ml Cytamen vial made up to a final concentration of 1ug/ml with dH_2O and stored at -80^{0}C as 750µl aliquots. The stock standard was then diluted to a final concentration of 50pg/ml on the day of assay. 75µl of sample was autoclaved at 121^{0}C for 10min at 15psi with 675µl B_{12} buffer, in order to precipitate any protein present. The plate was then centrifuged at 2200rpm for 15min. The supernatant was used for the microbiological assay. Samples for analysis were diluted using B_{12} buffer to the desired concentration in separate microvials and pipetted in the wells of the 96-well microtitre plate. A standard plate was prepared using B_{12} buffer and the working standard. Additions of this standard were made to the wells of the microtitre plate to give a range of concentrations from 0-50pg/well (0-100µl standard). All well volumes were made up with B_{12} buffer. All the 96-well microtitre plates were given 42h incubation at 37^{0}c and then read at 590nm spectrophotometrically (Labsystems Multiskan Plus). The data from the spectrophotometer was then extrapolated using Genesis Lite version 3.03 software (Medical Supply Company, Ireland).

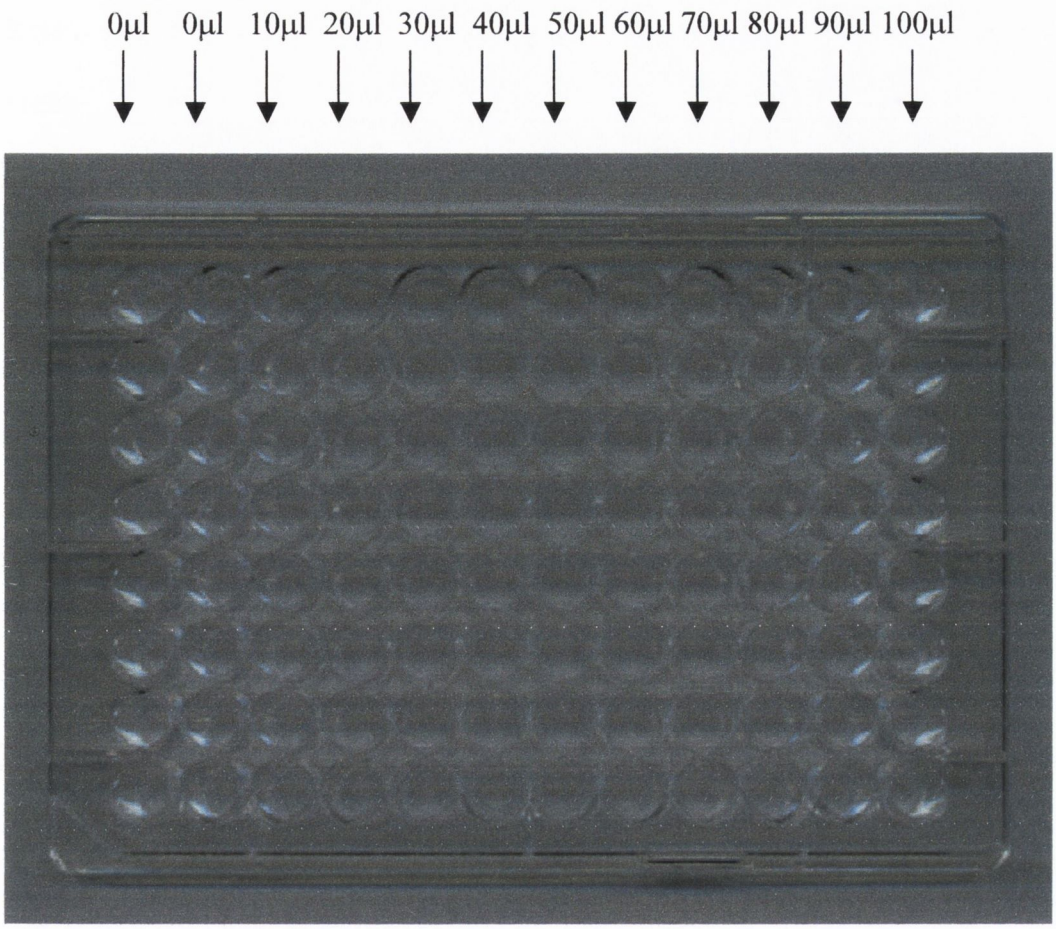


Figure 2.v **B_{12} standard microtitre plate, amounts in figure indicate the volume of standard that was added to each row and the remainder of the 100µl volume was made up using B_{12} buffer.**

vii. B_{12} Intrinisic Factor Assay

The Autozyme™ IFAB Anti-Intrinsic Factor Antibodies Assay (Cambridge Life Sciences, UK) is a solid phase (microwells) ELISA based on the "sandwich" principle. The wells are coated with purified porcine intrinsic factor. All reagents must equilibrate to room temperature before use for a minimum of 2h. All contents of the kit should be prepared according to instructions. Patient samples are diluted 1:50 using the sample diluent. The kit controls do not need dilution. The antigen-coated microwells were removed from their foil sachet. 100µl of all diluted samples and controls should be added. Incubate for 30min at room temperature to allow the samples to form an intrinsic factor/anti-intrinsic factor complex. The contents of the wells were flicked out over a sink and the wells were washed at least 3 times with wash buffer. The wells were then blotted with tissue. 100µl anti-IgG peroxidase conjugate was then added to each well and incubated for 30min at room temperature. The contents of the wells were flicked out over a sink and the wells were washed at least 3 times with wash buffer. 100 µl pale yellow substrate was then added and incubated for 30min at room temperature. The intensity of the green colour formed was proportional to the amount of anti-intrinsic factor antibodies bound in the first incubation. The reaction was stopped with stopping buffer, which was at a low pH. The absorbance was measured using a 405nm filter on a 96-well microplate reader. The results were once it was established that the controls were within the correct absorbance range. To calculate the result ratio the sample absorbance was divided by the cut-off absorbance. A sample was determined to be positive if the ratio was greater than 1.1, a negative result was less than 0.9 and any result in-between required a repeat test.

viii. MTHFR Genotyping

DNA was extracted from frozen whole blood samples using QIAamp™ DNA Mini Kit (QIAGEN Ltd., West Sussex, UK). 20µl proteinase K was pipetted into the bottom of a 1.5ml

microcentrifuge tube. 200µl of sample was added, 200µl Buffer AL was then added and mixed by pulse-vortexing for 15sec. The microcentrifuge tube was then incubated at 56^0C for 10min. The tube was then briefly centrifuged to remove any droplets from the lid. 200µl ethanol (96-100%) was added and the tube pulse-vortexed again. The brief centrifugation was repeated again. This mixture was applied to a QIAamp spin column with a 2ml collection tube and centrifuged for 1min at 8000rpm. The QIAamp column was placed in a fresh collection tube and 500µl buffer AW1 added. The centrifugation step above was repeated and the QIAamp spin column placed in another fresh collection tube.

500µl buffer AW2 was applied and the column spun for 3min at 14000rpm. The column was then placed on a clean 1.5ml microcentrifuge tube and 200µl buffer AE or dH_2O. The column was then incubated at room temperature for 5min and then centrifuged at 8000rpm for 1min. A subsequent 200µl buffer AE or dH_2O was added and the column centrifuged at 8000rpm for 1min.

PCR product was achieved through an established method using Sigma products (Dorset, UK). All work was done in a laminar flow hood. PCR mastermix was prepared as follows per reaction: 34.8µl PCR grade water, 4µl 2.5Mm dNTP's, 1µl forward MTHFR primer 5'-TGA AGG AGA AGG TGT CTG CGG GA-3' (MWG Biotech, Germany), 1µl reverse MTHFR primer 5'-AGG ACG GTG CGG TGA GAG TGG-3' (MWG Biotech, Germany), 4µl 25mM Magnesium Chloride, 5µl 10X PCR buffer and 0.2µl TAQ Polymerase. The mastermix solution was mixed thoroughly and 49µl mix added to each well of the PCR plate (ABgene, Surrey, UK). 1µl extracted DNA was then added to each well, negative (PCR mix only) and positive (known MTHFR genotype) controls were added to each plate to monitor contamination. Each row of the PCR plate was then sealed with a flat cap strip and the plate was placed in the DNA engine (Peltier Thermal Cycler PTC-200, MJ Research, USA). The program run for the MTHFR genotype was 94^0C for 5min ramp and then 94^0C for 1min,

62^0C for 1min and 72^0C for 1min, this was cycled 31 times. After completion, 10µl of PCR product was run out on 1.5% agarose gels for approximately 20min at 80V, 200mA to ensure that amplification of the target DNA had occurred.

If the reaction was successful then a restriction enzyme digestion was carried out. 9µl of PCR grade water was added to each well of a new PCR plate. 17µl PCR product was then pipetted into each well followed by 3µl digestion buffer and 1µl *HinF1* (ISIS Pharmaceuticals, USA). The wells were then sealed with domed cap strips and incubated at 37^0C for 24h. After digestion, the samples were run out on 2.5% agarose gels for at least 1h at 100V, 250mA. The gels were then read using the Gel Doc system.

ix. Discussion

The most important factor in the determination of cellular folates was ensuring that the method used was viable, accurate and reproducible. The epithelial cell isolation method was chosen as it permits the isolation of large numbers of viable epithelial cells from standard endoscopic biopsies, free of lamina propria contamination. This method had already been examined for its suitability for folate analysis (Meenan *et al.* 1996;Meenan *et al.* 1997) but not for single cell gel electrophoresis. The method was re-evaluated from this perspective over a course of experiments to optimise its use for the comet assay. The original method had used the epithelial cell isolates solely for cellular folate determination but the collaborative nature of the study required the cell yield to be divided equally between the comet and cellular folate assay. For this reason, a new method of expression was devised (pg folate / 10^5 cells) that would allow for standardisation of the folate results obtained from the microbiological assay.

Owing to the collaborative work that was being undertaken by both universities, it was important that the transportation of the isolated epithelial cells was carried out as quickly as possible while also ensuring that minimal disruption or damage to the cells was incurred. This

proved to be a long and arduous process as the reliability of couriers proved to be a regular problem, until a suitable company was found, as slides were either arriving late, damaged or simply disappearing. This factor had not been envisaged at the outset of this study. The method of transportation of the slides was also a critical step in the comet assay; numerous experiments were carried out to ensure that biopsy material was suitable to store for up to 72hrs, finding the best transport medium and that artifactual damage was not occurring to the cellular DNA while in transit. All other methodologies were well established and all necessary controls were put in place to ensure the standard of analysis was maintained.

CHAPTER 3

TISSUE FOLATE ANALYSIS

1. TISSUE FOLATE ANALYSIS

i. Introduction

Although the amount of publications in the area of folate and CRC were found to be vast, the level of publications that involved looking at tissue or localised folate concentrations in the large intestine were few. The most obvious reason for this was that obtaining samples of colonic tissue involved an invasive procedure, another reason was also that the folates from the tissue had to be extracted and there has been a long history of disagreement on the most suitable way to do this.

ii. Cellular Folate Determination

There have been two main methods published in the literature. The first method was devised by Joel Mason and colleagues in the US. In this method, a whole biopsy was homogenised and subsequently deconjugated for folate content analysis. In the original method, the biopsies were taken and placed into a foil packet and immediately immersed in liquid nitrogen and then transferred to -70^{0}C for subsequent analysis of colonic muosal tissue folate concentration. A rapid determination of biopsy weight was followed immediately by extraction of tissue folates in 20 volumes of freshly prepared folate extraction buffer [5mM β-mercaptoentanol and 0.1M sodium ascorbate in 0.1M bis(2-hydroxyethyl)imino-tris(hydroxymethyl) methane (pH 7.85)] for 20min at 95^{0}C then cooled on ice. Homogenisation took place using a handheld homogeniser (Ultra-Turrax T8, IKA-Werke, Germany) at high power for 5minutes. The resulting suspension was then centrifuged at 25000 G for 10min. The supernatent was transferred to a clean microfuge tube and frozen at –80^{0}c until deconjugation and microbiological analysis using *L.Casei.*

The other method was devised by John Meenan in Ireland. This method obtained a folate result for epithelial cell folate concentration. The epithelial cell population of colonic punch biopsies were obtained using the previously described ion-chelation separation

technique. Cell samples were stored at -40^0c in 500µl 1% sodium ascorbate PBS (pH 6.5). Prior to deconjugation, the cell membrane was required to be broken open to allow for the action of the conjugase to take effect on the intra-cellular polypglutamated folates. Sonication was chosen as a suitable technique and this was performed at power 5 for 30sec on ice (Cell Disruptor B15, Branson, USA). The samples were then boiled for 5min and cooled on ice pre-conjugase addition.

a. Conjugase Selection

The folate assay was based on the folate present being in the monoglutamate form, however, the folates present in the cells are polyglutamate in nature. Therefore, a deconjugation step was needed to provide a suitable substract for the microbiological assay. Initial experiments were carried out to identify and optimize a suitable conjugase (γ-glutamyldehydrogenase) to prepare the cells for microbiological analysis.

Fresh human epithelial cells from umbilical cord (kind gift of Fiona McCarthy, Biochemistry, TCD) were suspended in 8ml folate buffer. The cell suspension was then divided into 4 equal volumes of 2ml. Two of the cell suspension volumes were deconjugated using rat serum (animal donation) and the remaining two using chicken pancreas conjugase (CPC) (Difco, Becton-Dickinson, USA). The CPC solution was prepared by adding 10ml dH_2O to 0.05g CPC, this was mixed for 15min on a spiramix (Denley Instruments, UK) and later spun at 3000rpm for 10min. The supernatent was used and the solution was made fresh on each day of cellular folate analysis. The conjugase was added in a 1:30 dilution to the samples and incubated over a time course to observe which conjugase yielded the maximum cellular folates. Conjugase activity, after which time, was stopped by boil for 5min and cooling on ice. The solution was then spun for 5min at 10000rpm to remove any cellular debris that might affect the folate assay. The results of this experiment are shown in Figure 2.iv. The results

shown were the mean values from duplicate samples from each time point. The results suggest that CPC was the more effective conjugase on these epithelial cells.

However, CPC only deconjugated folates down to a diglutamate form and the microbiological assay was based on the monoglutamate form. A further experiment was required to look at the action of *L.Casei* on various polyglutamated forms of folate. 0.2265mmol solutions were made of monoglutamate, diglutamate and triglutamate folates. These were then prepared as per standard plates for the folate microbiological method and read 42h later. The results can be seen on Figure 2.v. where it has been shown that there is no significant difference between the responses of *L.Casei* to mono-, di- or tri-glutamate forms of folate.

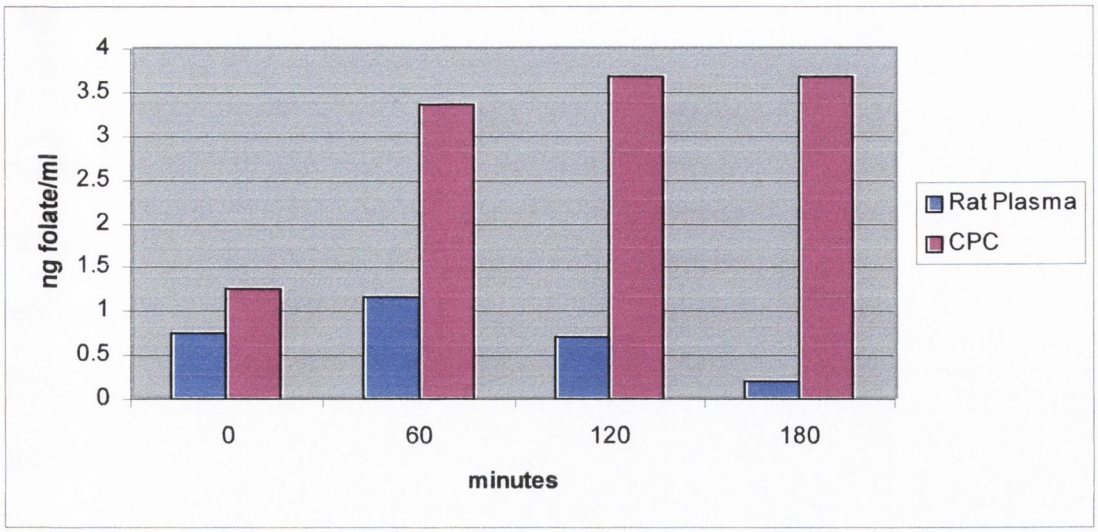


Figure 3.i Effect of different conjugase activity on cellular folate yields from human umbilical cord epithelial cells.

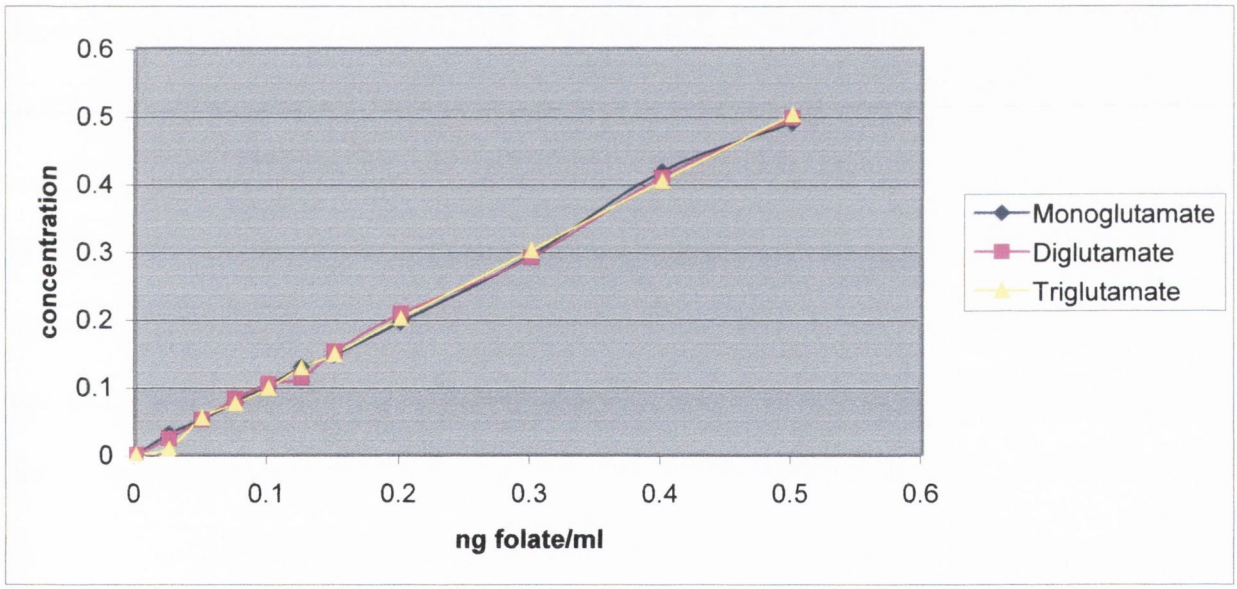


Figure 3.ii. Effect of *L.Casei* on various forms of folate

b. Optimisation of Conjugase Action

CPC was chosen as the suitable conjugase for the cells of interest and subsequent experiments were performed to examine the optimal conditions for the CPC action. The first experiment looked at the effect of time on CPC action. Cell culture work was performed to produce a suitable population of HT29 human adenocarcinoma epithelial cells (HT29 clone 19A; Augeron, 1984) for this work. Cells were grown to confluency over 5 days in $75cm^2$ culture flasks (Sigma, Dorset, UK) using a growth media of 500ml 5A McCoys (Biosciences, UK), 50ml heat inactivated foetal bovine serum (Sigma, Dorset, UK), 10ml culture cocktail containing L-glutamine and pencillin/streptomycin (Sigma, Dorset, UK). They were then trypsinised and resuspended in a buffer solution. HT29 cells were standardized by cell count (1×10^6) into duplicate volumes in 1% Sodium Ascorbate PBS. The cells then underwent deconjugation for varying amounts of time at 37^0C. The results of this experiment can be seen in figure 2.vi. where 2h was observed to be the optimal duration of incubation with CPC.

The second experiment looked at the effect of pH on the action of the conjugase. The HT29 cells were prepared in an identical manner as above except the pH of the buffer was altered to measure 4.5, 5.5, 6.5, 7.5. To these cell pH solutions, CPC was added and incubation occurred for 2h as before. The results as depicted in figure 2.vii and show that the optimal pH for CPC was 6.5, following this the cell buffer (1%NaAsc PBS) was brought to a pH of 6.5.

Another experiment was performed to examine the concentration of CPC needed for effective deconjugation. HT29 cells were once again used and prepared in duplicate for each volume of CPC. The volumes of CPC added were 10, 15, 17, 20µl to 500µl cell suspension; the overall total volume was adjusted where necessary with dH_2O. These were then incubated for 2h at 37^0C, followed by the boiling, cooling and centrifugation steps previously described. The results are shown in Table 2.i and show that concentrations greater than 17µl had no improvement on folate levels recovered.

c. Folate Recovery/Loss

Finally, an experiment was planned to examine any possible loss of folates that could occur during the folate deconjugation steps. SW-40 cell line was used in this experiment (kind gift of Eileen Looby, Immunology Department), which is a human colonic adenocarcinoma epithelial cell line. Trypsinised cells were resuspended in 5ml 1% sodium ascorbate PBS. A cell count was performed using ethidium bromide/acridine orange; cells were divided based on this into two 1ml volumes of 4 x 10^6 cells. One of the aliquots was stored at -40^0C. The remaining aliquot was sub-divided into 500µl volumes. These were sonicated at power 5 for 30 seconds on ice. 50µl H^3-folic acid (TRK212, specific activity 43Ci/mM folic acid; Amersham, UK) was added to each aliquot. This was mixed well by inversion and boiled for 5min, then cooled on ice. 100µl was removed for radiolabel counting. 17µl CPC was added to each aliquot and they were incubated for 2h at 37^0C. Following this stage, a further 100µl volume was removed and labeled. The original aliquots were boiled again for 5min and cooled on ice. Again 100µl was removed and labeled. The original aliquots were then centrifuged at 14,000G for 5min. The final stage was also sampled and stored.

All the 100µl samples were estimated using liquid scintillation. A 50µl of each sample was added to 3ml Scinti-Fluid (Eco-Lite, USA). Disintigrations per minute (dpm) of H^3-folic acid activity was measured (Liquid scintillation analyzer, TRICARB 1500, Packard, England). The results indicate that the deconjugation steps resulted in no significant loss of folates present in the cells (Table 3.ii)

d. Unit of Expression

The issue of the unit of expression arose mainly due to the collaborative nature of the study. The Meenan/modified ion-chelation method expressed the final folate results from the microbiological assay as pg/µg DNA, where the folate result was standardized with respect to spectrophotometric determination of the homogenate DNA content. However, this method

used quite a large volume of cells and this posed a major problem, as there was only a small volume of cells to work with for cellular folate determination.

Several experiments attempted to work with the original unit of expression either by protein content determination or DNA through fluorescence but these were not reliable. The comet assay required a cell count to be performed to determine the average amount of cells that were to be pipetted onto the comet slides and it was decided that this would be an ideal unit of standardization as it would provide an accurate figure of the amount of cells present. The unit of expression was decided to be pg folate / 1 x 10^5 cells.

e. Effect of Cell Viablilty

The effect of cell viability was examined with respect to epithelial cell folate content and the comet assay. Four endoscopic biopsies were taken from five endoscopically normal individuals. Two biopsies were processed as per the previously described methodology protocol. The remaining two biopsies were also processed as normal but 5mM Dithiothretol was added to the EDTA solution step to decrease viablility. Epithelial cell viability was assessed using ethidium bromide/acridine orange. The resulting cell isolates had their cellular folate assessed as per the previously described method.

The average viability for the normal group was 73.9 $\pm$ 11.4%, while the DTT-treated group was 3.5 $\pm$ 2.1%. The average folate content for the normal and the DTT-treated groups were 19.36 $\pm$ 5.01 pg/1 x 10^5 cells and 18.88 $\pm$ 3.9 pg/1 x 10^5 cells respectively. The comet parameters for % tail DNA were 32 $\pm$ 11 for the normal viability group and 39 $\pm$ 8 for the low viability one. The results of this experiment showed that the epithelial cells, regardless of viability, remained assayable for both folate status and comet assay parameters.

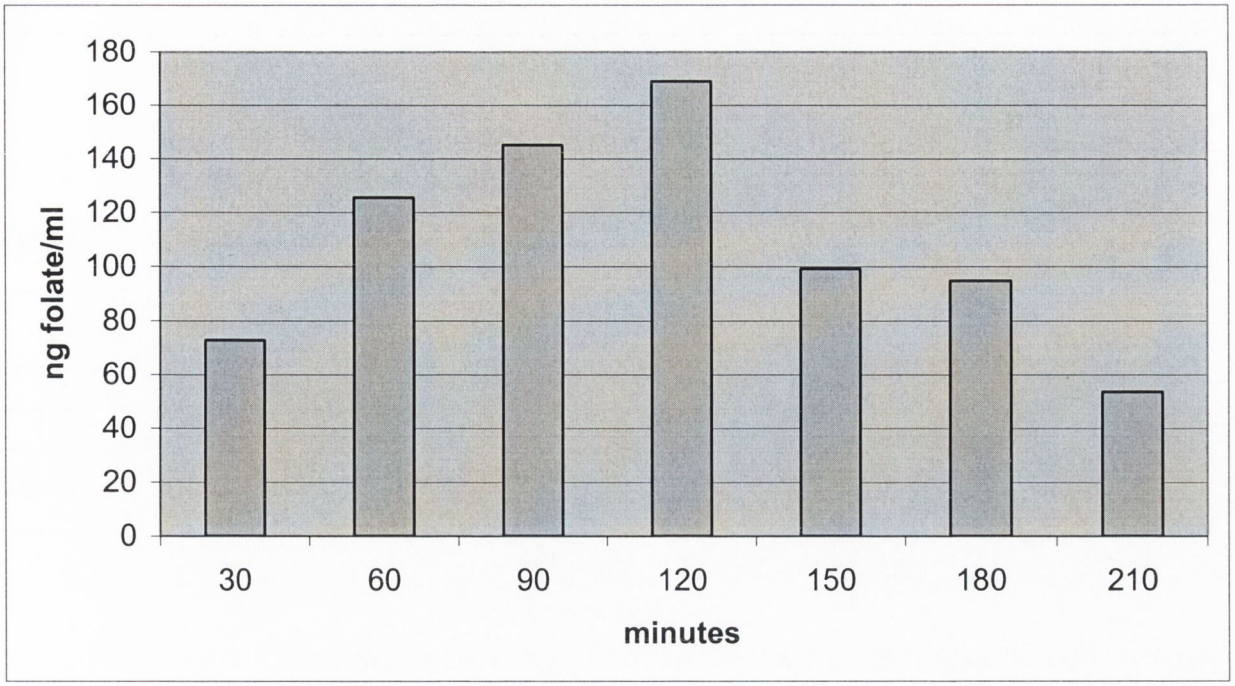


Figure 3.iii. Effect of length of incubation on CPC action on HT29 cell cultures

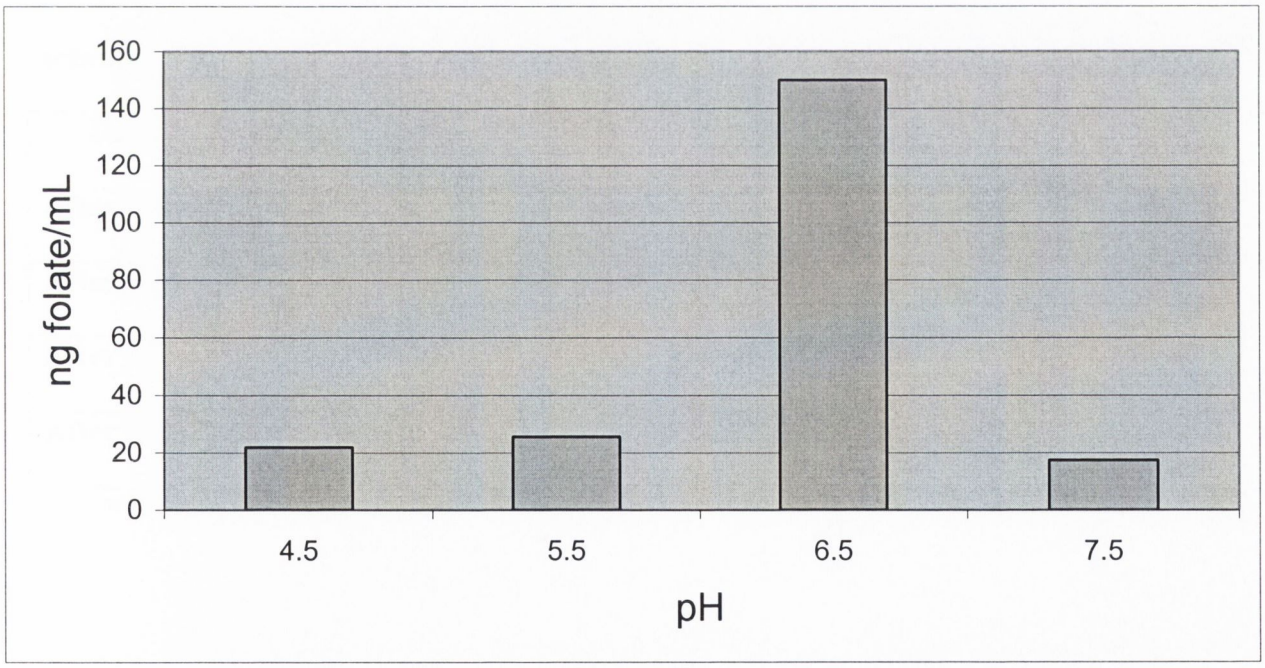


Figure 3.iv Effect of pH on CPC activity on HT29 cell cultures

Table 3.i Effect of concentration of CPC on folate deconjugation.

Concentration CPC	*Sample 1 (ng folate/ml)*	*Sample 2 (ng folate/ml)*
10µl	143.76	141.90
15µl	167.22	169.32
17µl	168.01	171.45
20µl	168.87	170.33

Table 3.ii Recovery experiment using SW-40 cell line and H^3-folic acid, measured by scintillation.

Deconjugation Step	*Sample 1 (dpm)*	*Sample 2 (dpm)*
After CPC addition	17182.9	21692.9
After first boiling	17238.7	22972.7
After second boiling	17957.7	23578.2
After centrifugation	16494.5	22470.1

iii. Validation of Epithelial Cell Folate Assay

It was important to examine the reproducibility and variation of the assay within individuals and between individuals. Initially, the reproducibility within individuals for the same site was examined.

a. Patient Selection

Twelve endoscopically normal patients were recruited. Exclusion criteria were active inflammatory bowel disease, a previous history of CRC, coeliac disease, previous bowel resection for non-malignant conditions, intestinal malabsorption, liver or renal disease, pregnancy, epilepsy, alcoholism, pernicious anemia and the use of antibiotics or anti-folate medication (sulphazalazine or methotrexate). Written informed consent was obtained from all patients. All patients had prepared for colonoscopy by taking orally administered colonic lavage solution (Kleanprep; Norgine, Harefield, UK).

b. Reproducibility of Results

From each patient, 4 descending colon biopsies were taken and placed into fresh HBSS. The biopsies were sub-divided into two sets of two biopsies in individual universal containers and assayed separately. The mean of the percent co-efficient of variation for the group was 3.49%. Subsequently, the data was analysed using release 10.0 SPSS for PC (SPSS UK, Surrey, UK). A strong positive correlation between the sets of biopsies results for epithelial cell folate concentrations was shown ($r = 0.898$, $P < 0.0001$), while a paired t-test confirmed that there was no significant difference between the concentration results (Figure 3.v).

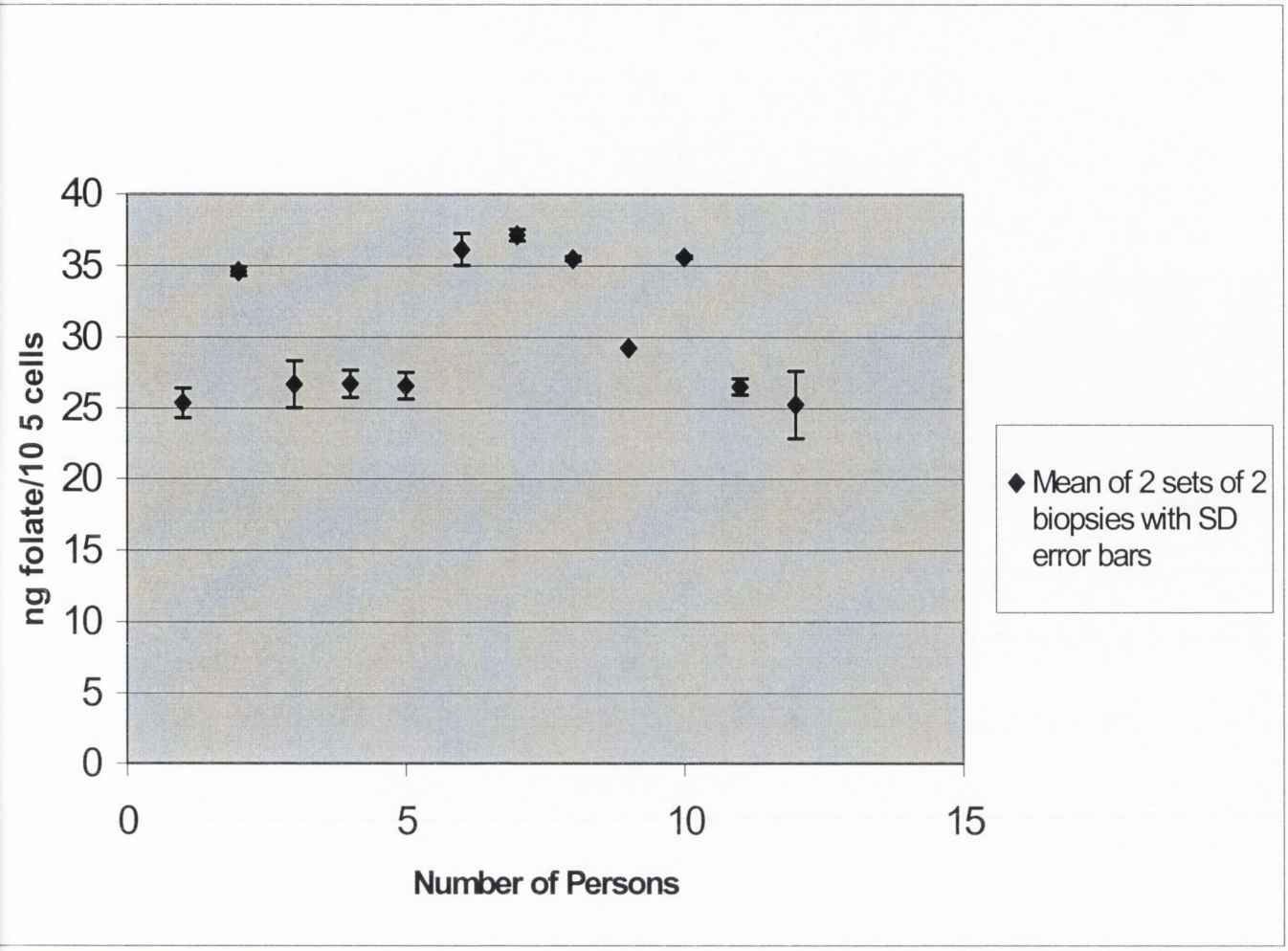


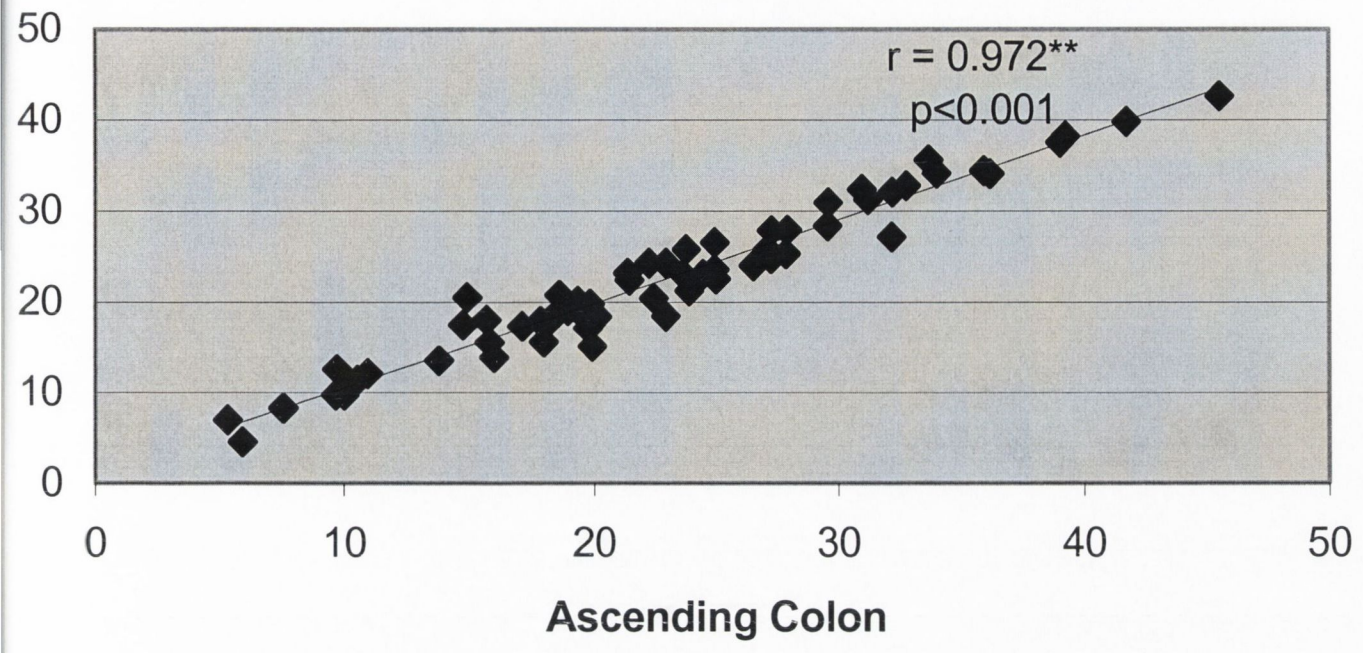
Figure 3.v Reproducibility of epithelial cell folate assay within a single site of separate individuals.

c. Correlation Between Sites

The following step was to investigate the relationship of different sites within the same individual and their epithelial cell folate concentrations. For this 61 endoscopically normal individuals were recruited as before. Six biopsies in total were taken from three anatomically defined areas – ascending, transverse and descending colon. Two biopsies were taken from each site and transferred into universal containers and assayed separately. The results from each individual were plotted on a scatterplot. A strong positive linear relationship was seen in each case (ascending versus transverse colon $r = 0.972$, $P < 0.001$; transverse versus descending $r = 0.975$, $P < 0.001$). One-way analysis of variance (ANOVA) was used to examine the difference between the concentrations between sites and this found no significant difference (Figure 3.vi).

d. Variability Between Individuals

Finally, inter-individual variation was examined for all above patients. In this case, no correlation was seen between individuals, as there was a distribution of concentrations across a range of values. The mean epithelial cell folate concentration was 23.6 ± 8.4 ng/10^5 cells and the values followed a normal distribution (Figure 3.vii).



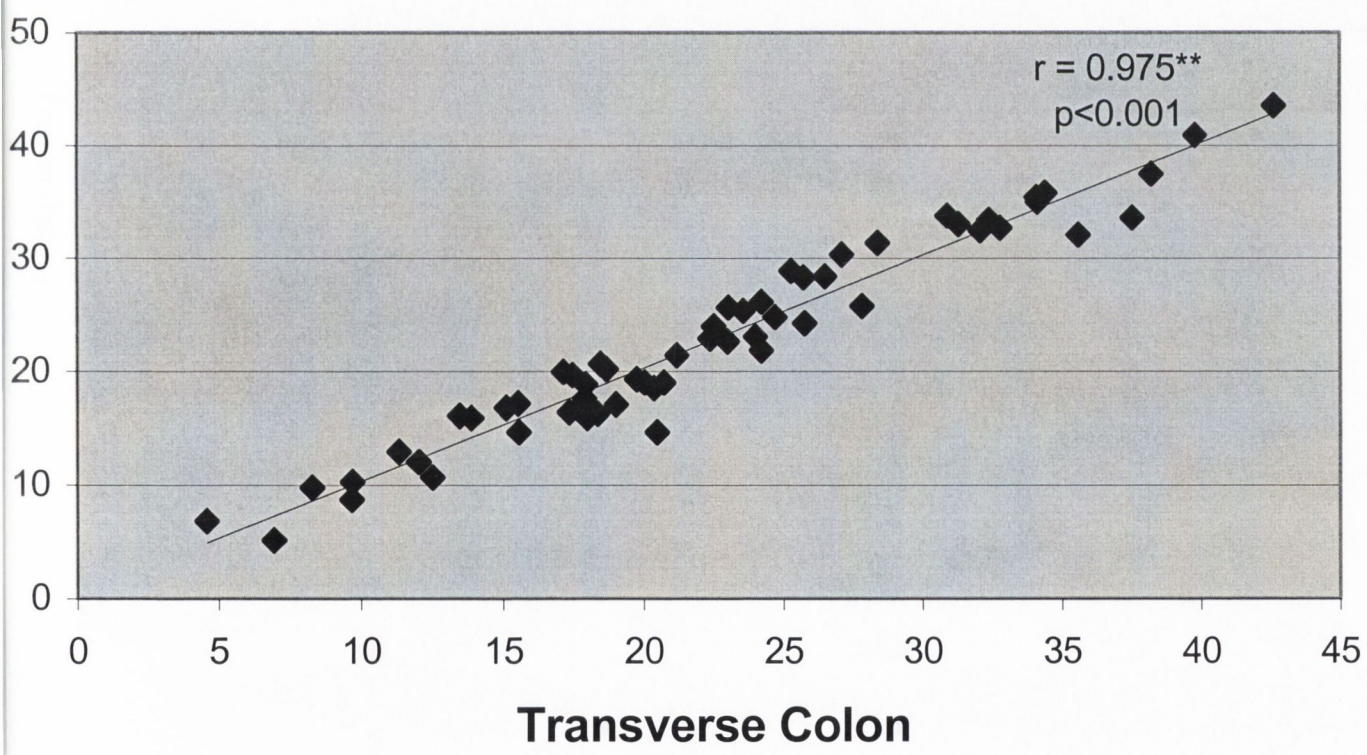


Figure 3.xi Scatterplots of three anatomical locations in the large intestine showing a strongly positive linear relationship with each other.

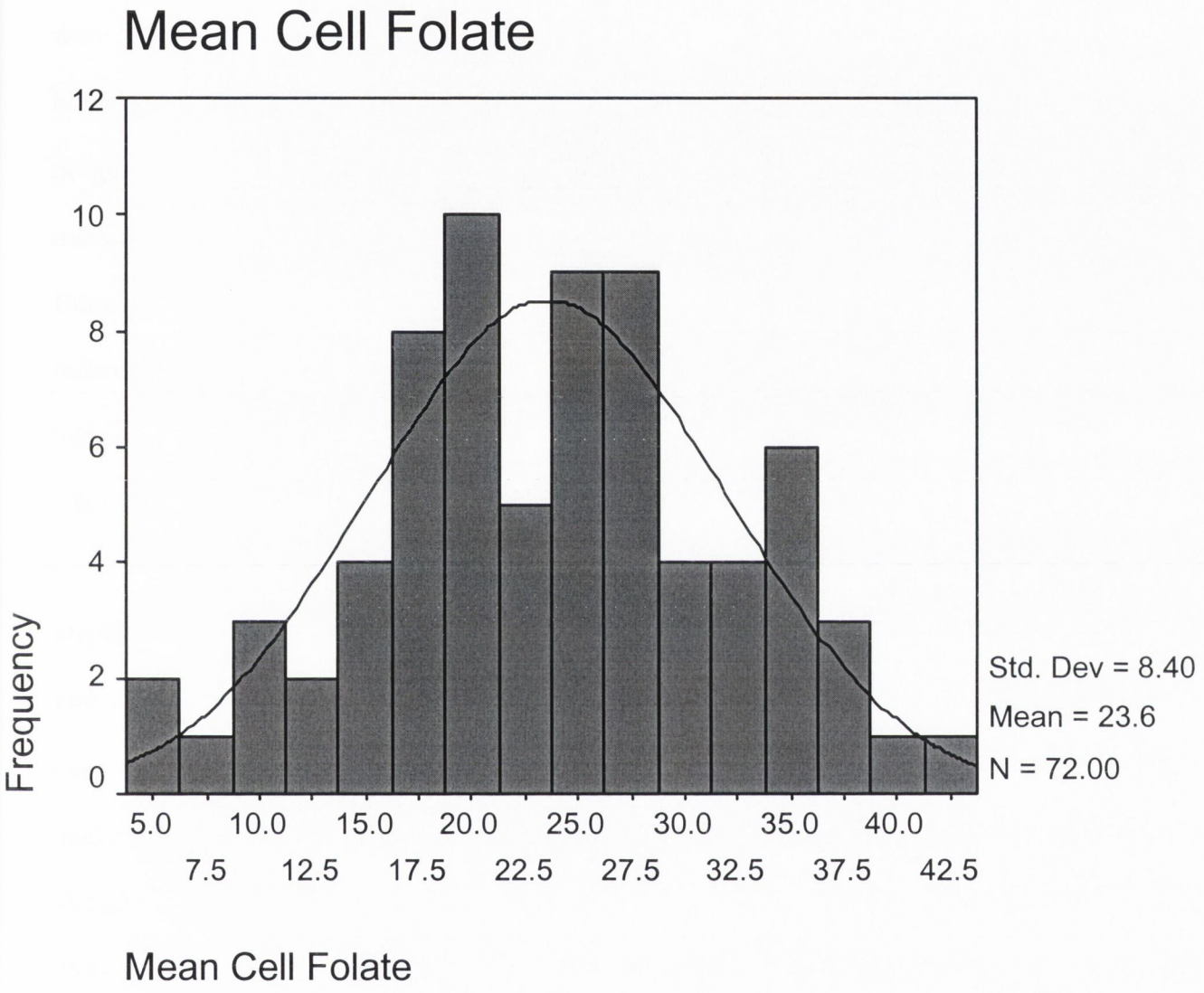


Figure 3.vii Distribution of epithelial cell folates between individuals

iv. Method Comparison

a. Patient Selection

To obtain a clearer picture of which method was the most appropriate for the analysis of tissue folate, a group of 27 endoscopically normal patients were recruited. Exclusion criteria were active inflammatory bowel disease, a previous history of CRC, coeliac disease, previous bowel resection for non-malignant conditions, intestinal malabsorption, liver or renal disease, pregnancy, epilepsy, alcoholism, pernicious anemia and the use of antibiotics or anti-folate medication (sulphazalazine or methotrexate). The study was approved by the Joint Research Ethics Committee of the Federated Dublin Voluntary Hospitals and St. James Hospital. Written informed consent was obtained from all patients. All patients had prepared for colonoscopy by taking orally administered colonic lavage solution (Kleanprep; Norgine, Harefield, UK).

b. Biopsy Selection, Patient Information and Blood Sampling

From each patient, four descending colon biopsies were taken and placed into fresh HBSS. A venous sample was collected into an ethylenediaminetetraacetic acid vacutainer for DNA extraction, erythrocyte folate and plasma homocysteine determination. A vacutainer containing no anticoagulant was used for the determination of serum folate and vitamin B_{12} analysis. For plasma Hcy, plasma was separated within 1h of collection and frozen at -40^0C. Weight and height were recorded and information on smoking habits, physical activity levels, alcohol intake, current medication, medical history, blood pressure and supplement use were gathered before colonoscopy. Subjects were defined as supplement users if they reported taking folic acid supplements or multivitamin preparations containing folic acid at any time in the previous year. One patient was excluded due to lack of venous access.

c. Whole Biopsy Folate Extraction Method

Upon return to the laboratory, two biopsies were removed from the container and processed using the homogenised method. The liquid nitrogen step was not performed as the possible contamination of the assay with faecal matter or residue from the bowel may have affected the tissue folate result. A wet weight was obtained and the biopsies were added to 20 volumes of extraction buffer. The samples were then boiled for 20min and cooled on ice. Homogenisation took place using a handheld homogeniser (Ultra-Turrax T8, IKA-Werke, Germany) at high power for 5minutes. The resulting suspension was centrifuged at 25000 G for 10min. The supernatent was transferred to a clean microfuge tube and frozen at -80^0c.

d. Epithelial Cell Isolation and Folate Analysis

The remaining 2 biopsies had the epithelial layer separated off using the methodology described previously and the resulting cell suspension was stored in 500µl 1% sodium ascorbate PBS (pH 6.5) at -40^0c until deconjugation and microbiological analysis using *L.Casei*. All tissue folates were determined within 4months of collection. The same deconjugation step and microbiological assay were carried out on the homogenisation method as the epithelial cell isolation method.

v. Method Comparison Results

Statistical analysis was performed using SPSS release 10 for PC (SPSS UK, Surrey, UK). Population data was compared using cross-tabulation tables and comparing means with subsequent Anova analysis to observe any significant differences in the group. Pearson's Correlation was used for comparison of the two methodologies.

a. Population Demographics

The population demographics are shown in Table 3.ii, which show no significant differences between the males and females recruited. Table 3.iii displays the local and systemic

folate concentration as well as the vitamin B_{12} concentrations. Again, there were no differences noted for sex.

b. Systemic and Local Folate

The MTHFR genotype appeared to affect the local folate distributions in both methodologies. The whole/homogenised biopsy method folate result does not appear to be predicted by systemic levels of the relevant nutrients but the epithelial cell folate appears to have a genotype interaction. In the case of the TT individuals, they are a replete group that have the highest mean cellular folate concentrations. There is a stepwise decrease through the remaining genotypes CT and TT. However, this difference was not statistically significant due to insufficient numbers.

As shown in Table 3.iv, colonic mucosal concentrations of folate using either method correlated directly with red cell folate and serum folate. A correlation was not seen with homocysteine in either method but this could be due to the sample population numbers. This result supports the epithelial cell folate as a viable marker for assessing colonic folate.

Table 3.iii Demographic characateristics of the study population

	Female (n=16)	*Males (n=11)*	*Total (n=27)*
Age	50.5±14.7	43.7±14.4	47.7±14.7
Body Mass Index	25.3±6.9	26.8±3.1	25.9±5.6
Indication for Colonoscopy			
Altered Bowel Habit	6 (37%)	3 (27%)	9 (33%)
Bleeding Per Rectum	1 (6%)	3 (27%)	4 (15%)
Abdominal Pain	3 (19%)	1 (9%)	4 (15%)
Anaemia	1 (6%)	1 (9%)	2 (7%)
Polyp Review	5 (31%)	3 (27%)	8 (30%)
Physical Activity			
Low	5 (31%)	4 (36%)	9 (33%)
Medium	11 (69%)	6 (55%)	17 (63%)
Very High		1 (9%)	1 (4%)
Alcohol			
Abstinence	4 (25%)	1 (9%)	5 (18%)
Drinkers – Beer	5 (31%)	7 (64%)	12 (44%)
Spirits	3 (19%)		3 (11%)
Wine	4 (25%)	3 (27%)	7 (26%)
Total Units / Week	6.5±6.6	11.4±9.3	8.7±8.2
Cigarettes			
Non-Smoker	6 (38%)	5 (46%)	11 (41%)
Ex-Smoker	5 (31%)	3 (27%)	8 (30%)
Smoker	5 (31%)	3 (27%)	8 (30%)
Duration - <10 years	1 (6%)		1 (4%)
< 20 years	7 (44%)	4 (36%)	11 (41%)
<30 years	2 (12%)	1 (9%)	3 (11%)
<40 years		1 (9%)	1 (4%)
Amount per day - <10	2 (12%)		2 (7%)
10-20	1 (6%)	3 (27%)	4 (15%)
>20	7 (44%)	3 (27%)	10 (37%)
Blood Pressure			
Systolic	135.1±29.3	124.7±9.9	130.9±23.6
Dystolic	79.25±17.3	82±9.5	80.3±14.5

Table 3.iv Descriptive Statistics of local and systemic folate and vitamin B_{12} status

	Female (n=16)	Male (n=10)	Total (n=26)
Epithelial Cell Folate pg/1 x 10^5	33.5±14.4	35.23±13	34.16±13.6
Whole Biopsy Folate ng/g tissue	539.1±230.7	502.59±192.7	525.05±213.6
Serum Folate	17.15±9.58	12.23±9.06	15.26±9.52
Red Cell Folate	408.99±163.4	411.09±162.24	409.8±159.7
Homocysteine	9.93±3.67	10.15±2.59	10.01±3.31
Vitamin B_{12}	353.88±166.14	356.73±179.94	354.98±167.99

Table 3.v Distribution of local and systemic folate and vitamin B_{12} by MTHFR genotype.

	CC (n=8)	CT (n=14)	TT (n=4)	Total (n=26)
Epithelial Cell Folate pg/1 x 10^5	31.86±14.03	34.24±13.63	38.51±15.75	34.16±13.65
Whole Biopsy Folate ng/g tissue	610.37±122.7	475.9±224.5	526.43±309.9	525.05±213.64
Serum Folate	11.46±7.23	16.83±10.52	17.36±9.87	15.26±9.51
Red Cell Folate	446.41±199.04	400.57±144.98	368.9±150.78	409.8±159.67
Homocysteine	10.75±4.23	9.7±2.76	9.65±3.78	10.02±3.31
Vitamin B_{12}	338.7±205.96	344.5±156.08	424.22±151.7	354.98±167.99

Table 3.vi Correlation of separate tissue folate determination methods with systemic folate status

	Epithelial tissue folate	Homogenised tissue folate	Serum folate	Red cell folate	Homocysteine
Epithelial tissue folate					
Pearson Correlation		.244	.404*	.477*	-.176
Significance (2-tailed)		.230	.040	.014	.390
Homogenised tissue folate					
Pearson Correlation	.244		.418*	.537**	-.099
Significance (2-tailed)	.230		.034	.005	.631
Serum folate					
Pearson Correlation	.404*	.418*		.734**	-.450*
Significance (2-tailed)	.040	.034		.000	.046
Red cell folate					
Pearson Correlation	.477*	.537**	.734**		-.413
Significance (2-tailed)	.014	.005	.000		.070
Homocysteine					
Pearson Correlation	-.176	-.099	-.450*	-.413	
Significance (2-tailed)	.390	.631	.046	.070	

* Correlation is significant at the 0.05 level (2-tailed)

** Correlation is significant at the 0.01 level (2-tailed)

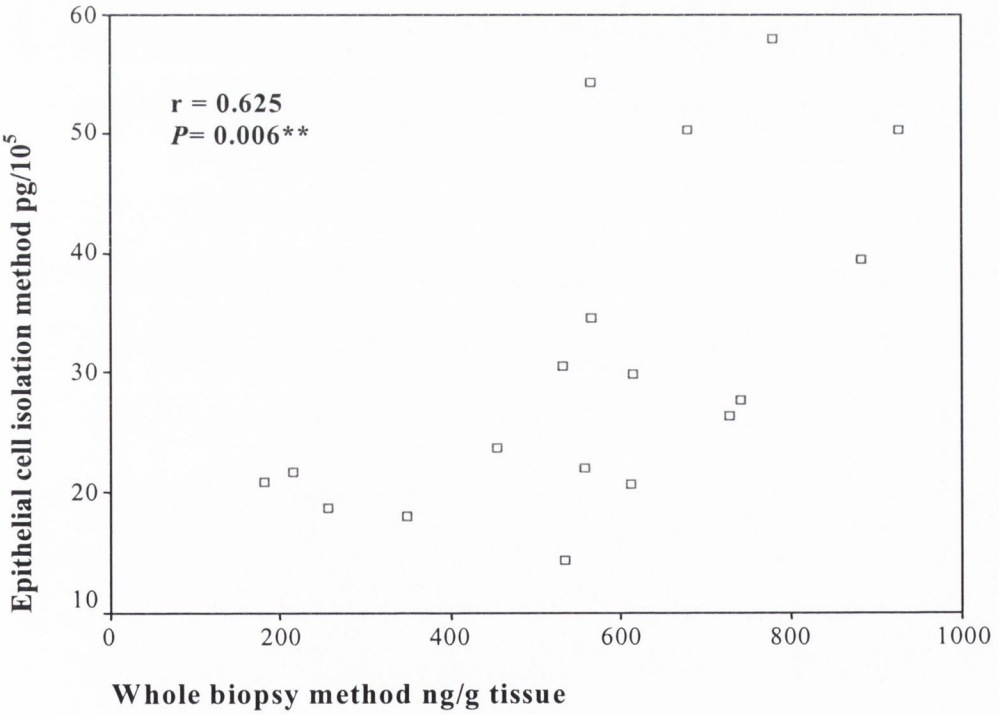


Figure 3.viii Scatterplot of epithelial cell folate and whole biopsy methods (2 patient outliers removed)

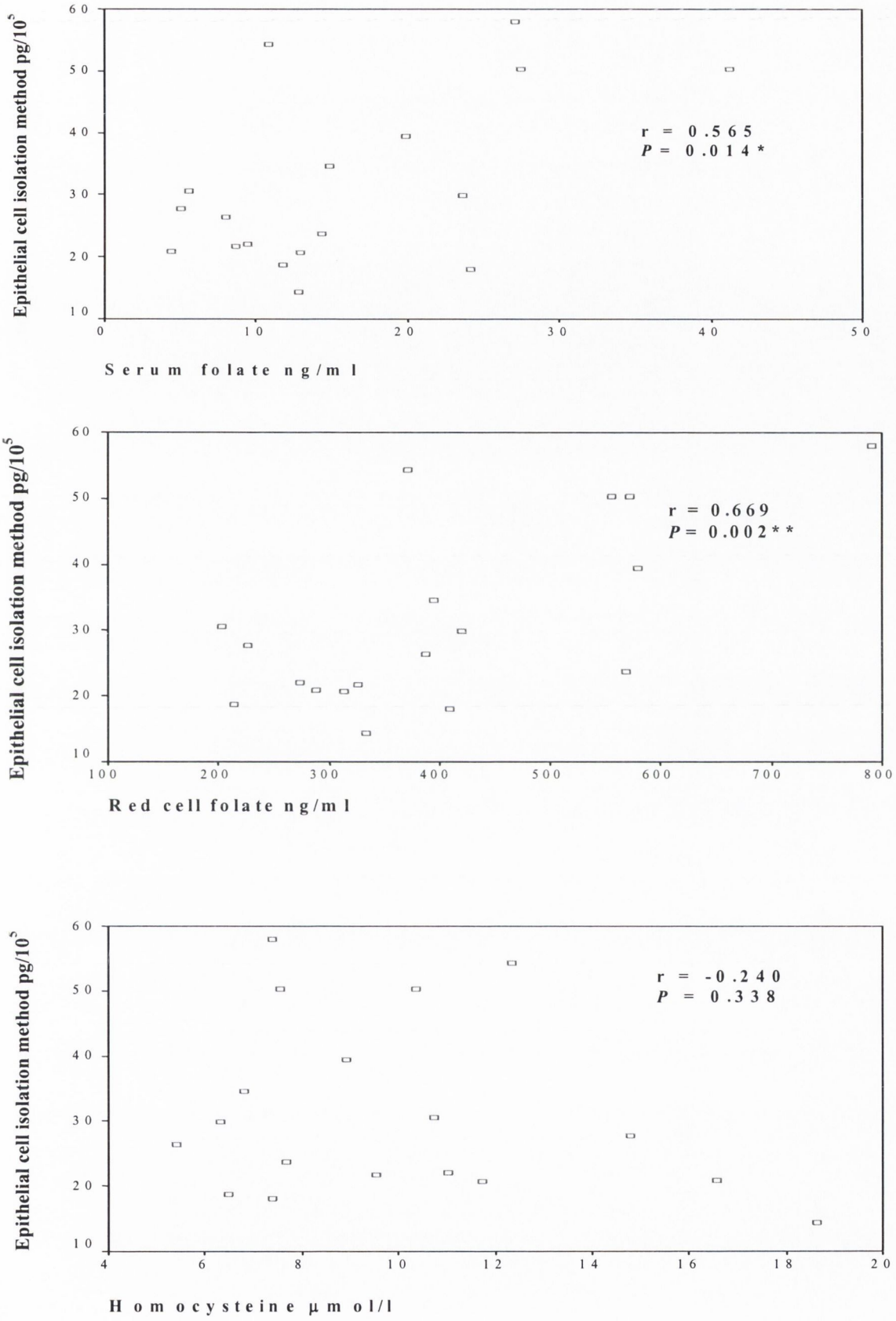


Figure 3.ix Scatterplots of correlations between systemic markers and epithelial cell isolation method (with 2 outliers removed).

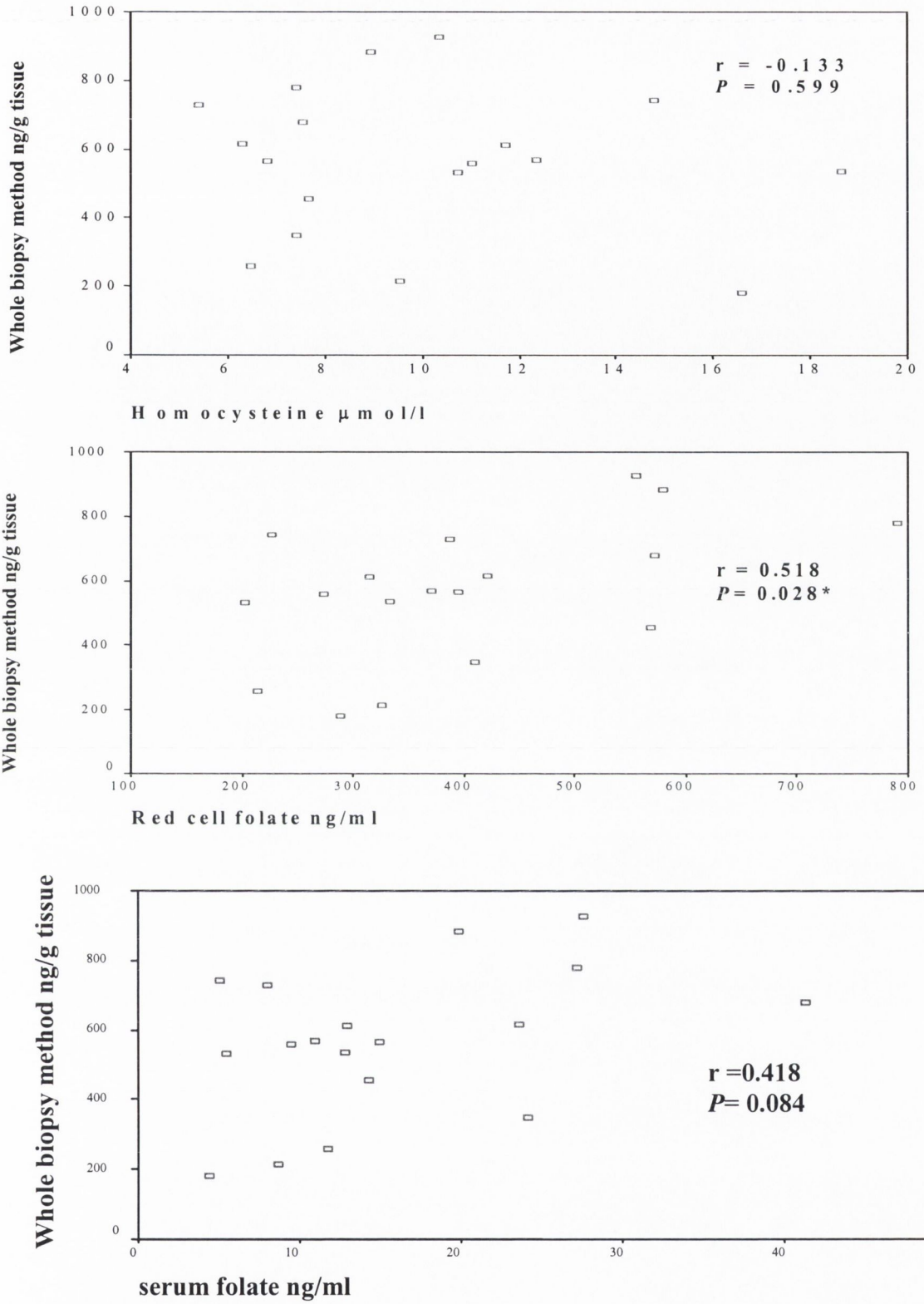


Figure 3.x Scatterplots of correlations between systemic folate values and whole biopsy method (2 outliers removed from analysis).

vi. Discussion

The deconjugation of the polyglutamated cellular folates was revisited from the original method to ensure that the conditions of the assay were optimised. Experiments were performed to identify the most suitable deconjugase for the assay (see figure 3.i) and once CPC was confirmed as the most suitable deconjugase, further experiments were done to ensure that the subsequent analysis of the cellular folate concentrations could take place (see figure 3.ii – 3.iv, table 3.i-3.ii). Validation of the assay was essential. Using repeat biopsies from the descending colon (n=12), it was possible to show that the assay was reproducible within the same anatomical site of the same individual with the mean percent co-efficient of variation of 3.49% (see figure 3.v). There was a strong positive linear relationship shown between anatomically defined sites within the same individual (n=61, ascending versus transverse colon r =0.972, P<0.001; transverse versus descending r =0.975, P<0.001). The variation seen in figure 3.vii between individuals appears to reflect the natural variance found in a normal population, as is seen with systemic markers of folate status.

The ongoing discrepancy between the two main methodologies (Meenan *et al.* 1996;Kim *et al.* 1996) used to analyse tissue folates was explored through investigating systemic folate status in normal individuals and correlating that with the results obtained using the two different methods on biopsy tissue from the same anatomical site. The debate regarding the two different methods stems from the folate source analysed. In the case of the method developed in the US, the whole biopsy is analysed and in the ion-chelation method, the epithelial cell folate is analysed. It can be argued that the whole biopsy method is inappropriate, as it will contain a variety of cells and tissue types. It could also be contaminated with faecal matter as the original method snap-freezes the biopsy once removed from the colon in liquid nitrogen and there is no washing phase. As faeces are a possible source of folate, the levels measured may be unduely affected by contamination. Another argument could be that

the whole biopsy method is not specific and does not analyse the cells of interest, the epithelial layer. However, these arguments aside, the results from the studies using this method have been able to show a relationship between systemic and localised tissue folates; though it must be added that this relationship was not consistent through the publications (Kim *et al.* 1998;Kim *et al.* 2001b). In the first publication examined a group of 30 patients who had a histologically confirmed polyp on colonoscopy (Kim *et al.* 1998). The colonic tissue folate concentration was determined from a normal biopsy from the rectosigmoid junction in all cases. A correlation was shown for all measures (red cell folate, serum folate and Hcy against colonic folate concentrations). However, in a later publication where a group of 20 individuals were followed-up in a double-blind, placebo-controlled trial for 1 year and the correlations were not seen. There was no correlation seen for the supplementation group at any time point and, in the placebo group, no correlation was seen for Hcy at the 6-month and 1-year follow-up time points. A point of note is that the statistics generated for the correlation between colonic mucosal concentrations of folate, serum and RBC folate and serum Hcy combined the placebo and treatment groups for analysis to derive significance for the initial results section (Kim *et al.* 2001b).

In the case of the ion-chelation method, while the method looks at the cell type of interest, the argument against this method is that the tissue may have its folate content altered over the time taken to disaggregate the epithelial cells. The results from an earlier study (Meenan *et al.* 1996) have been less conclusive from the ion-chelation method as there was a lack of correlation with the systemic markers of folate status but they did point towards a localised deficiency of folate in neoplastic tissues. A point of note about the ion-chelation publications is that there was no homocysteine analysis performed and this marker is now seen as the most sensitive marker of folate status (Selhub and Miller 1992). Also the effect of folate on the colorectal mucosa may not be entirely derived from blood folate as the colorectal

mucosa is exposed to intestinal luminal folate that is synthesized by intestinal microflora or that may have escaped small intestine absorption (Camilo *et al.* 1996). However, this seems less likely in light of the strong linear relationship seen between specific anatomical sites (see figure 3.xi). Yet, it may mean that a strong direct relationship between the biomarkers may not be relevant.

The results from the small study conducted in normal individuals found a significant association with either method for serum and red cell folate and not homocysteine, though it must also be pointed out that all previous tissue folate studies have been conducted on similarly small numbers (see table 3.vi). The whole biopsy method was more significiant than the epithelial cell isolate method for red cell folate before outliers were removed. The outliers were identified when examining the relationship between the two colonic folate determination methods. However, once the two outliers were removed as seen in figure 3.viii, there was a strong direct correlation between the two methods ($r = 0.625$, $P=0.006$). This also effected the overall correlations with the systemic markers, this was shown in figure 3.ix. In the case of serum folate, the strength of the correlation for the whole biopsy was weakened ($r = 0.418$, $P= 0.084$) while the epithelial cell isolate method's remained ($r =0.565$, $P= 0.014$). In the correlation between red cell folate and the two methods, the relationship between the epithelial cell isolate and red cell folate was strengthened ($r = 0.669$, $P= 0.002$) from and the whole biopsy method was weakened ($r = 0.518$, $P= 0.028$). The relationship for plasma homocysteine remained non-significant though a stronger trend immerged for each method (epithelial cell folate $r = -0.240$, $P= 0.338$; whole biopsy $r = -0.133$, $P= 0.599$). In further analysis, the colonic folate concentrations were broken down into quartiles and the relationship between them and Hcy was examined. For those below the median colonic folate concentration, a trend towards significance emerged for the epithelial cell isolation method ($r = -.577$, $P=.104$) and when the

lowest quartile was examined significance was seen ($r = -.999$, $P = .029$). No such relationship was seen for the whole biopsy method at any given quartile or above or below the median.

Looking at this chapter's results and taking into account the previous arguments for and against the two types of tissue folate analysis, it would appear that the epithelial cell folate is the more accurate marker within the study group and that it is a reproducible and valid assay for looking at the relationship between folate status and CRC.

CHAPTER 4

COMET ASSAY

1. COMET ASSAY

i. Introduction

The comet or single-cell gel electrophoresis assay has become a recognised method for looking at DNA damage (Ostling and Johanson 1984) and has been used to detect more complex DNA lesions (Duthie *et al.* 1996;McKelvey-Martin *et al.* 1998). The method has been contantly modified to allow for more specific areas of DNA damage and replication to be examined and the methods described below look at BrdUrd misincorporation, uracil misincorporation and hypomethylation in normal and abnormal colonic tissue. The theory behind these methods was described more fully in the introduction chapter. A simple flow chart of the method can be seen at figure 4.i.

ii. Bromodeoxyuridine Misincorporation Assay - Methods

a. Patient Selection

Patients were recruited from St. James Hospital Endoscopy Unit. Exclusion criteria were active inflammatory bowel disease, a previous history of CRC, coeliac disease, previous bowel resection for non-malignant conditions, intestinal malabsorption, liver or renal disease, pregnancy, epilepsy, alcoholism, pernicious anemia and the use of antibiotics or anti-folate medication (sulphazalazine or methotrexate). The study was approved by the Joint Research Ethics Committee of the Federated Dublin Voluntary Hospitals and St. James Hospital. All patients were recruited on the day of their colonoscopy, before the procedure. Written informed consent was obtained from all patients. All patients had prepared for colonoscopy by taking orally administered colonic lavage solution (Kleanprep; Norgine, Harefield, UK).

b. Biopsy Collection, BrdUrd Incorporation and Epithelial Layer Isolation

Two or four biopsies were taken from each site of interest into 8mL fresh HBSS. The biopsies were then transferred, after washing in fresh HBSS, into fresh HBSS oxygenated and

supplemented with 100μM BrdUrd (Sigma, Dorset, UK) pre-warmed to 37^0C. The biopsies were incubated for 15mins at this temperature prior to disaggregation; in chase experiments, biopsies were transferred into HBSS supplemented with 200μM thymidine, 2'-deoxycytidine, 2'-deoxyguanosine and 2'-deoxyadenosine (Sigma, Dorset, UK) for 1h at 37^0C before disaggregation.

The 2 biopsies per selected site had the epithelial layer separated off using the isolation methodology described previously and the resulting cell pellet was split into the sub-fraction for colonocyte folate analysis, which was stored in 500μl 1% sodium ascorbate PBS (pH 6.5) at -40^0c until deconjugation and microbiological analysis using *L.Casei* (all colonocyte folates were assayed within four months of isolation), and the other sub-fraction for Comet analysis. A cell viability assessment was carried out using ethidium bromide/acridine orange. The cell pellet was then suspended in phosphate-buffered saline at a concentration of 2×10^5 cells ml^{-1} prior to comet slide preparation.

c. Flow Cytometry

The purity of the cell preparation was confirmed using flow cytometry and a monoclonal antibody for the detection of epithelial cell antigen (Ber-Ep-4, Dako, Glostrup, Denmark). Aliquots of epithelial cell isolates were assessed for lamina propria contamination and intra-epithelial lymphocyte content by flow cytometry (FACScan; Becton Dickinson, Antwerp, Beligum) using the following monoclonal antibodies and fluorescent conjugates: Ber-Ep-4 (Dako, Glostrup, Denmark), CD3 (T cells) (Becton Dickinson), CD14 (monocytes) (Becton Dickinson), CD19 (B cells) (Becton Dickinson), rabbit anti-mouse fluoroscein isothiocyanate (Dako) and rabbit anti-mouse phycoerythrin (Dako). Control antibodies were used in each experiment (Simultest immunoglobulin IgG1 and IgG2a (Becton Dickinson)).Cells (2.5×10^6) were suspended in fluorescence-activated cellsorter buffer (PBS, 0.1% bovine serum albumin and 0.01% sodium azide) and washed. The cells were then

incubated with the various monoclonal antibodies for 30 min at 4°C. After two more washes, the cells were incubated with a second antibody or a fluorescent conjugate and washed twice. Flow cytometry was performed and 10 x 10^3 events were saved for analysis. The data was analysed with Lysys II software (Becton Dickinson).

d. Comet Slide Preparation and BrdUrd Comet Assay

A series of six slides were produced per site for comet analysis as described previously. The slides were transported in neutral lysis buffer containing proteinase K as before. Once the slides reached Coleraine, DNA unwinding (40 minutes) and electrophoresis (20 minutes at 25 volts and 300 milliamps) was performed under alkaline conditions (0.3M NaOH, 1mM EDTA, pH 13). Following electrophoresis, the gels were neutralized (0.4M Tris) and washed in PBS prior to immunostaining. The gels were incubated with 25µl mouse monoclonal anti-BrdUrd (10µg ml^{-1}, BO, Biosciences, Oxford, UK) per slide in the dark at room temperature for 1 hour. The primary antibody was gently washed off with three changes of PBS and one wash with 0.1% BSA PBS, before the addition of 25µl of the secondary antibody per gel (5µg ml^{-1} sheep anti-mouse IgG, fluorescein conjugated, Biosciences) which was incubated and washed off as before. The gels were then counterstained with 25µl of propidium iodide (0.75µg ml^{-1}, Sigma, Dorset, UK) and covered with a coverslip (22 x $50mm^2$) for image analysis.

Comet slides were analysed using a single-blind approach, in that the patient details were not made available until comet analysis was completed. Comet analysis was carried out using a final magnification of x400 (Nikon x40 Fluor Lens, Japan) and Komet 4.0 software (Kinetic Imaging Ltd., Liverpool, UK). The % DNA in the comet tail for each cell was measured. Results were expressed as mean % tail DNA in 25 cells on each duplicate comet slides. Data were collected as the mean and standard deviation of the 50 comets scored per

individual patient. The variance or dispersion from the mean % tail DNA of 50 comet measurements from each duplicate pair of slides was also noted.

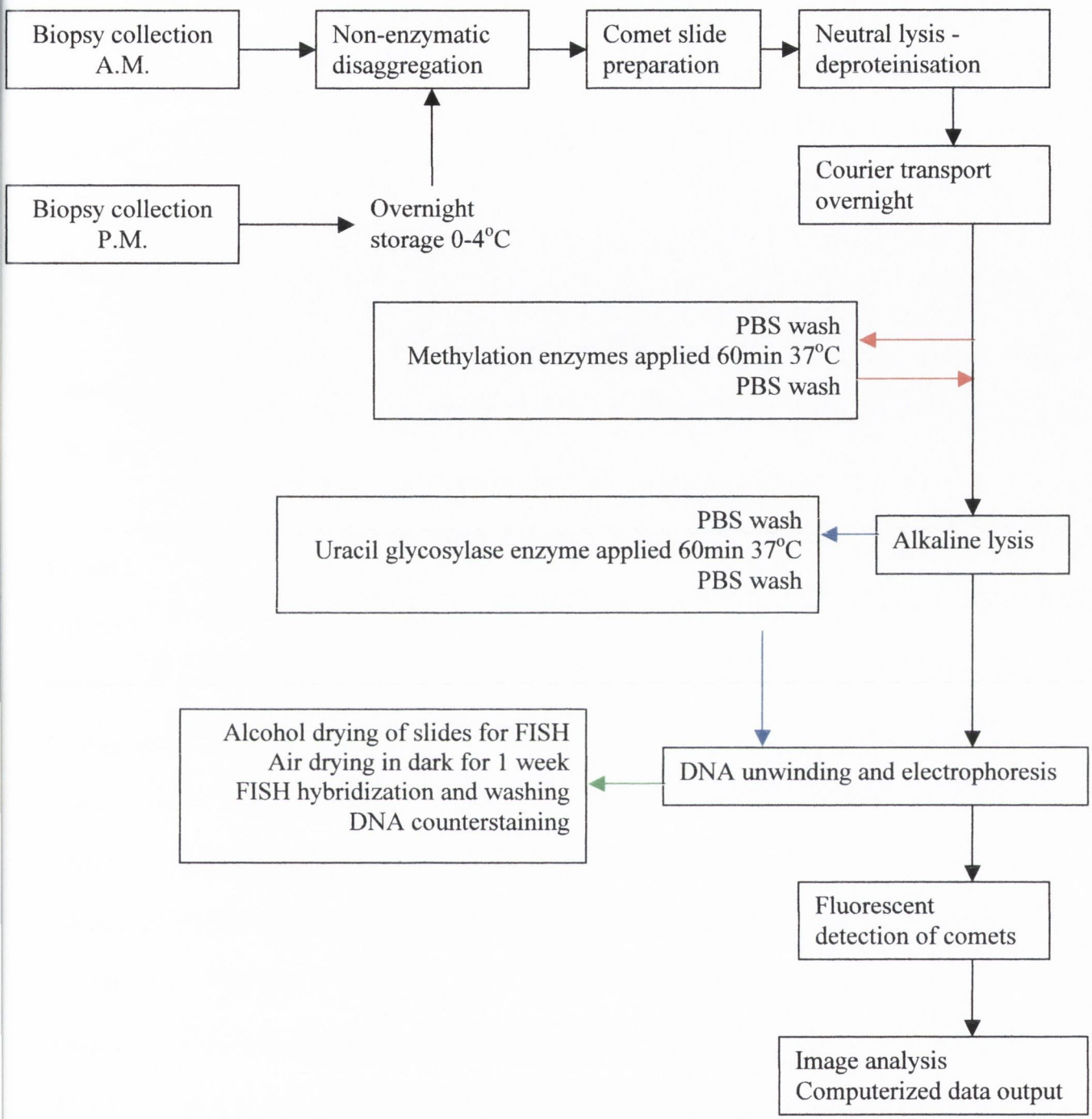


Figure 4.i Flow diagram of the protocol for the collection and processing of colonic biopsies for Comet analysis. Coloured arrows indicate steps that are carried out on sets of selected slides (enzyme digestions or FISH analysis), while all black arrows indicate common steps for all slides processed.

iii. Bromodeoxyurdine Comet Assay Results

a. Epithelial Cell Isolation

Using the ion-chelation method previously described, an epithelial cell population of greater than 95% purity was obtained, as assessed by flow cytometry using epithelial cell antigen positive expression (Figure 4.ii). Contaminating lymphocytes could be excluded on the basis of size during during the comet analysis.

Cell yield ranged from 1 to 2 x 10^5 cells per single biopsy. Cell viablilty, as assessed by acridine orange/ ethidium bromide, ranged from 60 to 80%.

b. Bromodeoxyuridine Comet Reproducibility

Inter- and intra-individual reproducibility was assessed for the BrdUrd comet assay. Comet measurements on duplicate endoscopic biopsies, which were collected from up to three different colorectal sites in each patient (n = 36 sites), showed no significant difference between sites, demonstrating the reproducibility of the assay (as shown in Figure 4.iii). Furthermore, a sequential follow-up specimen was obtained for one patient undergoing a repeat colonoscopy. Upon initial colonoscopy, the patient displayed adenomatous polyps and had a mean % comet tail of 50$\pm$11 in comets derived from normal descending colon. The repeat colonoscopy (four months later) had a mean % comet tail of 53$\pm$9 in comets derived from normal sigmoid colon. This indicated that even after a lapse of four months, reproducibility was possible. A total of 52 biopsies were collected from 35 patients who displayed no evidence of malignancy upon endoscopy. Little variation in mean % BrdUrd comet tail was observed in this sample group (mean % DNA comet tail = 46$\pm$7.1).

Pulse-chase experiments were performed to assess the DNA maturation (figure 4.iv-figure 4.v). A normal patient was examined and showed proficient DNA maturation after a chase period of 1 hour ($P<0.01$). However, epithelial cells from a patient with adenomatous polyps demonstrated a significant delay in DNA maturation compared to the normal patient

($P<0.05$, t-test). This was shown by the failure of the DNA comet tail to move towards the head as seen in figure 4.vi.

c. Bromodeoxyurdine Comet in Abnormal Tissue

A small number of sample data indicates that there was a significantly higher mean % comet tail DNA in biopsies from polyp or tumour tissue (mean of 63 ± 2.7, collective data for $n=4$, $P<0.001$) compared to those from normal subjects. This indicates that a reduction in DNA replication was detectable in these patients. The histologically normal tissue from patients with polyps ($n=15$) also showed a trend towards an elevated mean % comet tail DNA compared to patients with no polyps (mean comet result of 50 ± 7.1), although these results did not reach significance.

Multiple biopsies were collected from various sites within the colon of patients diagnosed with malignancy in order to assess the degree to which the reduction in DNA replication found in histologically abnormal samples extends into the histologically normal tissue surrounding a polyp/adenoma. In data obtained from three individuals harboring colonic tumours yielded interesting results. In these cases, biopsies obtained from the malignancy and the adjacent tissue showed elevated comet measurements as distinct from the histologically normal tissue 10cm away from the malignancy, which showed normal comet measurements (figure 4.vii).

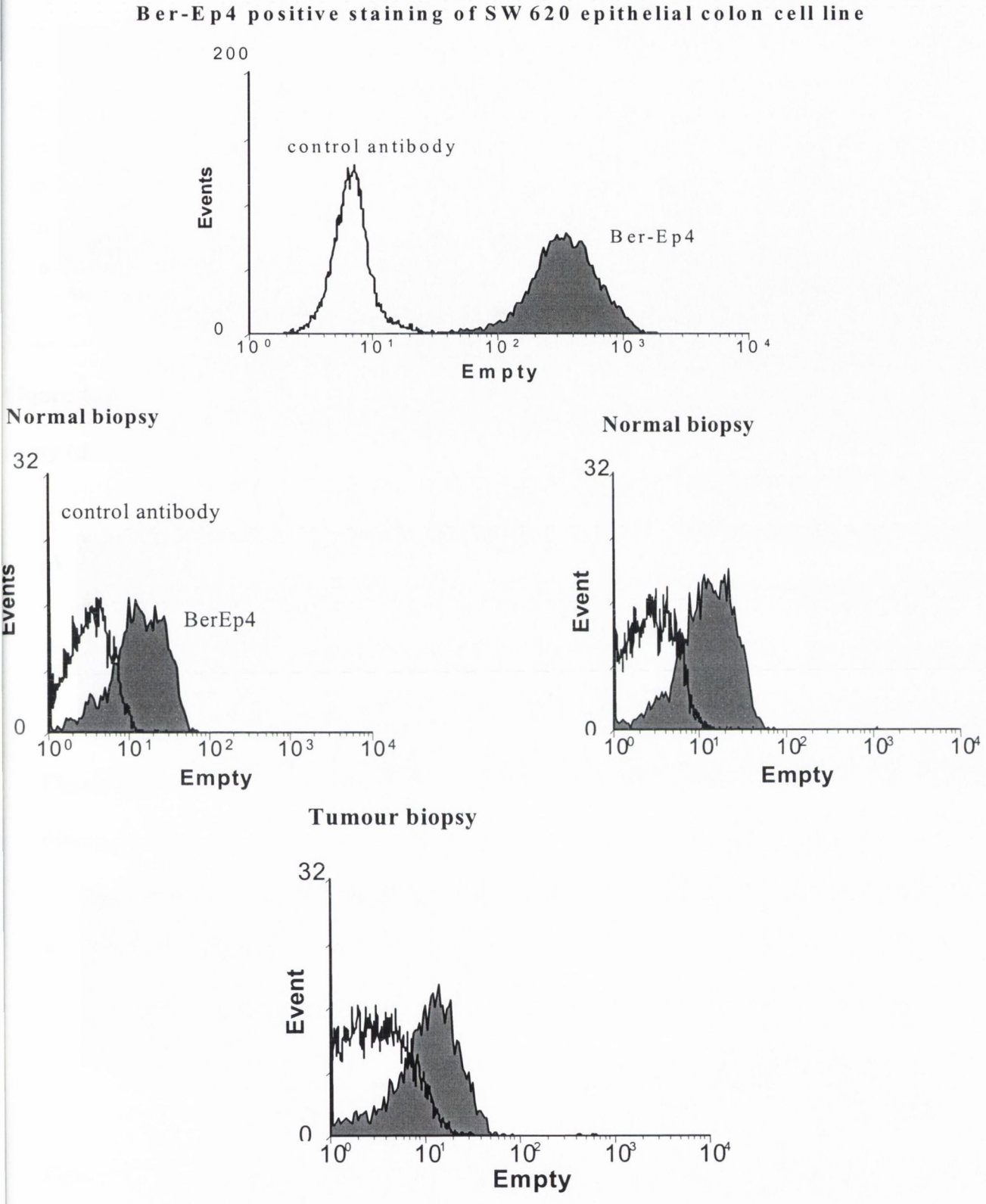


Figure 4.ii Epithelial antigen staining of human colonic disaggregated cells using Ber-Ep4 monclonal antibody using flow cytometry.

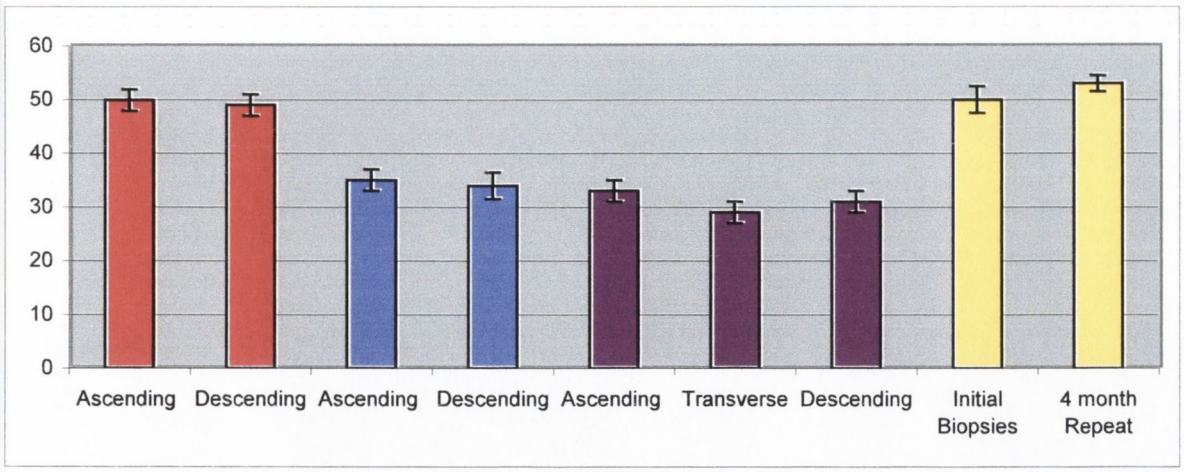


Figure 4.iii BrdUrd % mean tail DNA for normal patients illustrating reproducibility of the assay (different colours represent separate individuals).

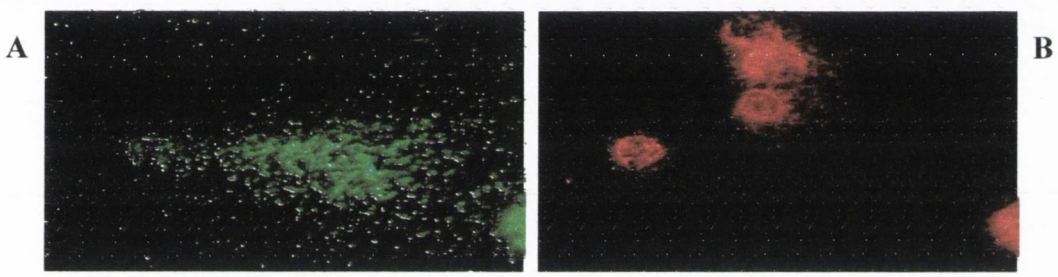


Figure 4.iv BrdUrd incorporation with BrdUrd label initially close to strand discontinuities in recently-replicated DNA (A) and PI staining of same slide location (B).

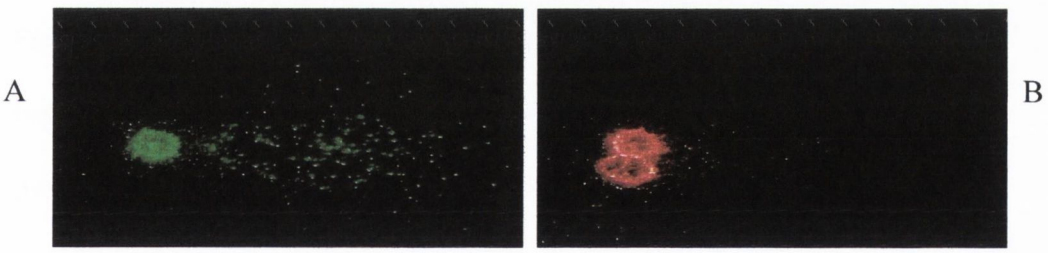


Figure 4.v BrdUrd post-chase (A) and PI staining of same cell location (B). The replicating fork moves away from labelled regions and there is disappearance of these discontinuities as newly-replicated DNA matures and the BrdUrd label is incorporated into high molecular weight continuous DNA in the head.

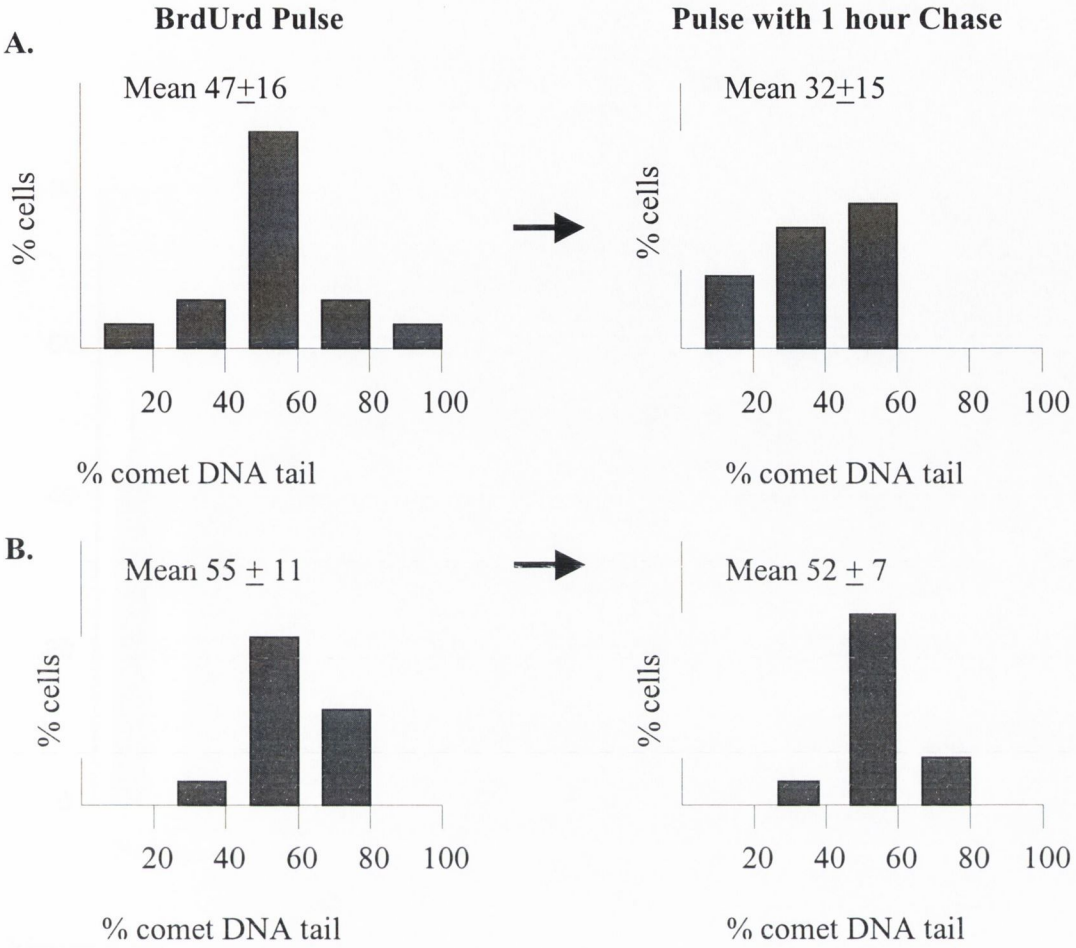


Figure 4.vi BrdUrd pulse-chase in colonic cells reveals inherent defects in DNA replicative integrity. Results were expressed as frequency distribution plots of BrdUrd percentage of tail DNA labelled as BrdUrd pulse alone or BrdUrd pulse with chase. One normal patient was analysed (A.) and a patient with adenomatous polyps and moderate dysplasia (B.).

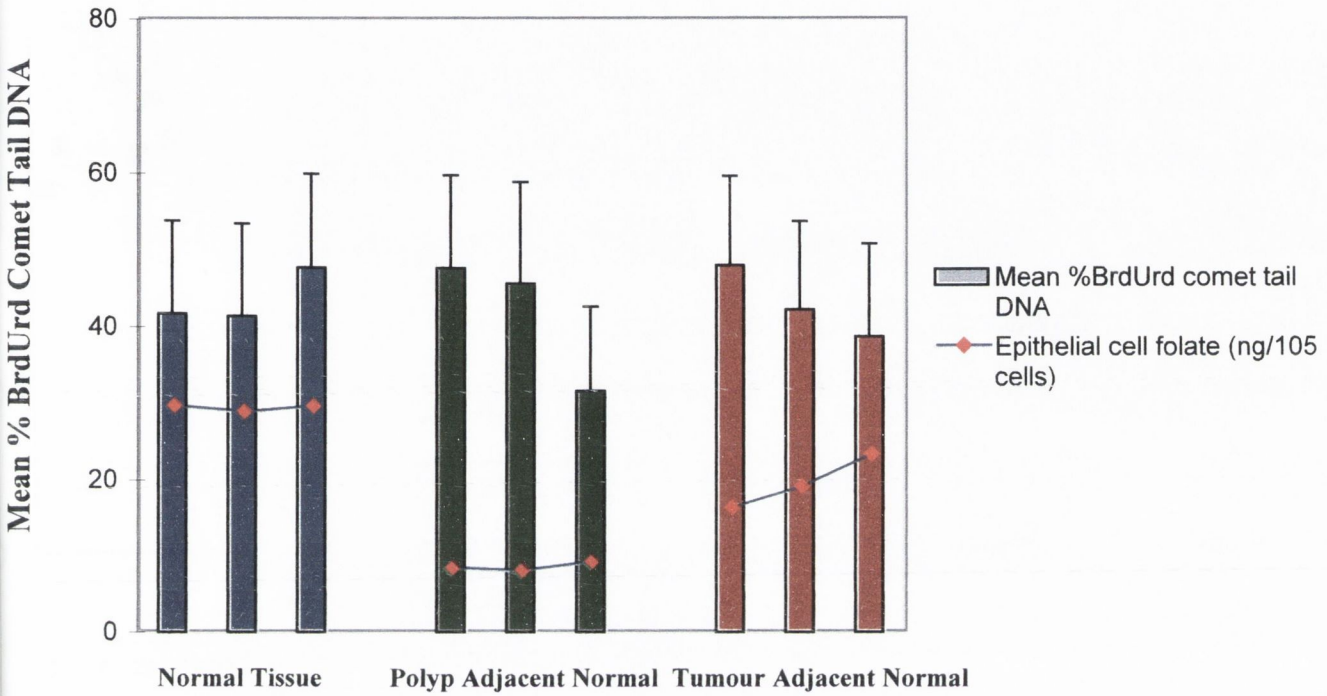
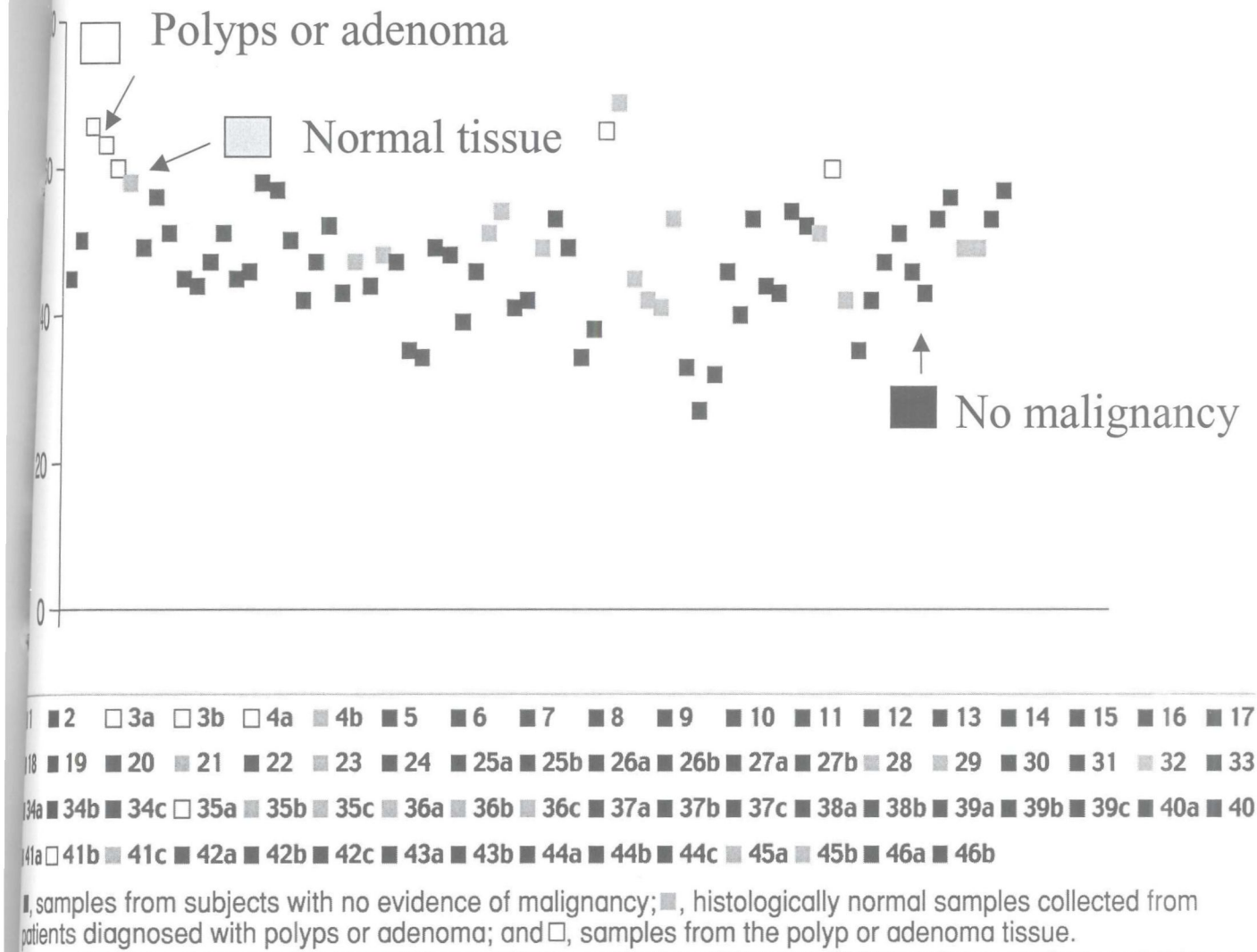


Figure 4.vii Comparison of BrdUrd mean % tail DNA and cellular folate concentrations from three sites in separate individuals.



1 ■2 □3a □3b □4a ■4b ■5 ■6 ■7 ■8 ■9 ■10 ■11 ■12 ■13 ■14 ■15 ■16 ■17
18 ■19 ■20 ■21 ■22 ■23 ■24 ■25a ■25b ■26a ■26b ■27a ■27b ■28 ■29 ■30 ■31 ■32 ■33
34a ■34b ■34c □35a ■35b ■35c ■36a ■36b ■36c ■37a ■37b ■37c ■38a ■38b ■39a ■39b ■39c ■40a ■40
41a □41b ■41c ■42a ■42b ■42c ■43a ■43b ■44a ■44b ■44c ■45a ■45b ■46a ■46b

■, samples from subjects with no evidence of malignancy; ■, histologically normal samples collected from patients diagnosed with polyps or adenoma; and □, samples from the polyp or adenoma tissue.

Figure 4.viii BrdUrd comet analysis of colonic epithelial cell isolates, results presented as a scatterplot of mean % BrdUrd comet tail DNA. Patient ID numbers (n=46) are given in the legend and multiple samples from the same patient are labelled *a*, *b* and *c*.

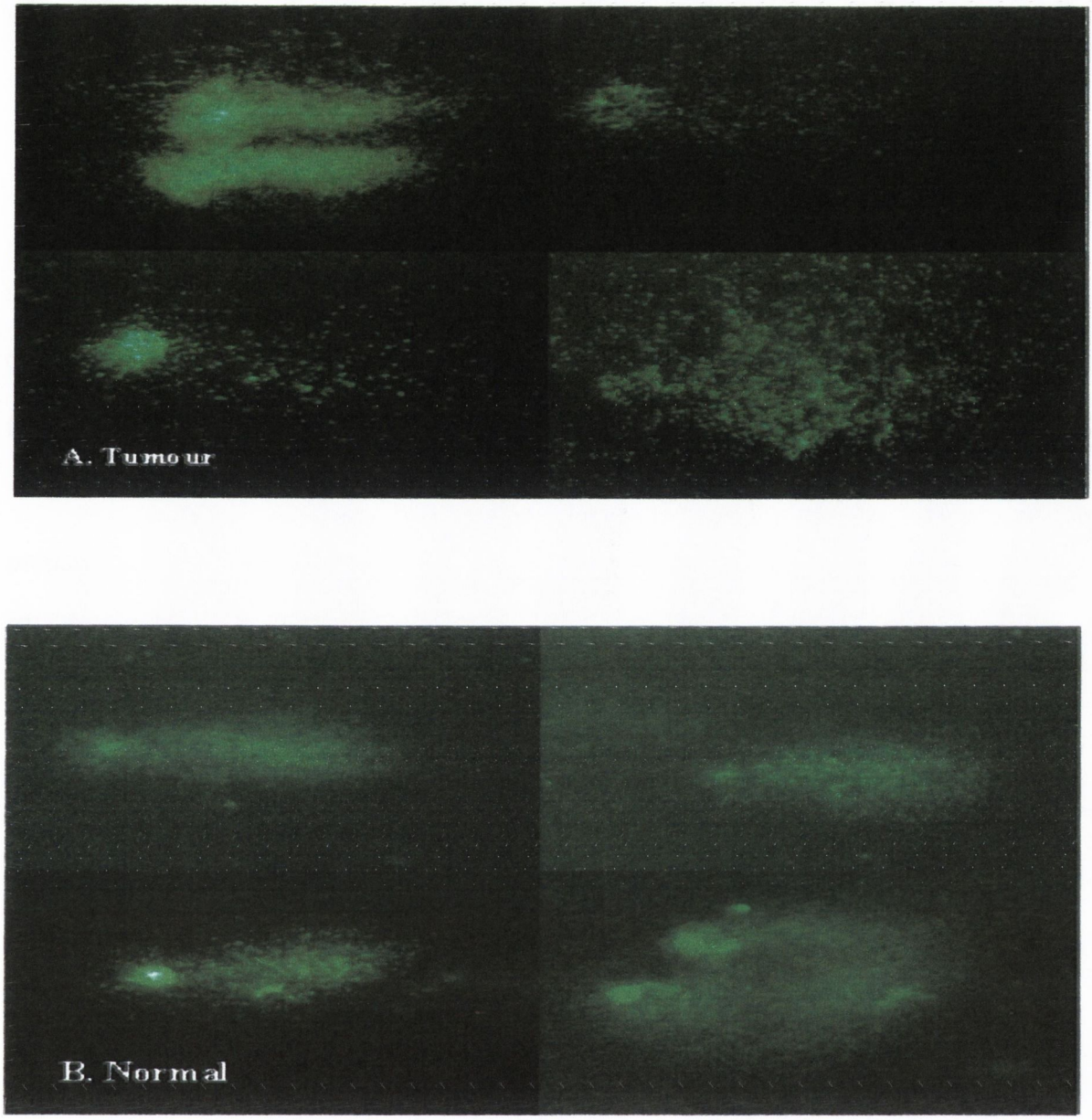


Figure 4.ix Variable appearance of BrdUrd comets from rectal tumour biopsy compared with adjacent tissue. BrdUrd comet images from a single slide prepared from a rectal tumour (A) compared with those from a single slide prepared from an adjacent normal tissue (B). The tumour-derived comets show increased variation in comet appearance in relation to head/tail DNA content, tail length and degree of BrdUrd incorporation as compared with the more homogenous normal comets.

v. Uracil Glycoslase and Hypomethylation Modified Comets – Methods

a. Slide Analysis

Biopsy collection and processing occurred as described above. A series of six or eight slides were produced per site for Comet analysis, slides *e-h* had a double layer of NMP agarose on each slide. The double layer of NMP agarose was used to decrease background fluorescence from the frosted slides when performing FISH-Comet analysis.

Following arrival at the Coleraine laboratory, the slides are immersed in freshly prepared alkaline lysis buffer (2.5M Sodium Chloride, 100mM EDTA, 10mM Tris, pH 10.0) (Sigma, Dorset, UK) with 1% Triton X-100 added just before use. The slides are immersed for 1h at 0-4^{0}C, after which an enzyme digestion may be conducted by adding the required enzyme in reaction buffer at a concentration of 1 unit per slide and incubating at 37^{0}C for 1h followed by washing in PBS (calcium and magnesium free) (Sigma, Dorest, UK).

The slides were removed from the lysing solution, drained and placed in a horizontal electrophoresis tank side by side with the gaps filled with blank slides. The tank was filled with fresh electrophoresis buffer (0.3M Sodium Hydroxide, 1mM EDTA) (Sigma, Dorset, UK) till just covering the slides and left for 20min to allow unwinding of the DNA before electrophoresis. Electrophoresis was carried out for 20min at 25V adjusting to 300mA by rising or lowering the buffer level in the tank.

After electrophoresis, the slides were drained, placed on a tray and flooded with three changes of neutralization buffer (0.4M Tris, Sigma, Dorset, UK) for 5min each time. This removes the alkalis and detergents that would interfere with the ethidium bromide staining. Slides were drained before staining with 50µl of 2µg/ml ethidium bromide and covered with a 22x50 coverslip (Sparks, Dublin). Slides can be stored for 1-2 days on damp tissue in a box shielded from the light. The slides were viewed using an epifluorescence microscope equipped

with a Chroma filter at excitation 540nm and emission 635nm. Analysis of the slides was done using an image analysis system and Komet 4.0 software. Figure 4.x displays the anticipated visual results, while figure 4.i gives a simplified flow chart of the protocol.

b. Uracil Glycosylase Modified Comet

Misincorporation of Uracil was measured using the bacterial enzyme, uracil glycosylase (UG), which specifically releases uracil from the DNA and creates a break in the DNA strand contributing to the creation of a Comet tail after electrophoresis. The % of DNA in the tail is therefore a measure of the amount of misincorporated uracil in each cell and this is compared to control cells (no enzyme). In brief, comet slides are prepared and the cells lysed in alkaline lysis solution. The free nucleotides are then exposed to the enzyme, which specifically releases uracil from the DNA, prior to gel electrophoresis and staining of DNA for image analysis.

The assay was first tested using a cell culture system in which HT29 colon cells were exposed to the anti-cancer drug 5-fluorouracil (0.1μM) for 48 hours prior to modified comet analysis. 5-fluorouracil is a fluoropyrimidine analogue, which inhibits the action of thymidylate synthase (TS) leading to depletion of dTTP pools and the misincorporation of uracil into DNA. The UG assay was applied to the epithelial cells isolated from colonic biopsies.

c. Hypomethylation Modified Comet

For measurement of hypomethylation of global DNA, the methylation specific endonucleases *HpaII* and *HhaI* (HH) were used. Each of these enzymes is capable of specifically cleaving DNA that is not methylated, giving rise to DNA strand breaks and a Comet tail after electrophoresis. The level of DNA in the tail is therefore indicative of the degree to which the DNA in that particular cell is hypomethylated. The results are expressed as

the ratio of mean % DNA in the tail in an enzyme-treated slide compared to that of a control slide (no enzymes).

Comet slides were prepared and exposed to the methylation sensitive enzymes following neutral lysis and prior to alkaline lysis and gel electrophoresis. Both of these enzymes are capable of cleaving double-stranded DNA at specific sites, but only if the DNA is unmethylated. An increase in DNA strand breaks and hence the comet tail is therefore indicative of DNA hypomethylation.

As a model, SW620 colon cell lines were cultured under various conditions designed to induce global hypomethylation. One such experiment involved exposure to the drug azacytidine (0.5μg/ml) for 48 hours before comet analysis, as this drug induces hypomethylation by blocking the action of DNA methyltransferases. Another subsequent experiment was various stages of depletion of folate in the culture medium. In this experiment, SW620 cells were grown in either folate-depleted or folate-replete medium for 14 days. A third group was created where the cells were grown in depleted media for 7 days and then folate-replete media for the next 7 days. In addition, the cells were also assessed for their cellular folate levels to confirm that the folate conditions of the media were, in fact, true. The HH assay was applied to multiple colonic epithelial cells derived from multiple sites of normal and abnormal colon.

d. Fluorscent *In-Situ* Hybridisation Comets

Comet slides were processed using the HH modified comet method. The slides were then dried down in an alcohol series for storage until FISH-comet probe hybridisation. The slides are rehydrated pre-hybridisation with the *p53* probe overnight. The experiments were performed using a commercial FISH probe directed towards a region including the *p53* gene

(probe size approximately 120kilobases) (Vysis Inc., USA). The slides are then washed in formamide and counter-stained using the DAPI (Vysis Inc.,USA). The comet analysis is then performed and the *p53* hybridisation "spots" in the comet head and tail are counted manually. Comparison of the number of FISH hybridisation spots in the comet head (unbroken methylated DNA) compared with those in the comet tail (digested hypomethlated DNA) gave a measure of the degree of hypomethyation in the *p53* region specific to the FISH probe.

The experiments have been carried out using folate-depleted colonic cell lines. A small number of biopsy samples from normal individuals with normal folate status were also tested.

v. Uracil Glycosylase and Hypomethylation Modified Comet - Results

a. Uracil Glycosylase Modified Comet Cell Culture

The cell culture experiments observed increase in comet tail DNA following enzyme treatment confirmed that misincorporation uracil could be detected specifically in colon cells. In the experiment where 5-fluorouracil was used to artificially recreate a situation of uracil misincorporation, the HT29 cells grown in the 5-fluorouracil media exhibited significantly higher levels of mean % comet DNA compared to those grown without the drug ($P = 0.03$) as shown in figure 4.xi.

b. Hypomethylation Modified Comet Cell Culture

Detection of global hypomethylation was examined under different cell culture conditions. The SW620 cells grown in either folate-replete or deplete medium for 14 days in one experiment that showed a significant increase in hypomethylation (Figure 4.xii) and another experiment, a third group that was grown in folate-depleted media for 7 days and repleted for a further 7 days was included as was analysis of the cellular folate content, showed a significant increase in hypomethylation in the group that was grown in the depleted media (P=0.02), the cellular level of folate was shown to be significantly lowered (Figure 4.xiii). Analysis shown for both HH modified comet assay and cellular

folate concentrations for each group. In the case of the SW620 cells exposed to azacytidine, there was a significant difference between the cells treated versus untreated (P=0.03) (Figure 4.xiv). These experiments confirm the HH modified comet assay is measuring hypomethylation in a quantitative fashion.

c. Human Colonic Biopsies

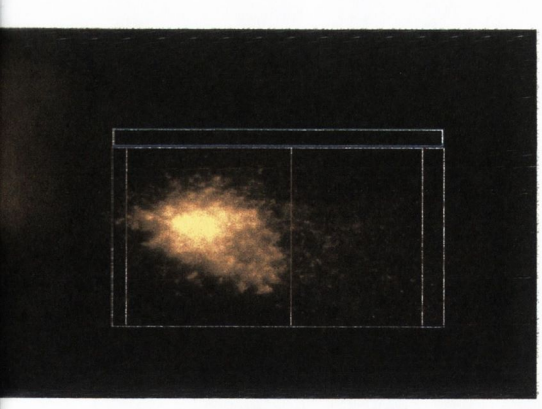
Colonic biopsies have been collected from 3 sites (ascending, transverse, descending colon) from a total of 40 non-polyp control individuals. A significantly higher mean % Comet tail DNA was found in the enzyme-treated slides (UG for uracil misincorporation, HH for hypomethylation) compared with the non-enzyme treated or buffer control slides (UGB for uracil misincorporation and HHB for hypomethylation) at each site for both parameters measured ($p<0.0001$). This indicates that the assay is effectively detecting hypomethylation at each colonic site in individuals without polyps. Results are expressed as the ratio of UGB:UG named as UGR and HHB:HH named as HHR (Figure 4.xv).

The next step was to determine the degree of variability in each biomarker by comparing results at three sites for each biomarker in all of the individuals analysed. Analysis of 3 site data for both uracil misincorporation and hypomethylation indicated no significant variation within or between individuals without polyps (analysed at 3 sites of normal colon in 33 individuals for UGR and 38 individuals for HHR). Results are shown graphically in Figure 4.xvi and Figure 4.xvii.

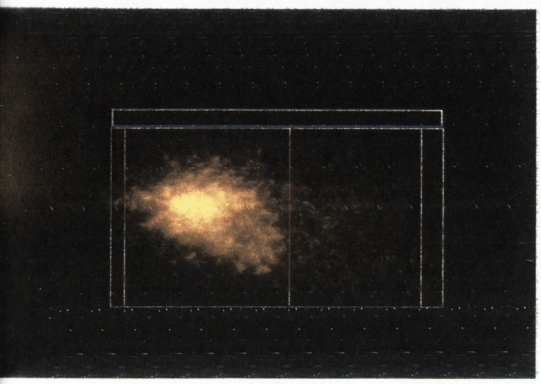
d. Flouorscent *In-Situ* Hybridisation Modified Comet

An increase in hypomethyation in the *p53* region in folate-depleted cells compared with folate–replete control cell lines has been shown (Figure 4.xviii and 4.xix). Analysis using the commercial probe is therefore sufficiently accurate to provide information on the methylation status of the *p53* gene in colon cells derived from human biopsies (Figure 4.xx). Furthermore,

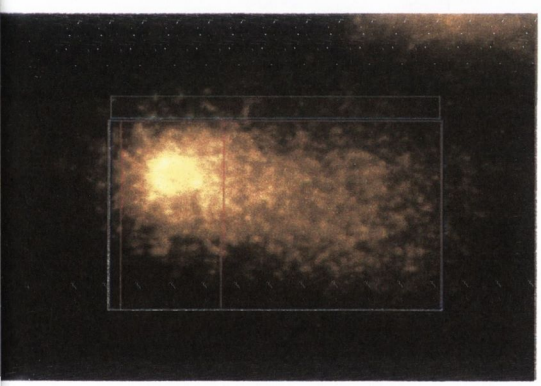
this modified FISH-methylation Comet assay is sensitive to the effects of folate levels in colon cells.



Buffer Treated Slide



Enzyme Treated Slide –
low **level of sites available for enzyme cleavage resulting in a small comet tail**



Enzyme Treated Slide –
High **level of sites available for enzyme cleavage resulting in an increase in the comet tail**

Figure 4.x For each enzymatic comet, a series of two slides are analysed (buffer alone or enzyme treated). This figure represents the possible outcomes depending on the health of the DNA present in the cells.

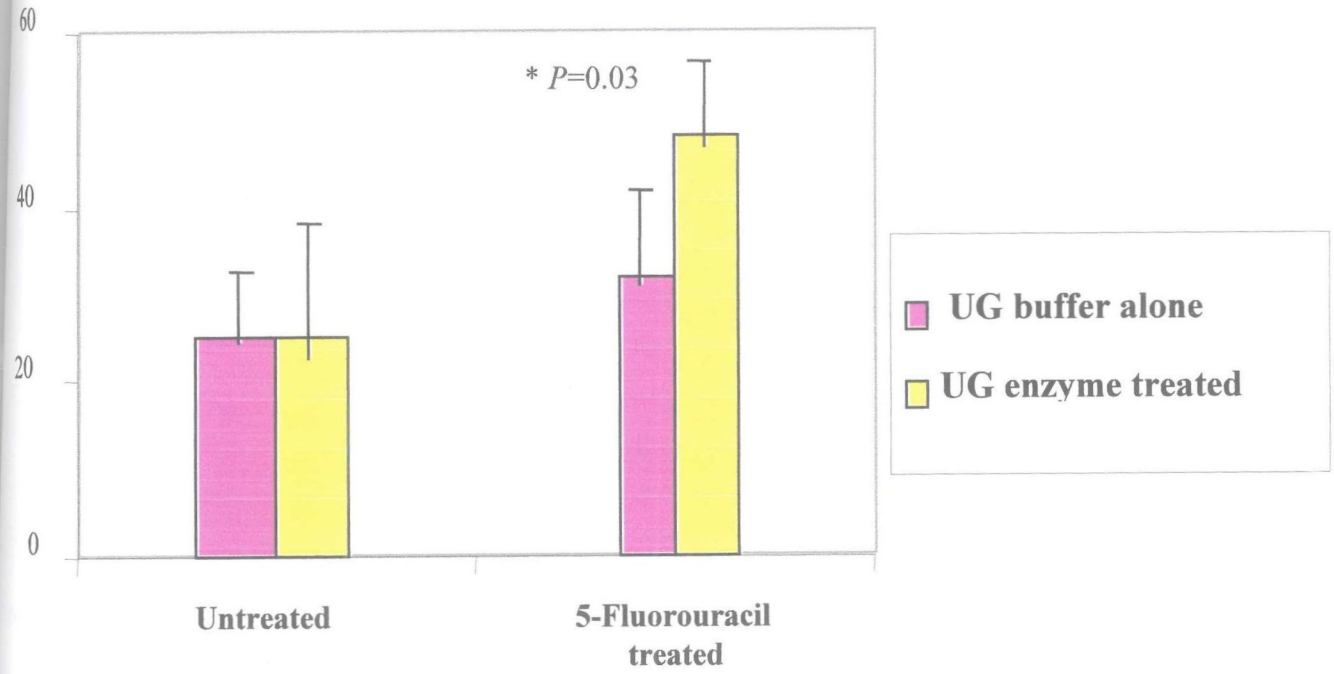


Figure 4.xi HT29 cells grown either with or without 5-Fluorouracil for 48h.

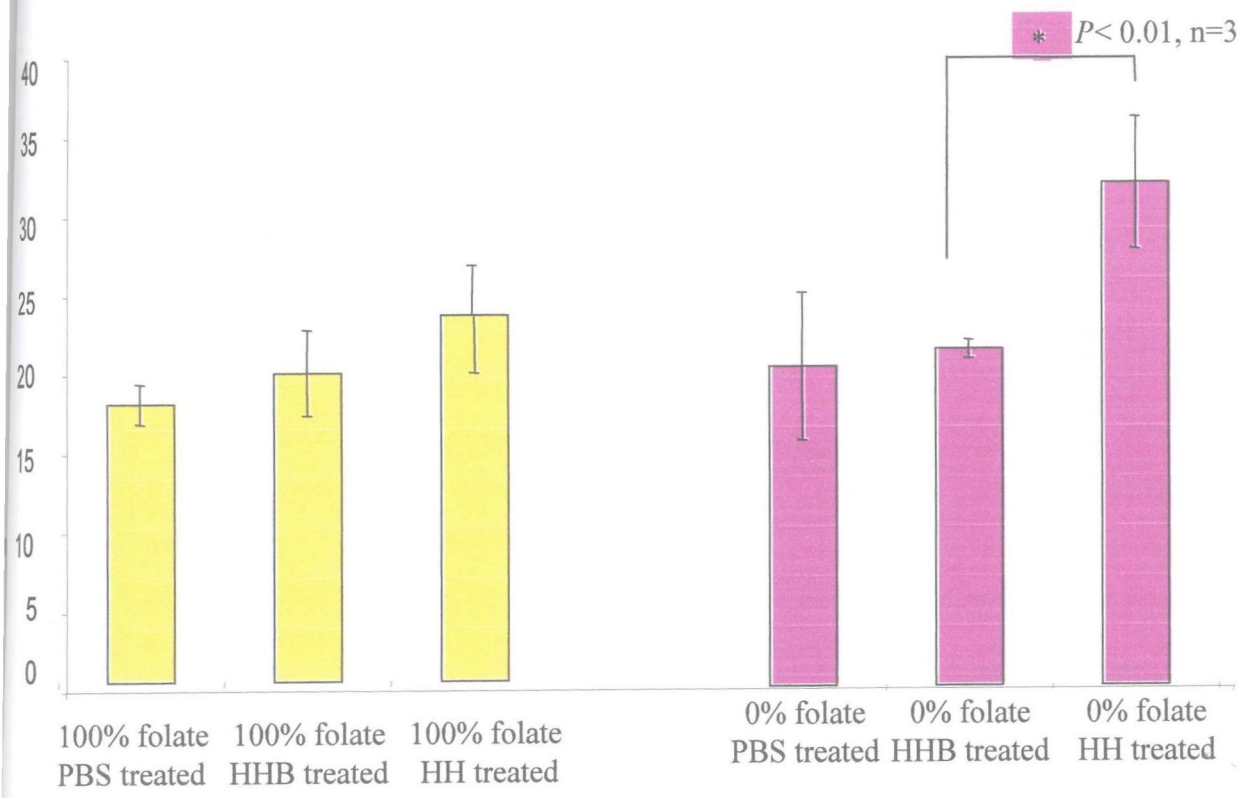


Figure 4.xii SW620 cells grown in either folate-replete or deficient medium over 14 days then analysed using the HH modified comet.

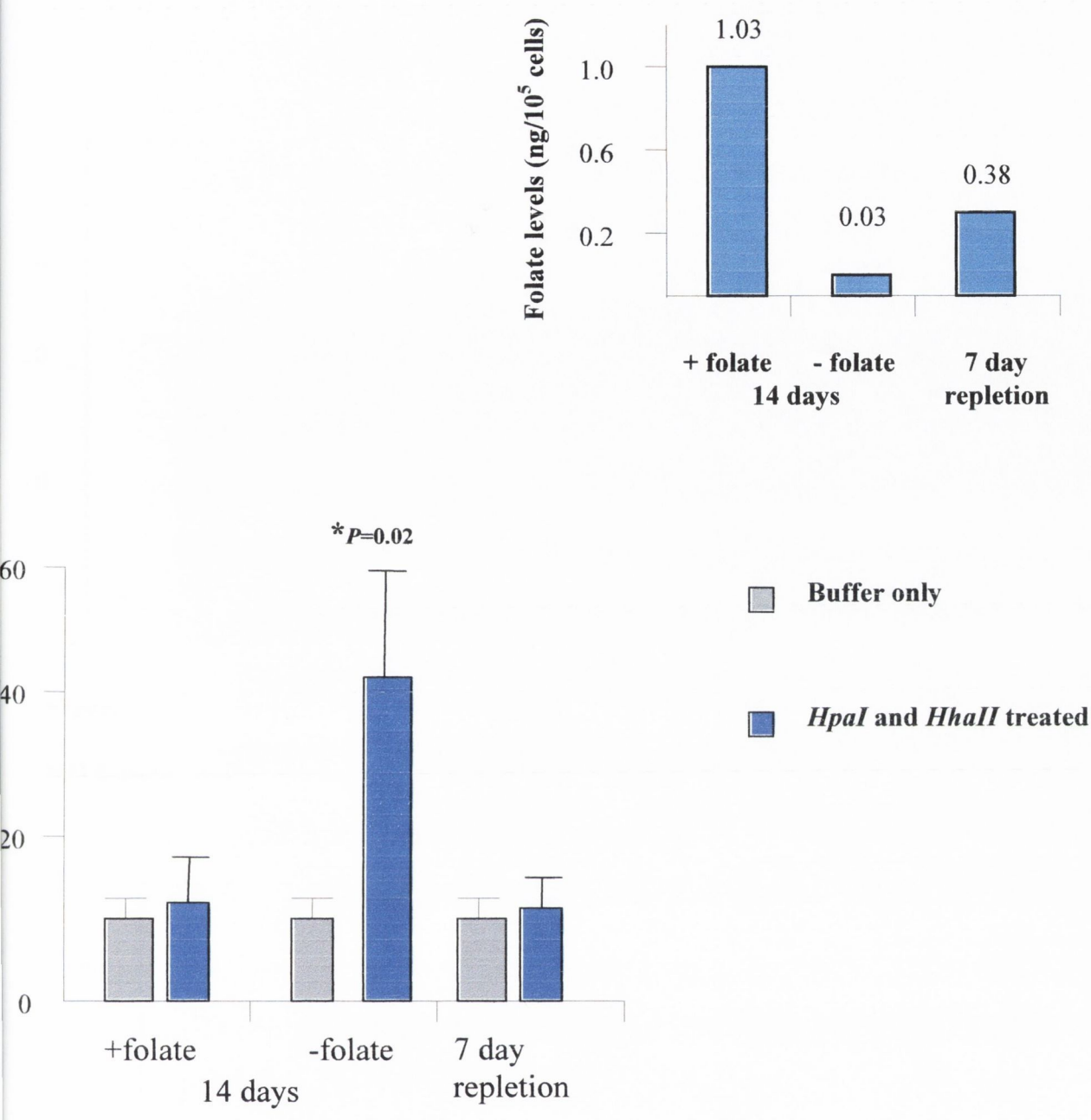


Figure 4.xiii SW620 cells grown in either folate-replete or deplete medium for 14 days, a third group was grown in folate-depleted media for 7 days and then repleted for a further 7 days. Analysis shown for both HH modified comet assay and cellular folate concentrations for each group.

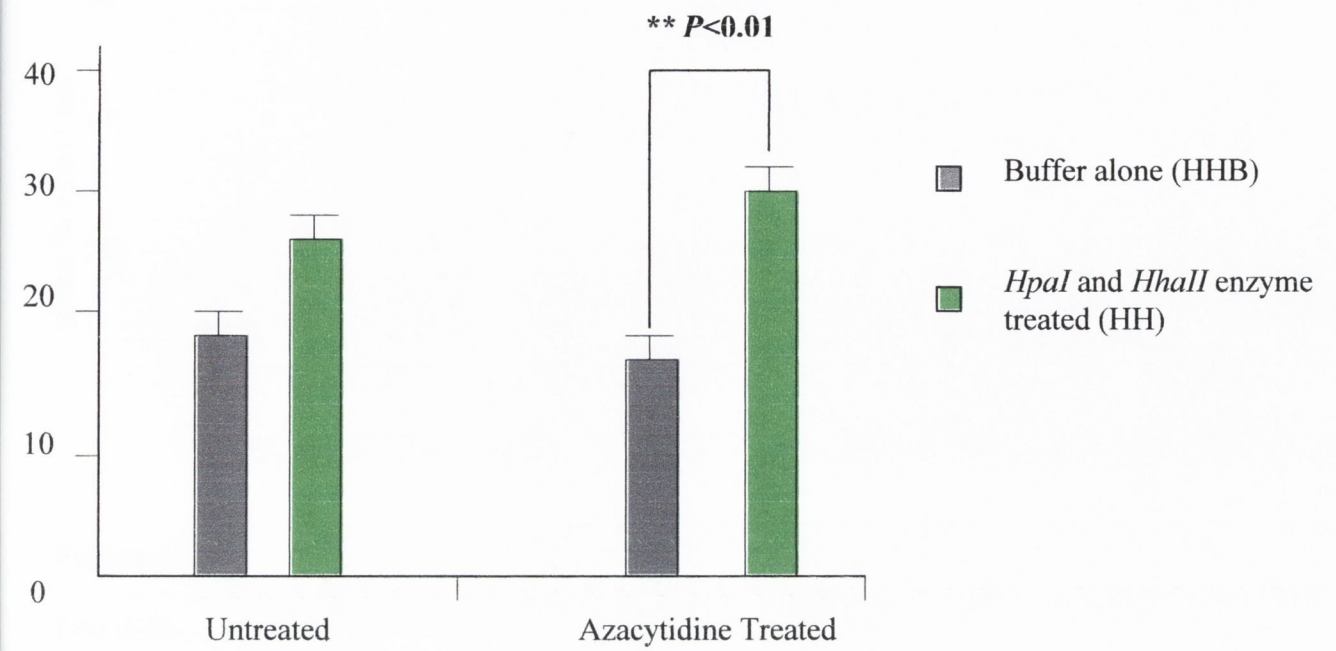


Figure 4.xiv Azacytidine-induced demethylation of global DNA in SW620 cells detected by the HH Comet assay.

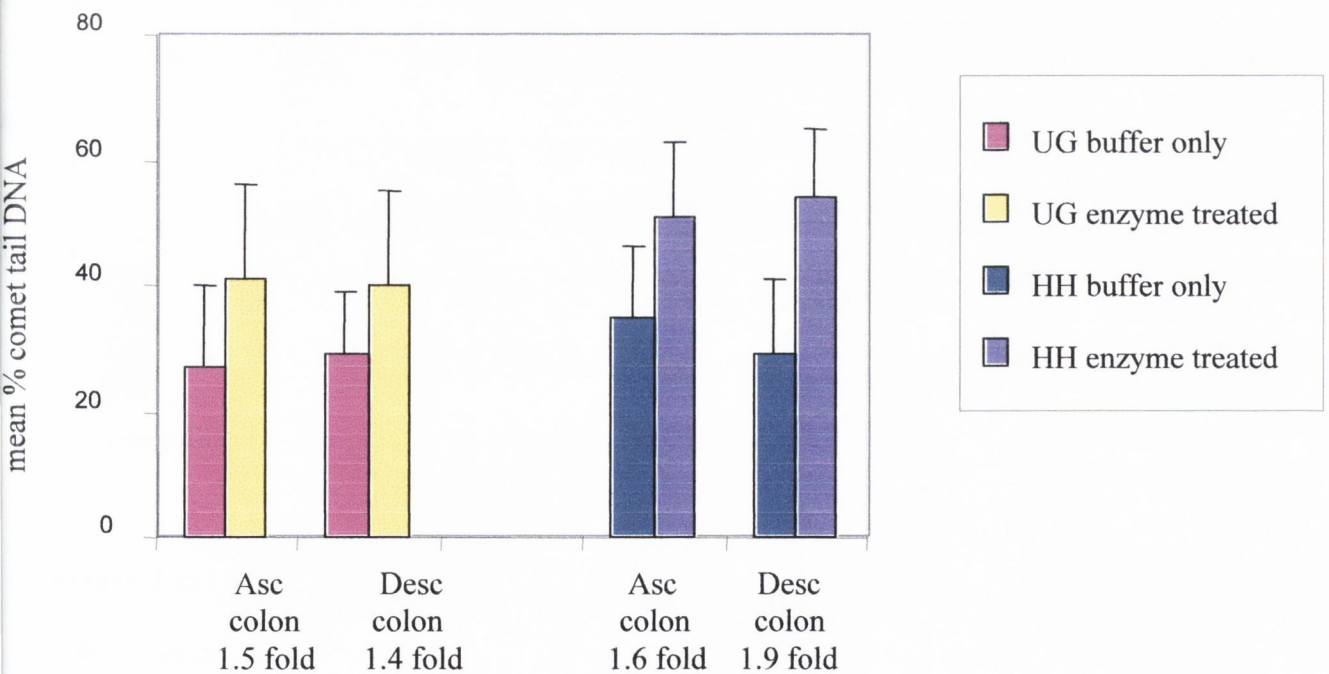


Figure 4.xv Detection of uracil misincorporation and hypomethylation, respectively, in two sites of a control/ normal individual.

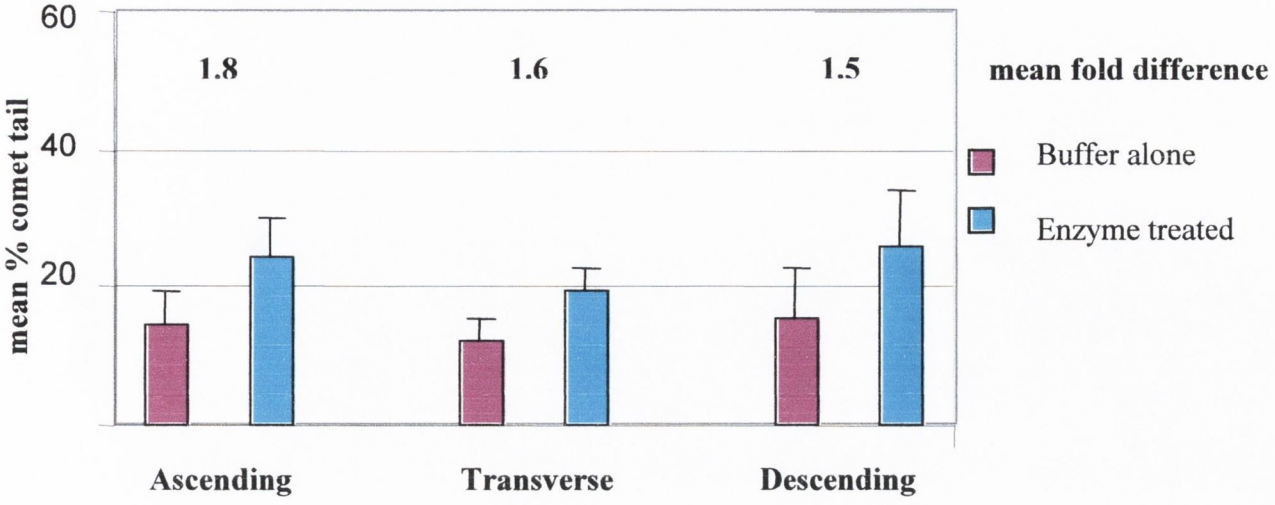


Figure 4.xvi Three locations within normal / control individuals (n=23) analysed for the mean fold difference between buffer treated and UG enzyme treated slides for each location.

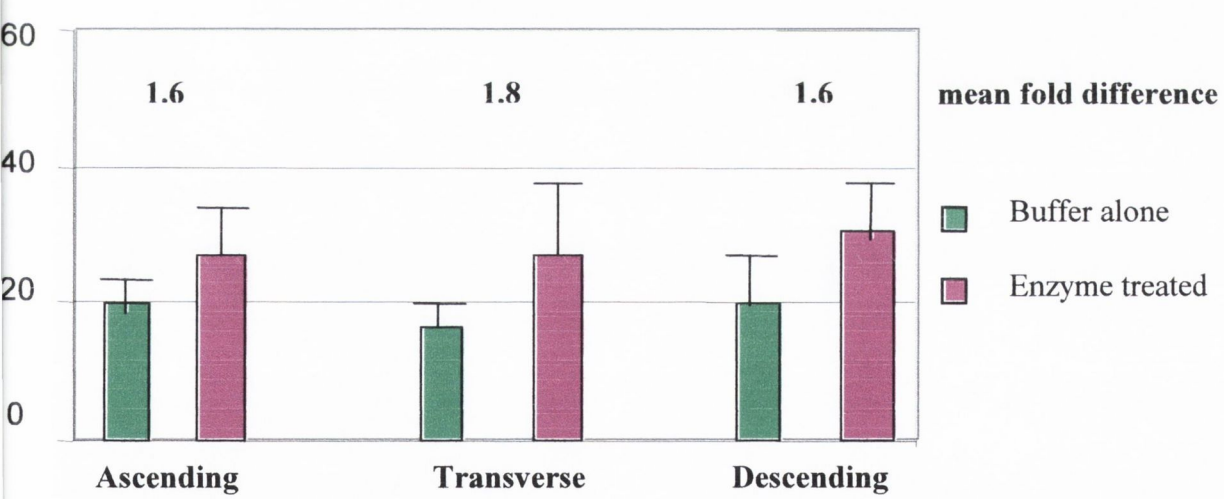


Figure 4.xvii Three locations within normal / control individuals (n=23) analysed for the mean fold difference between buffer treated and HH enzyme treated slides for each location.

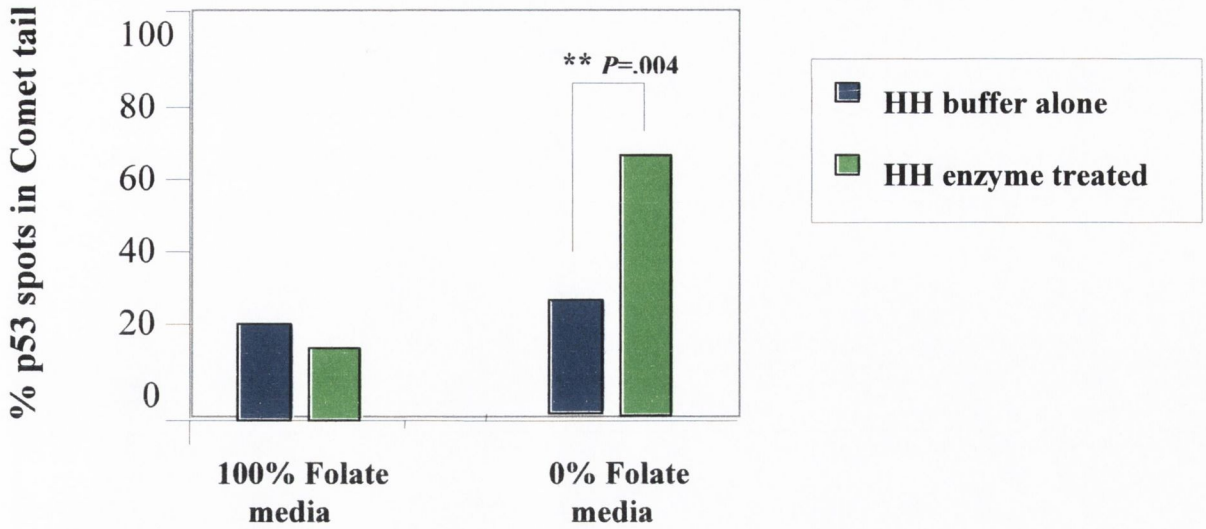


Figure 4.xviii SW620 cells grown in medium either depleted or replete in folic acid for 6 days prior to HH modified comet and subsequent FISH analysis.



Figure 4.xix Methylation-FISH comets in folate-depleted SW620 cells, note the presence of red FISH spots in the comet tail.

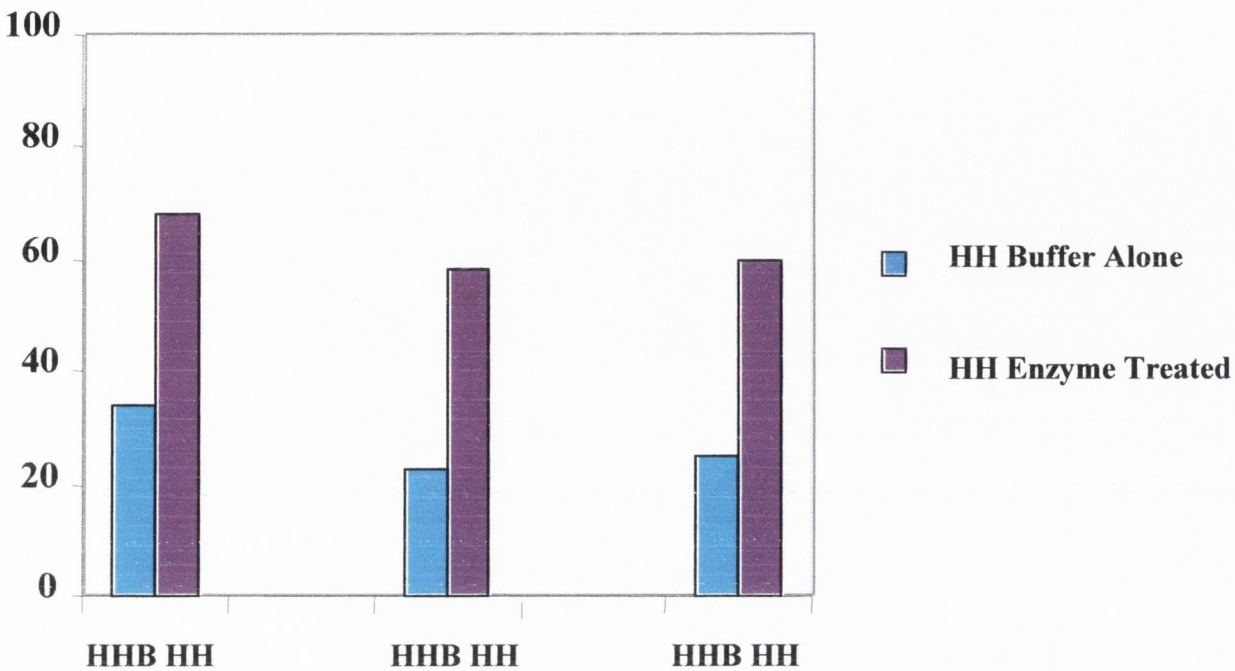


Figure 4.xx Methylation comets and *p53* FISH in normal / control biopsies from ascending colon (n=3). % *p53* spots in the comet tail in untreated (HHB) and enzyme treated (HH) comets of cells for each individual patient.

vi. Discussion

The comet assay in its modified forms provided a novel method of examining DNA damage in the context of misincorporation of BrdUrd in newly replicating DNA, misincorporation of uracil and global hypomethylation of DNA.

In the case of the BrdUrd comet assay, an increase in the comet tail length is indicative of a delay in DNA maturation, because of stalling of the replication fork at DNA lesions or defects in post-replicative repair. The BrdUrd comet assay proved to be suitable for the measurement of DNA maturation and integrity in small numbers of primary epithelial cells derived from colonic biopsies. Inter- and intra-individual reproducibility was assessed and comet measurements on duplicate endoscopic biopsies, which were collected from up to three different colorectal sites in each patient (n = 36 sites), showed no significant difference between sites, demonstrating the reproducibility of the assay (as shown in Figure 4.iii). Furthermore, the sequential follow-up specimen obtained from one patient undergoing a repeat colonoscopy showed an initial mean % comet tail of 50 ± 11 in comets derived from normal descending colon. The repeat colonoscopy (four months later) had a mean % comet tail of 53 ± 9 in comets derived from normal sigmoid colon. This indicated that even after a lapse of four months, reproducibility was possible. These results showed a low level of variability within the normal colonic tissue samples. It was possible to detect elevated BrdUrd comets from histologically abnormal tissue when compared to that of normal tissue and this seemed to indicate stalled maturation in the case of the abnormal tissue (see figure 4.vii). This may be due to a high level of intrinisic DNA damage or defective post-replication repair machinery in the tumour cells (Johnson *et al.* 1994). The pulse-chase experiments were performed to assess the DNA maturation (figure 4.iv-figure 4.v). A normal patient was examined and showed proficient DNA maturation after a chase period of 1 hour ($P<0.01$). However, epithelial cells from a patient with adenomatous polyps demonstrated a significant delay in DNA maturation

compared to the normal patient ($P<0.05$, t-test). This was shown by the failure of the DNA comet tail to move towards the head as seen in figure 4.vi. These experiments supported the ability of the BrdUrd comet assay to assess DNA maturation and determine the replicative integrity of human primary epithelial cells.

Uracil misincorporation is proposed to be one of the main pathways for folate in its role in CRC. By using the bacterial enzyme uracil DNA glycosylase (UG), it has become possible to detect uracil misincorporation in human DNA. The method has been previously validated in human lymphocytes and human immortalised colon epithelial cells (Duthie and Hawdon 1998;Duthie *et al.* 2000b). 5-fluorouracil was used to artificially recreate a situation of uracil misincorporation, the HT29 cells grown in the 5-fluorouracil media exhibited significantly higher levels of mean % comet DNA compared to those grown without the drug ($P = 0.03$) as shown in figure 4.xi. The UG assay was validated for this study in relation to its ability to detect uracil misincorporation in cell culture under the condition of drug-induced uracil misinicorporation and within cells isolated from human colonic biopsies.

No previous study has been performed on the modified comet assay looking at hypomethylation using the combination of *HpaI* and *HhaII*, though a study has been performed using the *Sss1* methylase enzyme (Duthie *et al.* 2000b) but the results were not conclusive for the assay used. In the initial stages validation stages, experiments were performed to examine the effect of the enzymes on suitable cell lines. The effect of drug-induced hypomethylation was examined as well as conditions of folate deprivation. SW620 cells grown in either folate-replete or deplete medium for 14 days in one experiment that showed a significant increase in hypomethylation (Figure 4.xii) and another experiment, a third group that was grown in folate-depleted media for 7 days and repleted for a further 7 days was included as was analysis of the cellular folate content, showed a significant increase in hypomethylation in the group that was grown in the depleted media ($P=0.02$), the cellular level of folate was shown to be significantly

lowered (Figure 4.xiii). Analysis shown for both HH modified comet assay and cellular folate concentrations for each group. In the case of the SW620 cells exposed to azacytidine, there was a significant difference between the cells treated versus untreated (P=0.03) (Figure 4.xiv). These experiments confirm the HH modified comet assay is measuring hypomethylation in a quantitative fashion.

Further validation work was performed on human epithelial cell biopsy isolates from normal colonic tissue from the same site and from different sites in the same individual. Colonic biopsies were collected from 3 sites (ascending, transverse, descending colon) from a total of 40 non-polyp control individuals. A significantly higher mean % Comet tail DNA was found in the enzyme-treated slides (UG for uracil misincorporation, HH for hypomethylation) compared with the non-enzyme treated or buffer control slides (UGB for uracil misincorporation and HHB for hypomethylation) at each site for both parameters measured ($p<0.0001$). This indicates that the assay is effectively detecting hypomethylation at each colonic site in individuals without polyps. Results are expressed as the ratio of UGB:UG named as UGR and HHB:HH named as HHR (Figure 4.xv).

The validation work performed on UG and HH modified comets, in suitable cell lines and human epithelial cell isolates. This proves that the methods are looking at uracil misincorporation and hypomethylation, respectively. Both methods are robust, rapidly and easily performed.

FISH-comet analysis was initially devised in University of Ulster, Coleraine (McKelvey-Martin *et al.* 1998). The FISH-comet assay uses fluorescently labeled DNA probes that hybridise to a specific gene sequence. Thus, the localization of a particular gene within a comet cell population can be established, offering the opportunity to follow gene-specific repair in response to DNA damage. The position of the fluorescent hybridization spots in the comet head or tail indicates whether the sequence of interest lies within or in the vicinity of a

damaged region of DNA. The fluorescently-labelled DNA probe used in this study was specific for a 200kb region of chromosome 17, this area includes the human *p53* gene. The probe was used in combination with the methylation-specific modified comet assay, which meant that detection of *p53*-specific areas of hypomethylation could be analysed. An increase in hypomethyation in the *p53* region in folate-depleted cells compared with folate–replete control cell lines has been shown (Figure 4.xviii and 4.xix). Analysis using the commercial probe is therefore sufficiently accurate to provide information on the methylation status of the *p53* gene in colon cells derived from human biopsies (Figure 4.xx). Furthermore, this modified FISH-methylation Comet assay is sensitive to the effects of folate levels in colon cells.

The above applications and techniques illustrate the additional benefits that can be obtained from an already powerful assay. The UG and HH modified comets enable research into the overall stability of the DNA, specifically at uracil misincorporation and hypomethylation. This can then be combined with assessment of the folate concentration within the epithelial cells, which will allow a more detailed, accurate analysis of the role of folate in CRC.

CHAPTER 5

CASE-CONTROL STUDY

1. CASE-CONTROL STUDY

The relationship between systemic markers of folate status and the risk of colorectal adenomas is still under debate. Prospective clinical studies have produced varying results, in some the serum folate was not significantly different between cases and controls (Paspatis *et al.* 1995;Meenan *et al.* 1996;Meenan *et al.* 1997;Kim *et al.* 1998) while in others significance was found (Kim *et al.* 2001b;Kim *et al.* 1998). The same results have been shown in RBC folate in the same papers; although Paspatis' group were unable to show significance, the RBC folate in the case group was 28% lower than the controls even though it was still within the normal range (536ng/mL) suggesting that a modest reduction in status can increase risk. Sample size seems to be the most common fault of all studies so finding no difference in systemic measures may be because of a type II error. Studies looking at higher numbers of cases (Bird *et al.* 1995;Kato *et al.* 1999) and controls have supported folates role in CRC. The Bird study group looked at 332 subjects and 350 controls and found a protective effect of RBC folate concentration against the development of colorectal polyps, at least in men (OR=0.53, 95%CI 0.32-0.87). The New Yorker University Women's Health Study Cohort (Kato *et al.* 1999) examined 105 cases and 523 matched controls and found the risk of CRC was almost twice as high in cases with below-median serum folate and above-median total alcohol intake compared with those with above-median serum folate and below-median alcohol consumption (OR=1.99, 95%CI 0.92-4.29). There have been few studies in the area of local colonic folate concentrations and CRC risk. All completed studies to date have suffered from small sample size as to obtain the colonic tissue folate concentration, either the patient must undergo a full colonoscopy or sigmoidoscopy and this has, in itself, has ethical considerations as it is an invasive procedure. Another problem comes from the different methods of analysis of the biopsy tissue. The methodology used in the U.S. looked at whole biopsy tissue folate while the Meenan method examined epithelial cell folate. Both methods have had criticism levelled at

them. For the whole biopsy method, it can be argued that the entire biopsy will be composed of a variety of cell types - the average punch biopsy will contain epithelium, lamina propria, lymphatic nodules, smooth muscle fibres, muscularis mucosa, some submucosa containing lymphatic vessels, venules and arterioles. The evidence for CRC and folate's involvement is derived from the epithelial layer being involved in the adenoma-to-carcinoma sequence and not from any other cell types so it can be argued that whole biopsy tissue folate may not be reflecting the true involvement of folate in CRC at tissue level. For the epithelial cell isolation method, it has been argued that the processing of the biopsy to isolate the epithelial cells may allow cellular folate to equilibrate from the time of biopsy to when the epithelial cells are isolated. The studies that examined whole biopsy folate have found, in general, correlations between tissue folate and systemic folate status (Kim *et al.* 1998;Kim *et al.* 2001b), which homocysteine being the most significant. However, in the latter study where supraphysiological folate supplementation was given for a year, these correlations were not seen and the correlations shown for the systemic markers against colonic tissue folate appear to be driven by the supplementation group. In contrast, the studies carried out by Meenan and co-workers were unable to show a relationship between serum or RBC folate and colonic epithelial cell folates (Meenan *et al.* 1996) It may also be worth noting that plasma Hcy was not measured in the Meenan studies. Another study showed that folate depletion occurred in the neoplastic but not the adjacent normal tissue ($p<0.02$). While adenomas showed lower levels of epithelial cell folates, this did not reach statistical significance ($p<0.06$) (Meenan *et al.* 1997). The effect of folate on the colorectal mucosa may not be entirely derived from the blood because the colorectal mucosa is exposed to luminal folate synthesized by intestinal microflora or that has escaped small intestine absorption (Camilo *et al.* 1996). Therefore, the absolute relevance of the systemic markers has been queried, particularly in light of studies which have shown a modest reduction in folate status resulted in increased risk.

Recent studies have been looking at the possible pathways through which folate status may be involved. Pufulete and colleagues have examined the influence of folate status, DNA methylation, and polymorphisms of methylenetetrahydrofolate reductase (MTHFR 677C-->T and 1298A-->C), methionine synthase (MS 2756A-->G), and cystathionine-beta-synthase (CBS 844ins68) on risk for developing colorectal neoplasia (Pufulete *et al.* 2003a). Thirty-five patients with adenoma, 28 patients with cancer, and 76 controls were recruited for a case control study. Blood samples were obtained for determination of blood folates, vitamin B_{12}, Hcy, DNA methylation, and genotypes. Tissue biopsy samples were obtained at colonoscopy for determination of DNA methylation in colonic mucosa. Constructing a score from estimates of dietary intake and serum and erythrocyte folate assessed folate status. Cancer patients had 26% lower folate status (95% CI 6%- 44%, P = 0.01) and 21% lower serum vitamin B_{12} concentration (95% CI -38% -1%, P = 0.06) compared with controls. [^{3}H] methyl incorporation into colonic DNA was 26% higher in patients with adenoma (95% CI 8%-56%, P = 0.009) and 30% higher in patients with cancer (95% CI -3%-48%, P = 0.08) compared with controls. High folate status was associated with decreased risk for cancer (P = 0.01 for trend). Colonic and leukocyte DNA hypomethylation were associated with increased risk for adenoma (P = 0.02 and P = 0.01 for trend, respectively) and a non-significantly increased risk for cancer (P = 0.09 and P = 0.08 for trend, respectively). Friso and co-workers were able to show genomic DNA directly correlated with folate status and inversely with plasma Hcy levels (P<0.01). TT genotypes had a diminished level of DNA methylation compared to those with CC genotype (P<0.0001). Moreover, in TT individuals DNA methylation status was correlated with the methylated proportion of RBC folate and was inversely correlated with formylated proportion of the red blood cells folate (P<0.03) that is known to be solely represented in these individuals. These results indicate that the C677T polymorphism influences DNA methylation status through an interaction with folate status (Friso *et al.* 2002). Reduced DNA repair

capacity and folate deficiency have been implicated in increased cancer risk. In a recent study (Wei *et al.* 2003) found that calorie-adjusted total folate intake was an independent predictor of DNA repair capacity ($P<0.001$). Additional stratification analysis indicated that this association was more pronounced in those who did not use folate supplements compared to those who did.

To investigate the relationship between folate status and the purported pathways involved in CRC development, a case-control study was proposed. This would allow for patients with and without polyps to be investigated in relation to their folate status and relevant biomarkers.

i. Methods

a. Patient selection

Endoscopically normal patients and patients found to be harbouring polyps were recruited from St. James' Hospital, Dublin. Exclusion criteria were active inflammatory bowel disease or coeliac disease, intestinal malabsorption syndromes, liver or renal disease, pregnancy, epilepsy, pernicious anemia and the use of antibiotics or anti-folate medication (sulphazalazine or methotrexate). The study was approved by the Joint Research Ethics Committee of the Federated Dublin Voluntary Hospitals and St. James Hospital. Written informed consent was obtained from all patients. All patients had prepared for colonoscopy by taking orally administered colonic lavage solution (Kleanprep; Norgine, Harefield, UK).

b. Blood Sampling and Patient Data Collection

A venous sample was collected into an ethylenediaminetetraacetic acid vacutainer for DNA extraction, erythrocyte folate and plasma homocysteine determination. A vacutainer containing no anticoagulant was used for the determination of serum folate and vitamin B_{12} analysis. For plasma Hcy, plasma was separated within 1h of collection and frozen at -40^0C. Weight and height were recorded and information on smoking habits, physical activity levels,

alcohol intake, current medication, medical history, blood pressure and supplement use were gathered before colonoscopy. Subjects were defined as supplement users if they reported taking folic acid supplements or multivitamin preparations containing folic acid at any time in the previous year. Hypothyroidism was identified in each population as this is a known medical condition that can increase plasma Hcy.

c. Biopsy Collection, Epithelial Cell Isolation and Modified Comet Preparation

From each patient, six colon biopsies were taken and placed into fresh HBSS. In the control patients, six colonic biopsies were taken from anatomically defined locations in sets of two (two ascending, transverse and descending biopsies) and placed into fresh HBSS for processing. Cases were defined as those with a histologically confirmed polyp upon colonoscopy. In all cases, 2 biopsies from the polyp, 2 biopsies from adjacent to the polyp and 2 biopsies from 10-15cm distal from the polyp were taken. However, in some cases a biopsy from the polyp area could not be obtained due to polyp location, size or loss of the polyp in the gut lumen when trying to retrieve it.

The two biopsies per selected site had the epithelial layer separated off using the isolation methodology described previously; the resulting cell pellet was split into the sub-fraction for colonocyte folate analysis. This was stored in 500µl 1% sodium ascorbate PBS (pH 6.5) at -40^{0}c until deconjugation and microbiological analysis using *L.Casei* (all colonocyte folates were assayed within four months of isolation), and the other sub-fraction for Comet analysis. A series of eight slides were produced per site for Comet analysis, slides *e-h* had a double layer of NMP agarose on each slide. The double layer of NMP agarose was used to decrease background fluorescence from the frosted slides when performing FISH-Comet analysis.

ii. Results

Statistical analysis was performed using SPSS release 10 for PC (SPSS UK, Surrey, UK). Data was mainly compared using Student's t-test (two tailed) for independent samples.

a. Demographic Data

The mean age of the control population was 52.5±16.1 years and the mean BMI was 26.6±5, which is in line with Ireland's current trend towards obesity. The most frequent indications for colonoscopy were altered bowel habit, bleeding per rectum and polyp review. In the population demographics, there were no significant differences seen between the sexes apart from alcohol choice and alcoholic units per week. These differences are expected ones as they are commonly seen in alcohol-related research. No significant difference was found between MTHFR genotypes when all systemic and local data was analysed with one-way analysis of variance (ANOVA).

Table 5.i Population demographics for control and case groups

	Controls (n=64)	*Cases (n=50)*	*Total (n=114)*
Sex – Female	33 (52%)	20 (40%)	53 (47%)
Male	31 (48%)	30 (60%)	61 (53%)
Age	52.5±16	64.6±12	57.8±16
Body Mass Index	26.6±5	26.6±4	26.6±4.5
Indication for Colonoscopy			
Altered Bowel Habit	15 (23%)	7 (14%)	22 (19%)
Bleeding Per Rectum	11 (17%)	7 (14%)	18 (16%)
Abdominal Pain	8 (13%)	1 (2%)	9 (8%)
Weight Loss	4 (6%)	2 (4%)	6 (5%)
Polyp Review	15 (23%)	27 (54%)	42 (37%)
Family History of CRC	8 (12%)		8 (7%)
Anaemia	3 (5%)	2 (4%)	5 (4%)
Query Mass		4 (8%)	4 (4%)
Physical Activity			
Low	30 (47%)	25 (50%)	55 (48%)
Medium	27 (42%)	20 (40%)	47 (41%)
High	6 (9%)	4 (8%)	10 (9%)
Very High	1 (2%)	1 (2%)	1 (2%)
Alcohol			
Abstainers	12 (19%)	11 (22%)	23 (20%)
Drinkers – Beer	27 (42%)	21 (42%)	48 (42%)
Spirits	16 (25%)	10 (20%)	26 (23%)
Wine	9 (14%)	8 (16%)	17 (15%)
Total Units / Week	15.6±21.4	21.7±29.7	18.2±25.3
Cigarettes			
Non-Smoker	26 (41%)	22 (44%)	48 (42%)
Ex-Smoker	21 (33%)	10 (20%)	31 (27%)
Smoker	17 (27%)	18 (36%)	35 (31%)
Duration – <5 years	1 (2%)	1 (2%)	2 (2%)
<10 years	5 (8%)	2 (4%)	7 (6%)
<20 years	23 (36%)	15 (30%)	38 (33%)
<30 years	6 (9%)	6 (12%)	12 (11%)
<40 years	1 (2%)	2 (4%)	3 (3%)
>40 years	2 (3%)	2 (4%)	4 (3%)
Amount per day – <10	16 (25%)	14 (28%)	30 (26%)
10-20	10 (16%)	6 (12%)	16 (14%)
>20	12 (19%)	8 (16%)	20 (17%)
Blood Pressure			
Systolic	134±25	143±23	138±24
Dystolic	82±17	80±12	81±15
Aspirin Users	9 (14%)	14 (28%)	23 (20%)
Hypothyroid	9 (14%)	14 (28%)	23 (20%)
Supplement			
None	46 (72%)	39 (78%)	85 (75%)
User – no folic acid	8 (12%)	7 (14%)	15 (13%)
User – folic acid	10 (16%)	4 (8%)	14 (12%)

Table 5.ii Polyp distribution, epithelial cell folate concentrations, modified comet ratios, MTHFR genotype, systemic folate and vitamin B_{12} concentrations for controls and cases.

	Controls (n=64)	*Cases (n=50)*		*Total (n=114)*
		Hyperplastic (n=14)	*Adenoma (n=36)*	
Polyp Type				
None	64 (100%)			64 (56%)
Hyperplastic		14 (100%)		14 (12%)
Adenoma – mild			30 (84%)	30 (26%)
Adenoma – moderate			3 (8%)	3 (7%)
Adenoma - severe			3 (8%)	3 (7%)
Adenoma				
None	64 (100%)	14 (100%)		78 (68%)
Serrated			1 (3%)	1 (1%)
Tubular			20 (56%)	20 (18%)
Tubulovillous			12 (33%)	12 (10%)
Villous			3 (8%)	3 (3%)
Polyp Location				
None	64 (100%)			64 (56%)
Caecal		4 (29%)	4 (11%)	8 (7%)
Ascending		1 (7%)	4 (11%)	5 (4%)
Hepatic Flexure		2 (14%)		2 (2%)
Transverse		2 (14%)	4 (11%)	6 (5%)
Descending		1 (7%)	3 (8%)	4 (3%)
Sigmoid		1 (7%)	12 (33%)	13 (11%)
Rectum		3 (21%)	9 (25%)	12 (11%)
Mean Epithelial Cell Folate (pg/10^5 cells)	23.3±8.5	18.82±11.18	15.3±7.19	20.1±9.2
Mean UG Ratio	1.49±0.39	1.23±0.33	1.62±0.44	1.5±0.42
Mean HH Ratio	1.62±0.47	1.55±0.6	1.67±0.48	1.62±0.49
Serum Folate (ng/mL)	18.18±9.9	18.09±9.04	18.3±9.41	18.23±9.6
Red Cell Folate (ng/mL)	447.9±164	615.9±265.9	538±170.4	499.6±189.2
Homocysteine (μmol/L)	9.39±2.28	10.6±2.2	11.2±3.3	10.16±2.77
MTHFR genotype				
CC	27 (42%)	6 (43%)	15 (42%)	48 (42%)
CT	27 (42%)	5 (36%)	18 (50%)	50 (44%)
TT	5 (8%)	2 (14%)	3 (8%)	10 (9%)
Vitamin B_{12} (pg/mL)	387.8±175	428.4±178.7	356.4±158	382.2±169.9

b. Epithelial Cell Folate Concentrations and UG/HH Modified Comet Ratios

For the control patients, when the comet UG and HH ratios were examined, a significant positive correlation was seen ($r = 0.816$, $P < 0.001$). However, no correlation was seen between epithelial cell folate and the comet ratios (Table 5.iv).

In the case group, there was a significant correlation seen between the comet UG and HH ratios ($r = 0.737$, $P<0.001$) but again no correlation with epithelial cell folate (Figure 5.iv).

Cases and controls were subsequently analysed using one-way analysis of variance (Anova) to examine inter-site relationships for epithelial cell folate and modified comet parameters (Table 5.iii). Highly significant differences were found between sites for cases ($P<0.001$) but not controls ($P=0.196$). When the cases were sub-divided it was the adenoma group that held the most significance ($P<0.001$) compared to the hyperplastic polyp group ($P=0.002$).

The same analysis was applied to the UG and HH modified comets. In the case group there was significance found for the UG comets ($P= 0.006$) but only trends were seen when the cases were sub-divided. In the HH comet analysis, near significance was seen for the case group ($P=0.051$) and again trends seen in the sub-groups of cases.

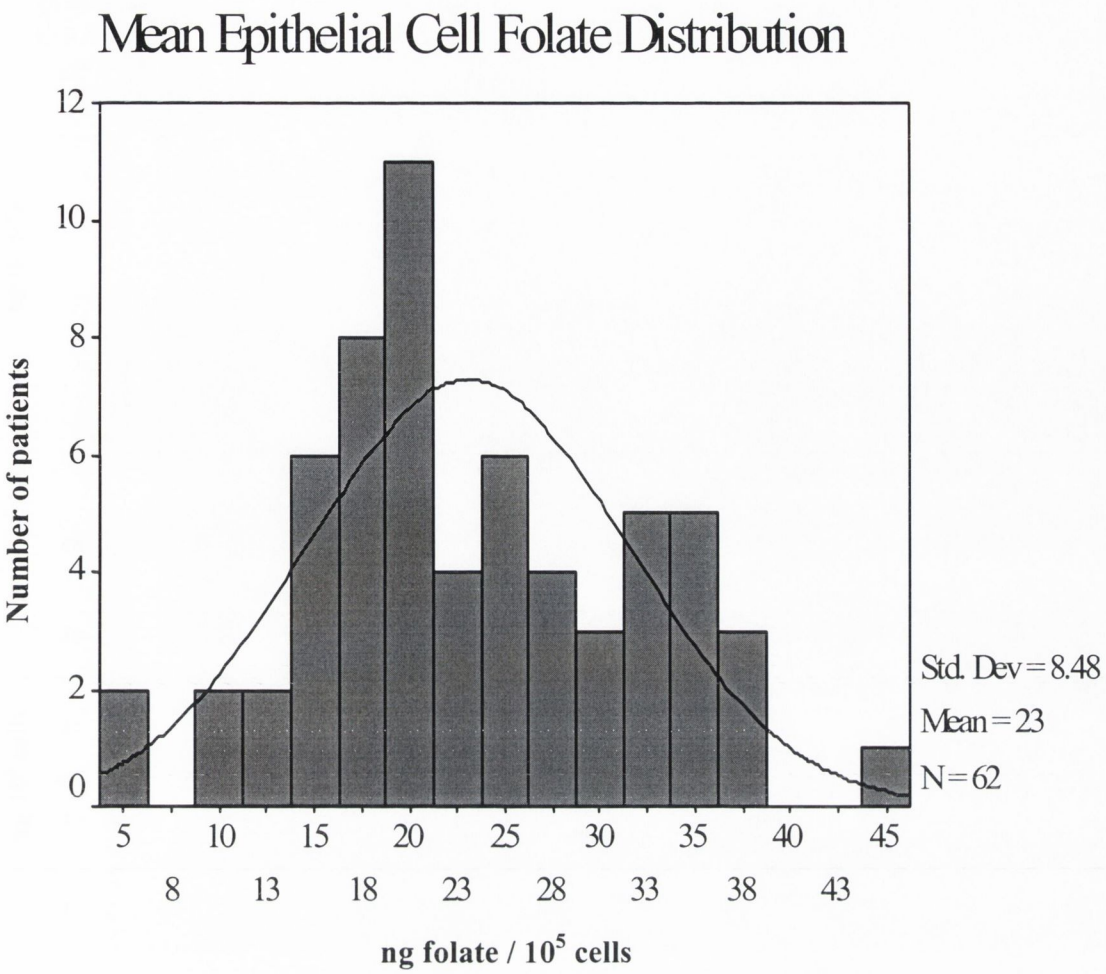


Figure 5.i Mean Distribution of Epithelial Cell Folate in the Control/Normal Population (n=62).

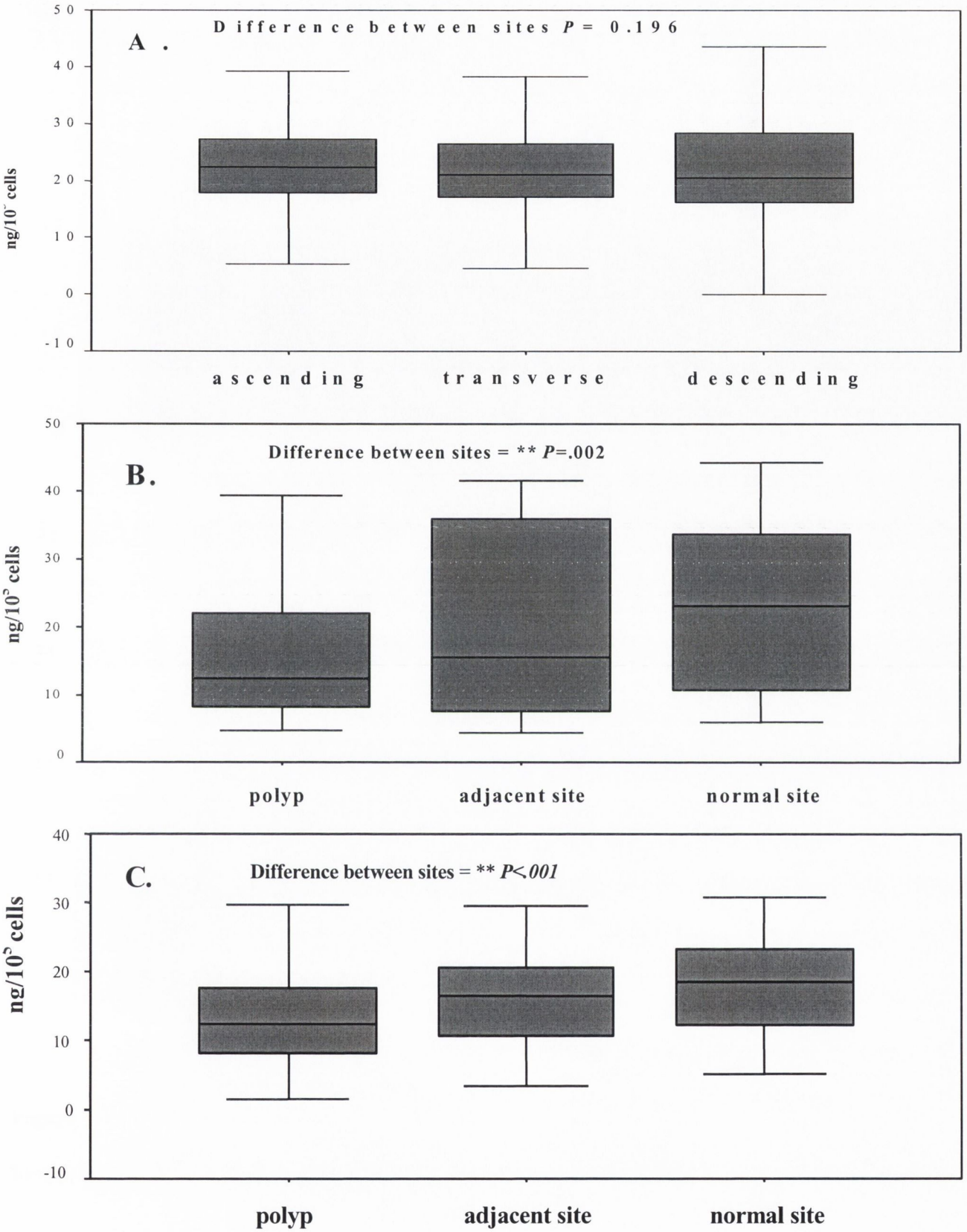


Figure 5.ii Boxplots, with error bars, of the three-biopsy sites examined per individual containing the results for epithelial cell folates in controls and cases.

A. Controls B. Cases – Hyperplastic C. Cases - Adenomas

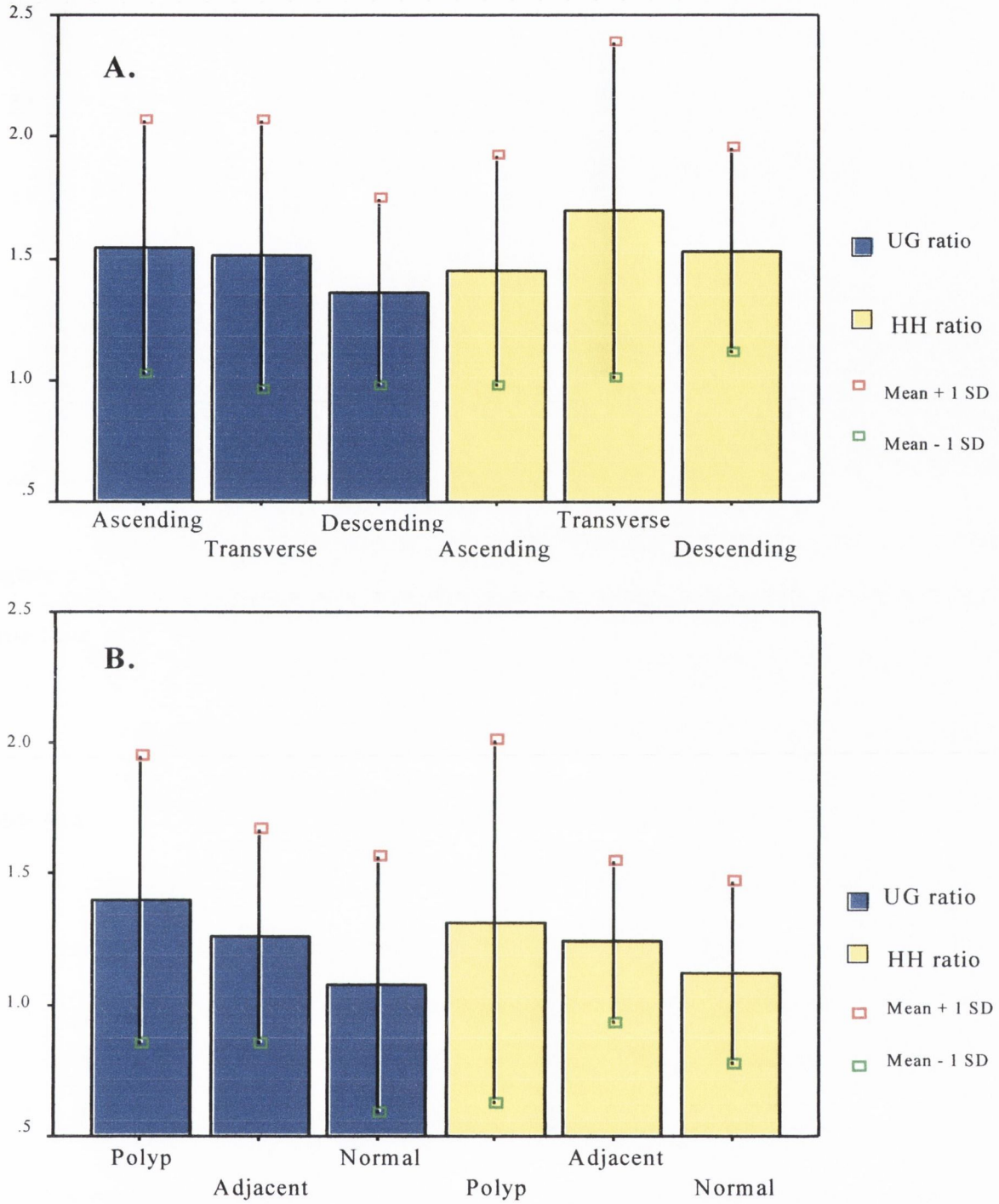


Figure 5.iii Histographic representation of UG and HH modified comet ratios, with error bars, for the three anatomically defined sites analysed per individual.

A. Controls B. Cases-Hyperplastic polyps

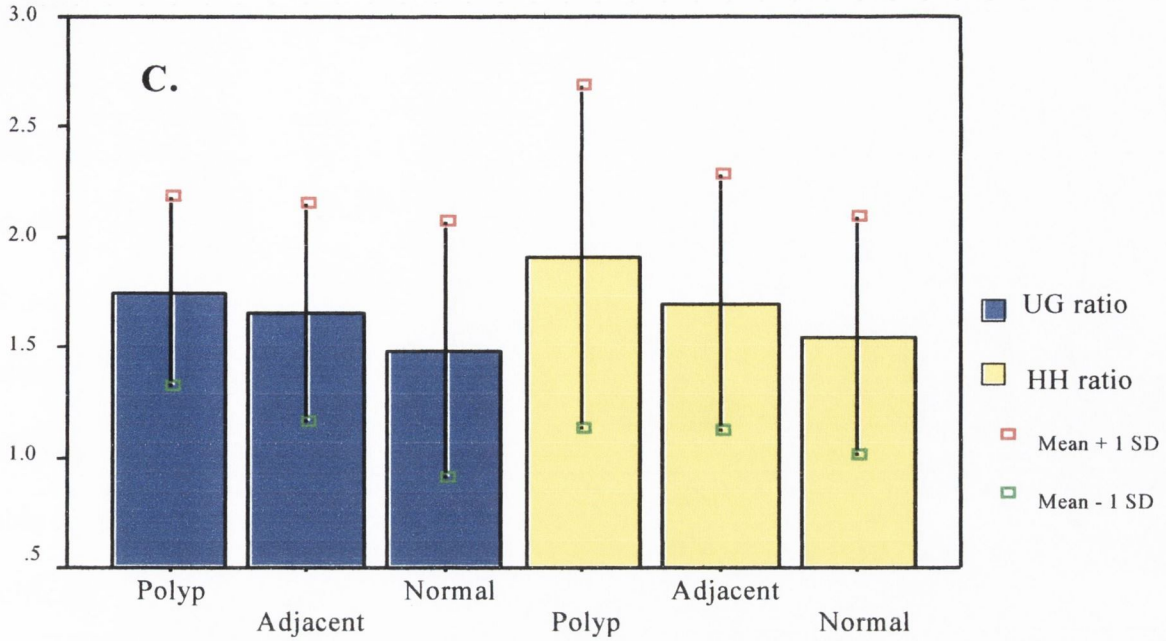


Figure 5.iii Histographic representation of UG and HH modified comet ratios, with error bars, for the three anatomically defined sites analysed per individual.

C. Cases-Adenomas

Table 5.iii Results of post-hoc Tukeys analysis for the difference between cases and controls, cases have been analysed as a whole group and as hyperplastic polyps and adenomas.

	Mean difference	*Standard error*	*Significance*
Epithelial cell folate			
Controls - Cases	6.93	1.4	<.001**
- Hyperplastic polyps	4.42	2.52	.002**
- Adenomas	8.01	1.79	<.001**
UG ratio			
Controls - Cases	0.16	0.14	.006**
- Hyperplastic polyps	0.26	0.13	.083
- Adenomas	-0.12	0.13	.068
HH ratio			
Controls - Cases	0.13	0.13	.051
- Hyperplastic polyps	0.07	0.16	.500
- Adenomas	-0.05	0.12	.089

c. Systemic Folate and Vitamin B_{12}

As expected, there were significant positive correlations between serum and red cell folate concentrations found in both the control group and the cases ($r = 0.488$, $P = 0.001$ for controls; $r = 0.526$, $P < 0.001$ for cases). Plasma tHcy concentration was inversely correlated with serum ($r = -0.481$, $P < 0.001$ for controls; $r = -0.463$, $P = 0.001$ for cases) and red cell folate ($r = -0.447$, $P = 0.001$ for controls; $r = -0.433$, $P = 0.002$ for cases). Plasma tHcy was negatively correlated with vitamin B_{12} in cases only ($r = 0.434$, $P = 0.002$) but not in controls (Table 5.iv). IFA, while significantly associated with vitamin B_{12} status ($P=0.012$), had no significant relationship with case or control status.

d. Biomarker Correlations

For controls, there was no significant correlation was seen between the epithelial cell folate and systemic folate concentrations (Table 5.iv). The epithelial cell folate was then divided through the median (22.2ng/10^5 cells) and analysed separately to see if those with lower cell folate would show an interaction. A negative correlation was seen with homocysteine and cellular folate ($r = -0.436$, $P = 0.033$) for those with lower epithelial cell folate status.

In the case group, significant correlations were seen between epithelial cell folate and serum folate ($r = 0.357$, $P =0.012$), red cell folate ($r = 0.428$, $P = 0.002$) and homocysteine ($r = -0.453$, $P = 0.001$). However, when the case group was sub-divided into hyperplastic polyps and adenomas (Table 5.vi); only the negative correlation with tHcy remained in both groups ($r = -0.710$, $P=0.007$ for hyperplastic polyps; $r =-0.399$, $P=0.016$ for adenomas). An unexpected negative correlation was seen between serum folate and the UG mean ratio of hyperplastic polyps ($r = -.610$, $P= 0.035$) and not in the adenomas ($r = -.064$, $P=0.732$). When the hyperplastic group was examined per site, the significance was driven by the polyp site ($r = -0.732$, $P= 0.025$), a negative trend towards significance was seen in the adjacent site ($r = -$

0.479, P= 0.162) and a loose negative relationship seen with the normal site (r = -.255, P= 0.425) (Table 5.vii). Trends towards significance were seen for UG modified comet ratios in the hyperplastic polyp group for red cell folate (r = -0.496, P= 0.101) and homocysteine (r = 0.51, P= 0.09) also. In the adenoma group or in HH modified comets, these relationships were not seen.

There was a highly significant difference between cases and controls for the epithelial cell folate concentrations (P<0.001), red cell folate (P=0.002) and homocysteine (P = 0.002). For all other measures (serum folate, vitamin B_{12}, UG and HH comets) there was no significant difference seen by Independent t-test analysis (Table 5.v).

Table 5.iv Correlation of epithelial cell folate, modified comet assay and systemic folate concentrations for cases and controls.

CONTROLS	Epithelial tissue folate	UG mean ratio	HH mean ratio	Serum folate	Red cell folate	Homocysteine
pithelial tissue folate						
earson Correlation		.069	.232	.122	.199	-.070
ignificance (2-tailed)		.667	.150	.379	.157	.625
G mean ratio						
earson Correlation	.069		.737**	-.089	.076	-.045
ignificance (2-tailed)	.667		.000	.595	.656	.794
H mean ratio						
earson Correlation	.232	.737**		.112	.313	-.058
ignificance (2-tailed)	.150	.000		.509	.063	.739
erum folate						
earson Correlation	.122	-.089	.112		.448**	-.481**
ignificance (2-tailed)	.379	.595	.509		.001	.000
ed cell folate						
earson Correlation	.199	.076	.313	.448**		-.447**
ignificance (2-tailed)	.157	.656	.063	.001		.070
Homocysteine						
Pearson Correlation	-.070	-.045	-.058	-.481**	-.447**	
Significance (2-tailed)	.625	.794	.739	.000	.070	

* Correlation is significant at the 0.05 level (2-tailed)

** Correlation is significant at the 0.01 level (2-tailed)

CASES	Cell folate	UG mean ratio	HH mean ratio	Serum folate	Red cell folate	Homocysteine
Epithelial tissue folate						
Pearson Correlation		-.158	-.200	.357*	.428**	-.453**
Significance (2-tailed)		.304	.198	.012	.002	.001
UG mean ratio						
Pearson Correlation	-.158		.737**	-.089	-.162	.285
Significance (2-tailed)	.304		.000	.595	.299	.064
HH mean ratio						
Pearson Correlation	-.200	.737**		.112	-.060	.136
Significance (2-tailed)	.198	.000		.509	.704	.391
Serum folate						
Pearson Correlation	.357*	-.089	.112		.526**	-.463**
Significance (2-tailed)	.012	.595	.509		.000	.001
Red cell folate						
Pearson Correlation	.428**	-.162	-.060	.526**		-.433**
Significance (2-tailed)	.002	.299	.704	.000		.002
Homocysteine						
Pearson Correlation	-.453**	.285	.136	-.463**	-.433**	
Significance (2-tailed)	.001	.064	.391	.001	.002	

* Correlation is significant at the 0.05 level (2-tailed)

** Correlation is significant at the 0.01 level (2-tailed)

Table 5.v **Independent t-test of cases versus controls for mean epithelial cell folate, modified comet assay, systemic folate and vitamin B_{12} concentrations. The mean difference between the cases and controls was obtained and whether this was significant was determined.**

	Mean difference	*Standard error*	*Significance (2-tailed)*
Mean epithelial cell folate	6.79	1.65	.000**
Mean UG ratio	0.02	-0.06	.907
Mean HH ratio	-0.02	-0.04	.906
Serum folate	1.08	1.87	.957
Red cell folate	-108.18	35.76	.002**
Homocysteine	-1.76	0.52	.002**
Vitamin B_{12}	17.09	33.08	.711

Table 5.vi Correlation of the mean epithelial cell folate, modified comet assay and systemic folate concentrations for cases sub-divided into hyperplastic polyps and adenomas.

HYPERPLASTIC	Cell folate	UG mean ratio	HH mean ratio	Serum folate	Red cell folate	Homocysteine
Epithelial tissue folate						
Pearson Correlation		-.163	-.229	.563*	.624*	-.710**
Significance (2-tailed)		.594	.493	.045	.023	.007
UG mean ratio						
Pearson Correlation	-.163		.518	-.610*	-.496	.510
Significance (2-tailed)	.594		.070	.035	.101	.090
HH mean ratio						
Pearson Correlation	-.229	.518		.159	-.173	.115
Significance (2-tailed)	.493	.070		.623	.591	.722
Serum folate						
Pearson Correlation	.563*	-.610*	.159		.663*	-.690**
Significance (2-tailed)	.045	.035	.623		.013	.009
Red cell folate						
Pearson Correlation	.624*	-.496	-.173	.663*		-.766**
Significance (2-tailed)	.023	.101	.591	.013		.002
Homocysteine						
Pearson Correlation	-.710**	.510	.115	-.690**	-.766**	
Significance (2-tailed)	.007	.090	.722	.009	.002	

ADENOMAS	Cell folate	UG mean ratio	HH mean ratio	Serum folate	Red cell folate	Homocysteine
Epithelial tissue folate						
Pearson Correlation		-.090	-.156	.285	.222	-.399*
Significance (2-tailed)		.630	.411	.093	.193	.016
UG mean ratio						
Pearson Correlation	-.090		.672**	-.064	.042	.226
Significance (2-tailed)	.630		.000	.732	.821	.221
HH mean ratio						
Pearson Correlation	-.156	.672**		.104	.049	.141
Significance (2-tailed)	.411	.000		.584	.799	.458
Serum folate						
Pearson Correlation	.285	-.064	.104		.494**	-.423*
Significance (2-tailed)	.093	.732	.584		.002	.010
Red cell folate						
Pearson Correlation	.222	.042	.049	.494**		-.349*
Significance (2-tailed)	.193	.821	.799	.002		.037
Homocysteine						
Pearson Correlation	-.399*	.226	.141	-.423*	-.349*	
Significance (2-tailed)	.016	.221	.458	.010	.037	

* Correlation is significant at the 0.05 level (2-tailed)

** Correlation is significant at the 0.01 level (2-tailed)

Table 5.vii Correlation between modified comet assay ratios per site with serum folate concentrations.

	Serum Folate
Hyperplastic Polyps	
UG polyp site – pearsons correlation	-.732*
significance (2-tailed)	.025
UG adjacent site – pearsons correlation	-.479
significance (2-tailed)	.162
UG normal site – pearsons correlation	-.255
significance (2-tailed)	.425
Adenomas	
UG polyp site – pearsons correlation	.131
significance (2-tailed)	.532
UG adjacent site – pearsons correlation	-.120
significance(2-tailed)	.542
UG normal site – pearsons correlation	-.152
Significance (2-tailed)	.44
Hyperplastic Polyps	
HH polyp site – pearsons correlation	-.141
significance (2-tailed)	.738
HH adjacent site – pearsons correlation	-.419
significance (2-tailed)	.199
HH normal site – pearsons correlation	-.252
Significance (2-tailed)	.455
Adenomas	
HH polyp site – pearsons correlation	.149
significance (2-tailed)	.498
HH adjacent site – pearsons correlation	-.077
significance (2-tailed)	.696
HH normal site – pearsons correlation	.122
significance (2-tailed)	.535

iii. Discussion

An accumulating body of evidence over the past decade suggests that folate may be involved in the development and prevention of several malignancies including cancer of the CRC, lungs, pancreas, oesophagus, stomach, cervix and breast (Choi and Mason 2002). These studies collectively suggest an inverse association between folate status and the risk of these malignancies. Most prospective and case-control studies of serum folate, red cell folate or reported dietary or total folate intake are compatible with inverse associations with colon cancer and adenoma (Sharp and Little 2003).

The role of folate was examined in relation to systemic markers, epithelial cell folate concentrations and modified comet ratios in this chapter. The relationship between the modified comet biomarkers when examined showed that they had a strong, positive relationship in both cases and controls. For the control patients, when the comet UG and HH ratios were examined, a significant positive correlation was seen ($r = 0.737$, $P < 0.001$) as shown in table 5.iv. In the case group, there was a significant correlation seen between the comet UG and HH ratios ($r = 0.737$, $P<0.001$). The pathways of uracil misinicorporation and hypomethylation of DNA appear to be involved in a complementary fashion; this may be due to the central role of folate in both pathways. There was a lack of significant correlation between the UG and HH comets and the epithelial cell folate concentration for both groups but there were negative trends seen for both UG ($r = -0.158$, $P= 0.304$) and HH comets ($r = -0.2$, $P= 0.198$) in cases that was not seen for the controls.

The results from the Anova analysis examining the difference between values obtained for each site per patient group showed interesting results in table 5.iii. While there was no significant relationship seen in the control group intersite, the case groups as a whole exhibited a significant difference between sites for UG comet ratios ($P= 0.006$) and strong trends when sub-divided into hyperplastic ($P = 0.083$) and adenomas ($P = 0.068$). In the HH comet ratios,

the cases exhibited near significance (P = 0.051) and adenoma group also (P = 0.089) but the hyperplastic group failed to show a relationship (P = 0.5). The modified comet ratios are displayed histographically in figure 5.iii. Overall, the data illustrated a higher level of DNA damage was seen in the abnormal compared to the normal tissue as has been found in other comet work (Duthie and Hawdon 1998).

The results obtained from the epithelial cell folate work yielded interesting results,displayed in figure 5.ii. The picture that resulted from the Anova analysis of intersite variation showed an inverse result to the modified comet ratios. There was a stepwise decrease in the epithelial cell folate seen in the cases from the normal tissue through to the polyp biopsies. The difference was more pronounced in the adenoma group than the hyperplastic group ($P<0.001$ compared to $P=0.002$ respectively). This result supports the previous work done on the epithelial cell folate method (Meenan *et al.* 1997). The cases as a group had a significantly lower mean epithelial cell folate concentration than the controls. There was also a difference seen in the mean epithelial cell folate concentrations of hyperplastic polyps versus adenomatous ones when the case group was sub-divided, with the adenomatous groups having the lowest mean concentration. This was of interest as it agreed with a previous publication showing a global reduction in tissue folate in colonic tissue of patients with adenomatous polyps. In that publication, the alternative method of tissue folate estimation was used (Kim *et al.* 1998), suggesting that the reduced folate concentration in the mucosa of patients with adenomatous polyps may coincide with a metabolic defect in folate metabolism.

The relationship between systemic measures of folate status and tissue biomarkers was examined in this case-control study. No relationship was seen between the control population's systemic markers and epithelial cell folate concentrations initially, but when the group was divided through the median, a negative correlation was seen between epithelial cell folate concentrations and homocysteine for those in the lower median group ($r = -0.436$, $P= 0.033$).

The concentration of epithelial cell folate in the below median group was closer to the mean value obtained from the cases, where a negative correlation was also seen with plasma Hcy (r = -0.453, P= 0.001). These results would suggest that normal healthy colonic mucosa may not necessarily derive all of their folate content entirely from the blood; at least part may be absorbed from the intestinal lumen. While this result seems at odds with the previous publications using the whole biopsy tissue folate extraction method (Kim *et al.* 1998), it may be argued that the epithelial cell folate may be looking at a more biologically relevant interaction.

When the case group was examined as a whole, a strong positive relationship was seen with serum (r = 0.357, P =0.012) and red cell folate (r = 0.428, P = 0.002), a strongly negative correlation was also seen for Hcy (r = -0.453, P = 0.001) as seen in table 5.iv. The case group was then sub-divided to examine hyperplastic and adenomatous polyps separately (table 5.vi). The hyperplastic polyp group maintained the above pattern of significant positive correlations for serum and red cell folate, and a highly significant negative correlation for Hcy. The adenomatous polyp group displayed only a significant negative correlation for Hcy, with trends towards significance in the serum and red cell folate concentrations. It may be possible that the hyperplastic polyps sampled in this study were, in fact, pre-neoplastic tissue. Recent publications have examined the malignant potential of hyperplastic polyps, which were historically believed to be completely benign. The malignant potential of serrated adenomas has been identified in pathological studies (Longacre and Fenoglio-Preiser 1990b;Jass 2001b) but the potential for malignancy in hyperplastic polyps was much more in doubt. Genetic alterations of the type seen in neoplasms have been reported in hyperplastic polyps (Rashid *et al.* 2000b) and epidemiological evidence shows that the risk factors for hyperplastic polyps were also risk factors for adenomas and carcinomas (Martinez *et al.* 1997a). It also appears that not all hyperplastic polyps have this malignant potential, right-sided and large hyperplastic

ones are the polyps of concern (Rashid *et al.* 2000b;Hawkins and Ward 2001a;Morimoto *et al.* 2002a). The hyperplastic polyps sampled in this study were predominantly large in size and a large percentage came from the right side of the colon, so it may be possible that the relationship between epithelial cell folate and systemic markers was stronger in this group owing to the importance of folate in the very early stages of neoplasia.

This hypothesis was further strengthened when a positive correlation was found between the UG comet ratio and the serum folate concentrations as well as strong trends towards significance with red cell folate and Hcy in the hyperplastic polyp group (see table 5.vii). A previous study using a folate- and methyl-deficient rat model were able to demonstrate that increased uracil misincorporation was preceded by an increase in DNA strand breaks and abasic sites in preneoplastic liver (Pogribny *et al.* 1997b). Also, recent reports of uracil in DNA of folate-deficient human lymphocytes (Blount *et al.* 1997) confirmed earlier reports of uracil misincorporation associated with increased intracellular dUTP/dTTP (Goulian *et al.* 1980). An increase in dUTP/dTTP in vitro has been shown to promote folate fragile site expression, DNA strand breakage, error-prone DNA repair and mutagenesis (Kunz 1988). It is possible that UG comets in hyperplastic polyps as a biomarker were measuring a premalignant process that is of biological relevance in early DNA damage, especially as the polyp site drove the negative correlation.

The hypomethylation biomarker, HH modified comet assay, did not yield any direct correlations with the groups investigated. However, as mentioned previously, near significance was achieved when the variation between sites in the case group was examined, this was driven by the adenoma group. This would suggest that the alterations in methylation status occur later in the adenoma-to-carcinoma sequence than misincorporation of uracil. This has been examined in a recent publication (James *et al.* 2003) that hypothesised due to DNA methyltransferase's ancestral role as a DNA repair enzyme, it will bind with higher affinity to

DNA strand breaks, gaps, abasic sites and uracil than it does to its hemimethylated CpG sites (Kim *et al.* 1997). High-affinity binding of the maintenance DNA methyltransferase to unrepaired lesions in DNA may sequester the available enzyme away from the replication fork and promote passive replication-dependent demethylation. Experiments were performed that found lesion-containing DNA was less efficiently methylated than lesion-free DNA from folate/methyl-deficient lymphocytes and that an increase in DNA strand breaks preceded DNA hypomethylation (Pogribna *et al.* 1999).

In summary, significant differences were found between the case and the control group in relation to systemic and localised biomarkers. The differences suggest that folate does have a role in CRC development but that larger numbers would have benefited the final result.

CHAPTER 6

INTERVENTION TRIAL

1. INTERVENTION TRIAL

Several intervention studies have been carried out but again to date these have all had small numbers of participants. Cravo and colleagues have shown that folic acid supplementation at 10mg/d for 6 months in patients with either colonic adenoma or cancer (n=22) followed by removal of these lesions can significantly reverse genomic hypomethylation in normal rectal mucosa. During the washout period, it was shown that DNA methylation values moved toward the initial values in most cases (Cravo *et al.* 1994). In another study, 60 subjects with colorectal adenoma were randomised to receive either 1mg/day folic acid or placebo for 2 years after removal of the adenoma. Although adenoma recurrence was reduced by 40% to 46% in the folic acid supplementation group compared to the placebo group at year 1 and 2 colonoscopic follow-up, respectively, the results fell short of statistical significance (Paspatis and Karamanolis 1994). An intervention study in Portugal looked at the effect of a 3-month supplementation of 5mg/day in 20 subjects post-adenoma removal. The subjects with only one adenoma responded to the folic acid supplementation with a 40% increase in genomic DNA methylation ($p=0.05$), whereas those with greater than one adenoma showed no increase (Cravo *et al.* 1998). Another study ran an intervention for 1 year using 5mg/day in a placebo-controlled manner in colonic adenoma patients. The folate supplementation group and placebo showed increased genomic DNA methylation and decreased strand breaks in exons 5 through 8 of the *p53* tumour suppressor gene ($p<0.01$) (Kim *et al.* 2001a), suggesting that factors other than folate supplementation were responsible for the parallel increase in methylation of DNA and increased stability of the *p53* gene. An Australian group investigated the effect of the combination of 700μg folic acid and 7μg vitamin B_{12} per day over a 12 week period and then 2mg folic acid and 20μg vitamin B_{12} over an additional 12 weeks in relation to DNA methylation and damage as measured by micronucleated cell frequency in healthy volunteers (18-32 years of age) (Fenech *et al.* 1998). The micronucleated

cell frequency was significantly reduced by 25.4% in those individuals who were initially in the high 50th percentile ($p<0.01$) but overall the genomic DNA methylation status was not altered in the supplementation group. It has been shown in a trial of patients with colonic polyps that a 3 month supplementation of 5mg folic acid /day resulted in a 35% decrease in abnormally high ornithine decarboxylase activity in polyps that was accompanied by a 43% increase in SAM content in polyps (Bukin *et al.* 2001). An Italian study was able to show that a dose of 15mg/day folic acid significantly reduced cell proliferation in the upper 40% of the colonic crypts (Biasco *et al.* 1997) of UC patients; an expansion of the proliferative compartment from the lower 60% into the upper 40% of the crypts has been observed in patients with CRC (Bostick *et al.* 1997). A further study examined the same end point using a supplement dose of 2mg folic acid for 3 months duration in patients with recurrent adenomatous polyps (n=6) and a control group receiving placebo (n=5). Rectal biopsies were taken at commencement, 4, 12 and 18 weeks. The biopsies at each time point were immediately incubated in culture medium enriched with BrdUrd. The S phase cells, which incorporated the BrdUrd into their DNA, were identified following immunohistochemical staining. BrdUrd labelling indices (LIs) were calculated for the entire crypt and for each of five equal compartments running consequently from the base to the luminal surface, 25 orientated crypts were identified for each time point and the number and position of BrdUrd positive and negative cells were counted. The LIs of the treatment group were lowered after 12 weeks of supplementation (9.1 (6.7-12.3) v 7.4 (5.3-9.6)). The LIs for compartments within the crypt showed that the most significant drop in number of proliferating cells was in the upper most regions of the crypt. This study indicated that folate supplementation decreased the colonic mucosal cell proliferation and that the most significant reduction was in the luminal aspect of the crypt (Khosraviani *et al.* 2002).

This study proposed to examine the relationship between the intermediate endpoint of colorectal cancer, colorectal adenomas, and folate status pre- and post-intervention. The intervention period was for six months on either placebo or 600μg folic acid. The parameters to be examined were those defined by the Comet assay (uracil misincorporation and hypomethylation) and folate status systemic and local.

i. Methods

a. Patient Selection

Patients were recruited from St. James Hospital Endoscopy Unit. Exclusion criteria were active inflammatory bowel disease, a previous history of CRC, coeliac disease, previous bowel resection for non-malignant conditions, intestinal malabsorption, liver or renal disease, pregnancy, epilepsy, alcoholism, pernicious anemia and the use of antibiotics or anti-folate medication (sulphazalazine or methotrexate). The study was approved by the Joint Research Ethics Committee of the Federated Dublin Voluntary Hospitals and St. James Hospital. All patients were recruited on the day of their colonoscopy, before the procedure. Written informed consent was obtained from all patients. All patients had prepared for colonoscopy by taking orally administered colonic lavage solution (Kleanprep; Norgine, Harefield, UK). Patients were considered eligible for inclusion on the trial if they had a polyp discovered at the time of the colonoscopy that was subsequently confirmed histologically to be an adenoma.

b. Biopsy Collection, Epithelial Cell Isolation and Modified Comet Preparation

In all cases, 2 biopsies from the polyp, 2 biopsies from adjacent to the polyp and 2 biopsies from 10-15cm distal from the polyp were taken. However, in some cases a biopsy from the polyp area was not possible due to size, location or loss of the polyp upon removal into the gut lumen. The two biopsies per selected site had the epithelial layer separated off using the isolation methodology described previously. The resulting cell pellet was split into the sub-fraction for colonocyte folate analysis, which was stored in 500μl 1% sodium ascorbate

PBS (pH 6.5) at -40^{0}c until deconjugation and microbiological analysis using *L.Casei* (all colonocyte folates were assayed within four months of isolation), and the other sub-fraction for Comet analysis. A series of eight slides were produced per site for Comet analysis, slides *e-h* had a double layer of NMP agarose on each slide. The double layer of NMP agarose was used to decrease background fluorescence from the frosted slides when performing FISH-Comet analysis.

c. Blood Sampling and Patient Data Collection

A venous sample was collected into an ethylenediaminetetraacetic acid vacutainer for DNA extraction, erythrocyte folate and plasma homocysteine determination. MTHFR genotype was determined following DNA extraction. A vacutainer containing no anticoagulant was used for the determination of serum folate and vitamin B_{12} analysis. For plasma Hcy, plasma was separated within 1h of collection and frozen at -40^{0}C. Weight and height were recorded and information on smoking habits, physical activity levels, alcohol intake, current medication, medical history, blood pressure and supplement use were gathered before colonoscopy.

d. Intervention trial

Following the colonoscopy, confirmation of the histology from the polyp was awaited before contacting the patient about the intervention trial. Only those with a histologically confirmed adenoma were considered eligible. All patients who agreed to take part in the trial completed the study. The intervention was designed to be placebo-controlled, randomised and double-blind. The supplement tablet contained 600μg folic acid while the placebo was of the same size, shape and colour. Patient compliance was encouraged through regular monthly contacts with the patients via phone or post. The intervention tablets were posted on a monthly basis to each patient in a "days-of-the-week" pillbox, any untaken tablets from the previous month were returned by post after the new month's supply arrived. Tablet counts were performed to monitor compliance.

At the end of the six-month intervention period, those involved agreed to return to St. James Hospital Endoscopy Unit to have a repeat colonoscopy. During the procedure, four repeat biopsies were taken. Two biopsies were taken from the original location of the polyp found on initial colonoscopy and two biopsies from the normal area 10-15cm distal to the polyp location. These were placed into HBSS-containing universal containers and analysed as before. Blood sampling was also repeated and all biomarkers were re-evaluated.

ii. Results

Statistical analysis was performed using SPSS release 10 for PC (SPSS UK, Surrey, UK). Data was compared using Student's t-test (two tailed) for paired data.

a. Demographic Data

The demographic characteristics of the study and variables measured at baseline are shown in Table 6.i. Tablet counts indicate good compliance, defined as omitting less than two tablets per month for the six-month intervention period. In this study group, compliance was 100% as they were followed closely and well motivated as a group. The supplements were well tolerated and safe: no treatment-related adverse side effects were observed. Values of the investigated haematological variables before and after treatment were generally within their respective reference ranges, although some values deviated from the range but they were not clinically relevant. Five of the nine participants were wild-type for MTHFR (55%) and 4 were heterozygous (45%), there were no homozygous individuals. Polyp location was 11% proximal, 33% distal and 56% rectal for the group. There was no significant difference between the placebo and folic acid as tested by ANOVA.

Table 6.i Intervention demographics divided into placebo and treatment sub-groups.

	Placebo (n=3)	*Folic Acid (n=5)*	*Total (n=9)*
Sex – Female	1 (33%)	4 (67%)	5 (44%)
Male	2 (67%)	1 (33%)	4 (56%)
Age	58±9.2	61.2±6.5	60.1±7
Body Mass Index	27.2±1.9	27.7±3	27.5±2.6
Indication for Colonoscopy			
Altered Bowel Habit		1 (17%)	1 (11%)
Bleeding Per Rectum		1 (17%)	1 (11%)
Abdominal Pain		1 (17%)	1 (11%)
Polyp Review	3 (100%)	3 (50%)	6 (67%)
Physical Activity			
Low	1 (33%)	4 (67%)	5 (56%)
Medium	2 (67%)	2 (33%)	4 (44%)
Alcohol			
Drinkers – Beer	2 (67%)	1 (17%)	3 (33%)
Spirits	1 (33%)	3 (50%)	4 (44%)
Wine		2 (33%)	2 (22%)
Total Units / Week	20.7±20.1	10.3±20.4	13.8±19.7
Cigarettes			
Non-Smoker	1 (33%)	4 (67%)	5 (56%)
Ex-Smoker	1 (33%)	1 (17%)	2 (22%)
Smoker	1 (33%)	1 (17%)	2 (22%)
Duration - <20 years	1 (33%)	1 (17%)	2 (22%)
<30 years		1 (17%)	1 (11%)
<40 years	1 (33%)		1 (11%)
Amount per day - 10-20	1 (33%)	2 (33%)	3 (33%)
>20	1 (33%)		1 (11%)
Blood Pressure			
Systolic	165.3±29.1	149.2±21.8	154.6±23.9
Dystolic	85.3±10.8	77±5.3	79.8±8
Aspirin Users	1 (33%)		1 (11%)

Table 6.ii Intervention group demographics for polyp location and histological diagnosis.

	Placebo (n=3)	*Folic Acid (n=6)*	*Total (n=9)*
Polyp location			
transverse		1 (17%)	1 (11%)
descending		1 (17%)	1 (11%)
sigmoid	1 (33%)	1 (17%)	2 (22%)
rectum	2 (67%)	3 (50%)	5 (56%)
Adenoma			
serrated		1 (17%)	1 (11%)
tubular	1 (33%)	4 (67%)	5 (56%)
tubulovillous	2 (67%)	1 (17%)	3 (33%)
Dysplasia - mild	3 (100%)	5 (83%)	8 (89%)
severe		1 (17%)	1 (11%)

b. Epithelial Cell Folate Concentrations and UG/HH Modified Comet Ratios

Following the intervention period, the placebo group had no significant difference between the pre- and post-intervention biomarkers except for the polyp site UG ratio (P = 0.017) and the HH ratio of the adjacent site (P = 0.049). However, in the case of the group that received the folic acid supplementation, significance was found in most biomarkers (Table 6.ii and Table 6.iii).

The site where the polyp had originally been located was significantly increased to bring the concentrations in line with the normal site (mean increase = 6.72 ng/10^5 cells, P = 0.011). The UG ratio for the same location was significantly reduced, indicating that the misincorporation of uracil was reduced following folic acid supplementation (mean ratio decrease = 1.21, P <0.001). The mean value for HH modified comets was also reduced though not significantly (ratio reduction = 1.14, P = 0.179) but this was most likely due to a large standard deviation in the initial ratio mean. The site that was adjacent to the polyp was compared to the anatomical site that matched the polyp location on repeat colonoscopy, as this was the original site for both tissue samples. However, there was no significance found in the change (mean increase = 4.01ng/10^5 cells, P = 0.186). This may have been due to the variance found in the original values for the biopsies obtained and, possibly, the actual biopsy site being either adenomatous or normal tissue. In the case of UG and HH modified comet ratios for this site, these were both significantly reduced (mean UG ratio reduction = 0.44, P = 0.043; mean HH ratio reduction = 0.78, P = 0.015). The normal tissue site post-intervention was not significantly different to pre-intervention (mean increase = 0.96, P = 0.564), the same holds true for the UG and HH ratios (UG mean ratio reduction = 0.5, P = 0.564, mean HH ration reduction = 0.43, P = 0.113).

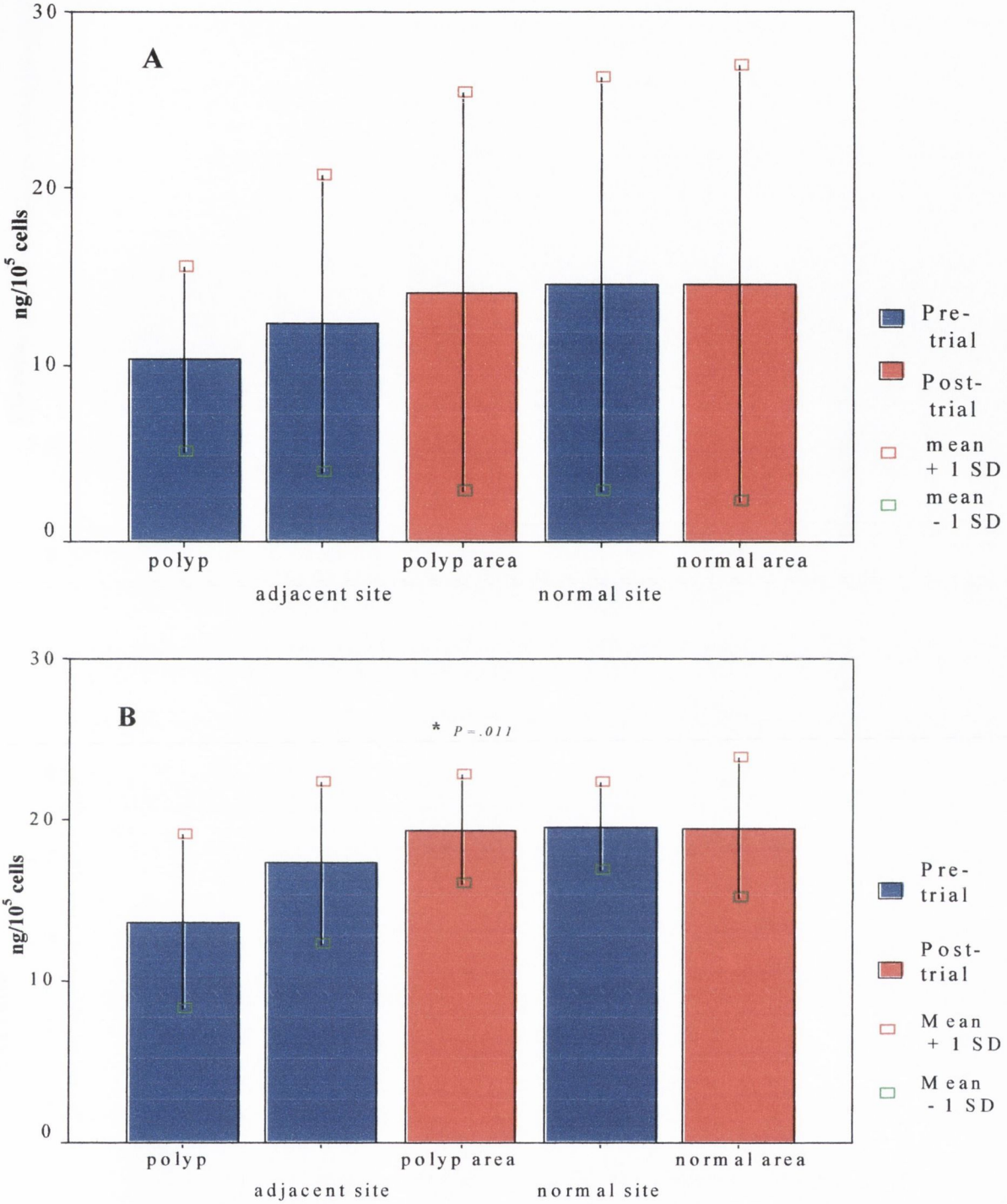
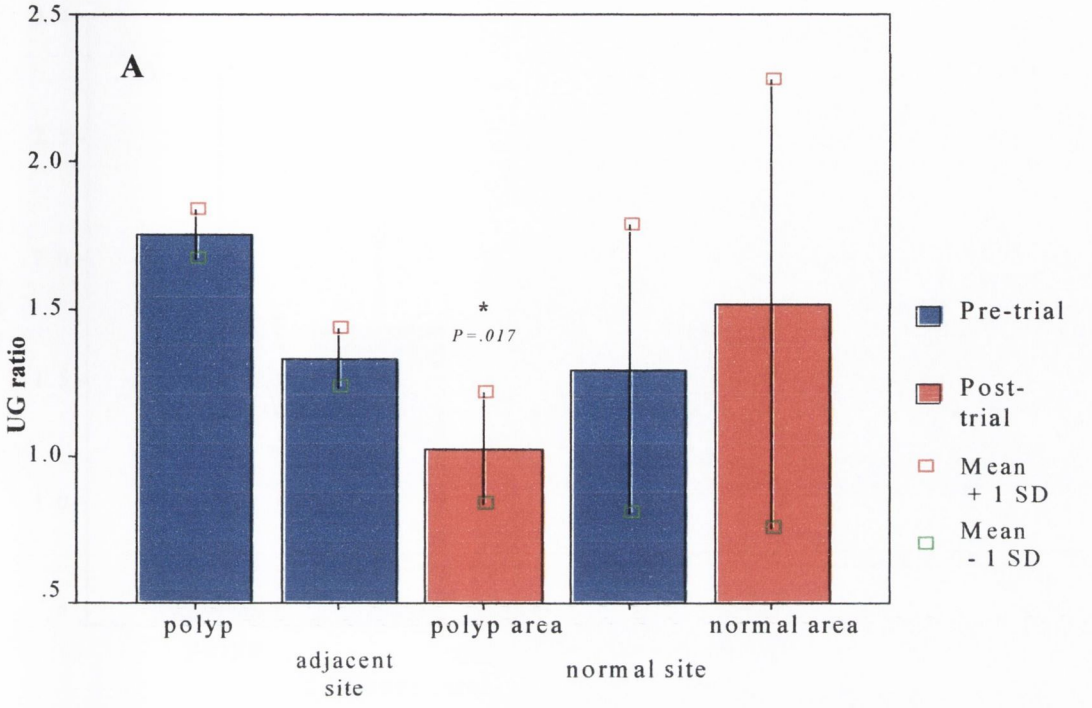


Figure 6.i Epithelial cell folate concentrations pre- and post-intervention period represented as histographs with error bars per site.

A. placebo group B. folic acid supplementation group



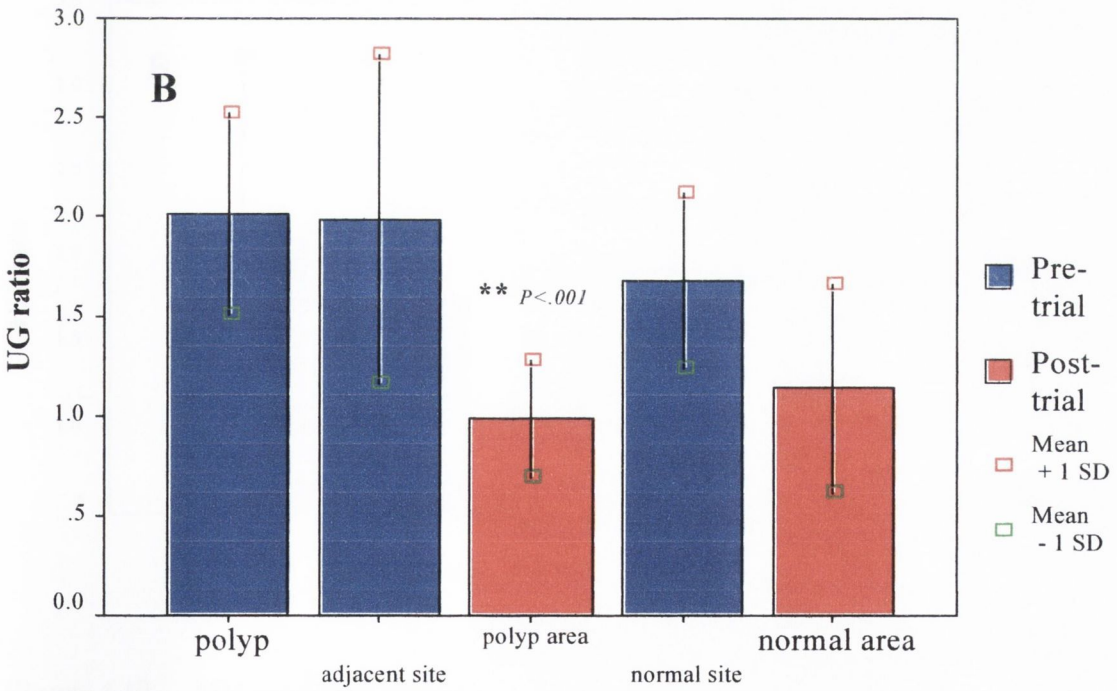
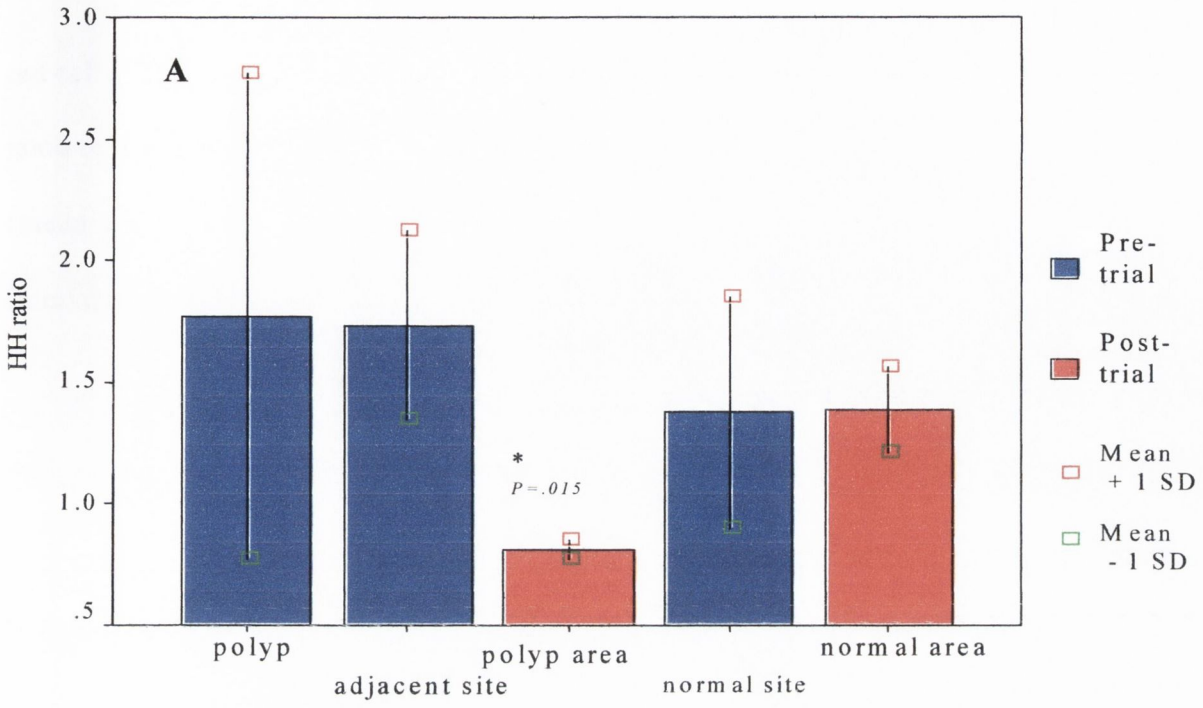


Figure 6.ii UG modified comet ratios pre- and post-intervention period represented as histographs with error bars per site.

A. placebo group B. folic acid supplement group



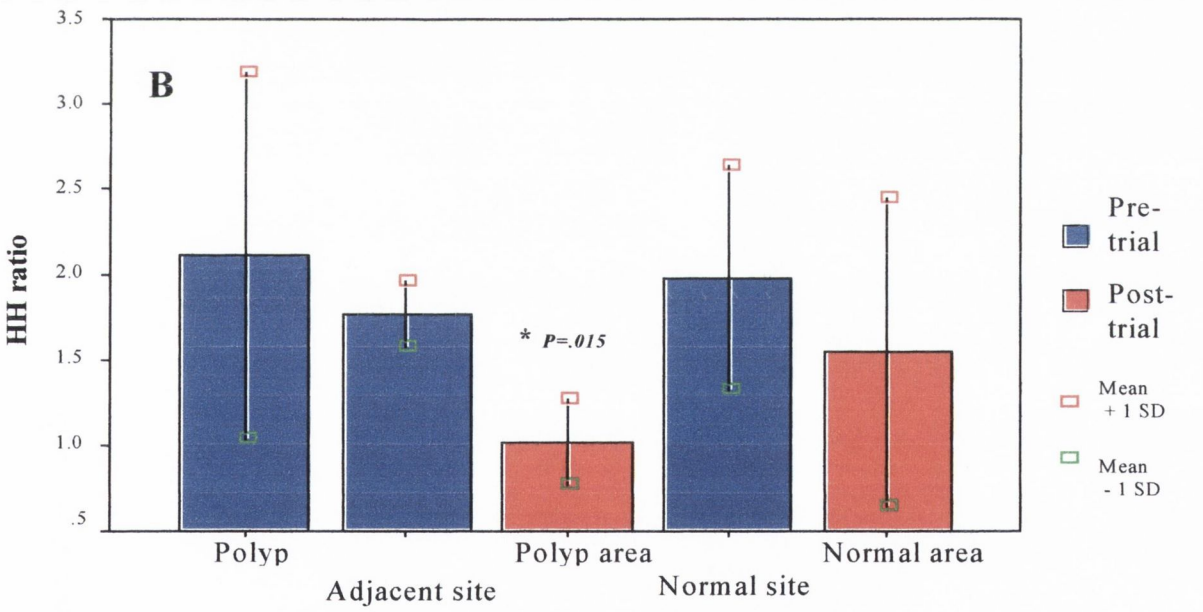


Figure 6.iii HH modified comet ratio pre- and post-intervention period represented as histographs with error bars per site.

A. placebo group B. folic acid supplement group

c. Systemic Folate and Vitamin B_{12}

Serum folate increased significantly (mean increase = 17.1ng/mL, $P < 0.001$) as did the red cell folate (mean increase = 359.11ng/mL, $P = 0.013$), this confirms that compliance was good in the folic acid group. Homocysteine was significantly reduced in the supplement group (mean decrease = 1.8μmol/L, $P = 0.032$). Vitamin B_{12} was unaltered by the folic acid intervention (mean difference = 5.53pg/mL, $P = 0.899$).

Table 6.iii Mean results for epithelial cell folate and modified comet ratios per site as well as the systemic folate and vitamin B_{12} pre- and post-intervention.

	Placebo (n=3)		*Folic Acid (n=6)*	
	Pre	**Post**	**Pre**	**Post**
Serum Folate	21.63±9.05	24.46±7.94	21.87±11.09	38.97±11.25
Red Cell Folate	435.47±88.56	405.22±141.26	511.29±126.26	870.4±287.3
Homocysteine	11.63±5.19	12.06±5.76	12±4.61	10.2±3.72
Vitamin B_{12}	403.93±201.51	415.31±218.28	308.49±238.06	314.03±166.01
Polyp Site Cell Folate	10.31±5.26	14.1±11.26	14.7±5.26	21.42±5.4
Polyp Site UG Ratio	1.75±0.76	1.03±0.19	2.2±0.34	0.99±0.34
Polyp Site HH Ratio	1.77±1	0.81±1.03	2.11±1.08	0.97±0.31
Ajacent Site Cell Folate[1]	12.33±8.38	14.1±11.26	14.76±7.19	18.77±3.3
Ajacent Site UG Ratio[1]	1.33±0.47	1.03±0.19	2.21±0.77	0.87±0.17
Ajacent Site HH Ratio[1]	1.73±0.39	0.81±0.97	1.77±0.19	0.99±0.27
Normal Site Cell Folate	14.59±11.71	14.6±12.34	18.73±7.48	19.69±5.46
Normal Site UG Ratio	1.29±0.49	1.39±1.03	1.64±0.49	1.14±0.52
Normal Site HH Ratio	1.38±0.47	1.29±0.78	1.98±0.66	1.55±0.9

[1] Ajacent site to polyp was compared to the polyp site repeat biopsies as was the same anatomical area.

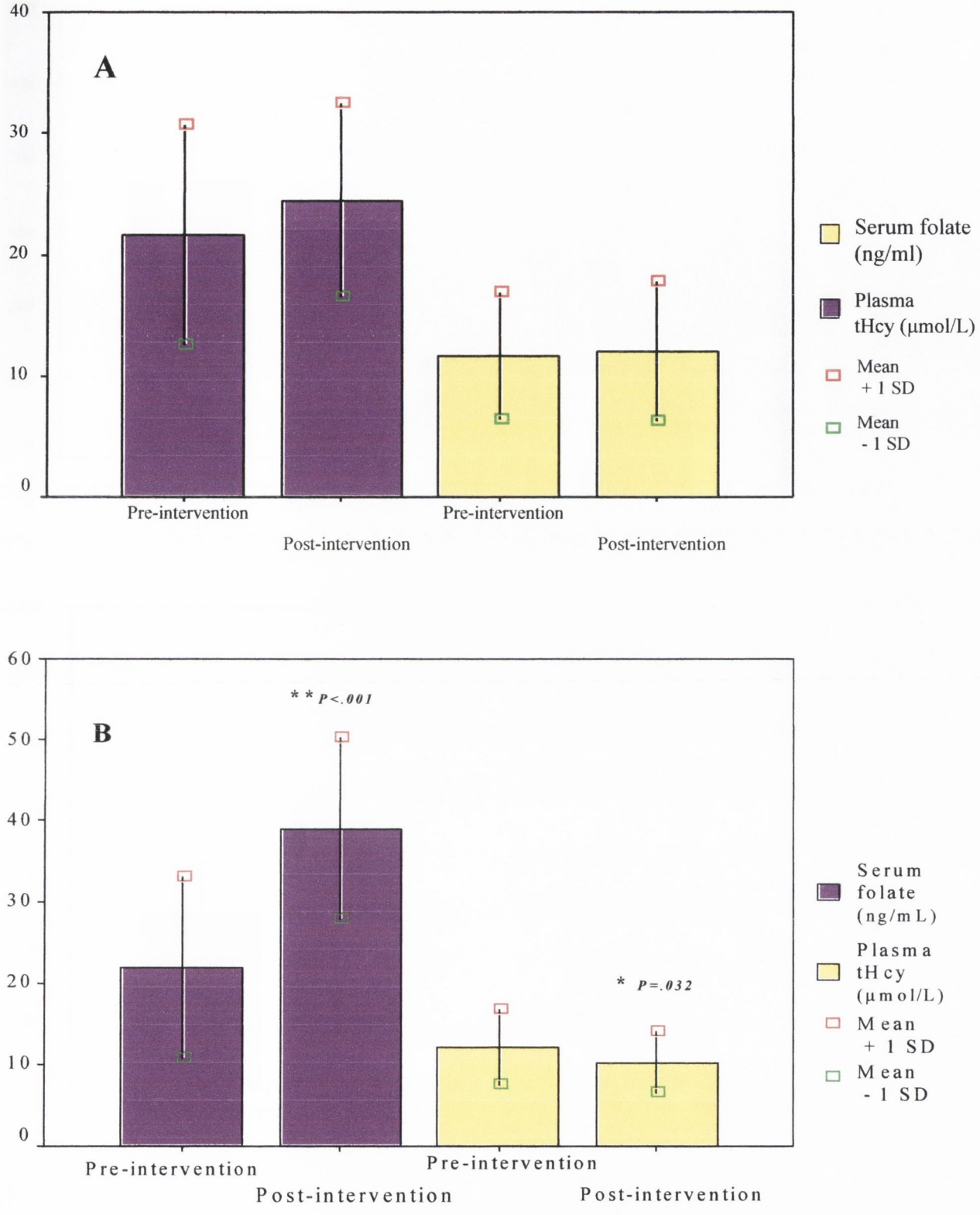


Figure 6.iv Serum folate and plasma homocysteine pre- and post-intervention represented as histographs with error bars.

A. placebo group B. folic acid supplement group

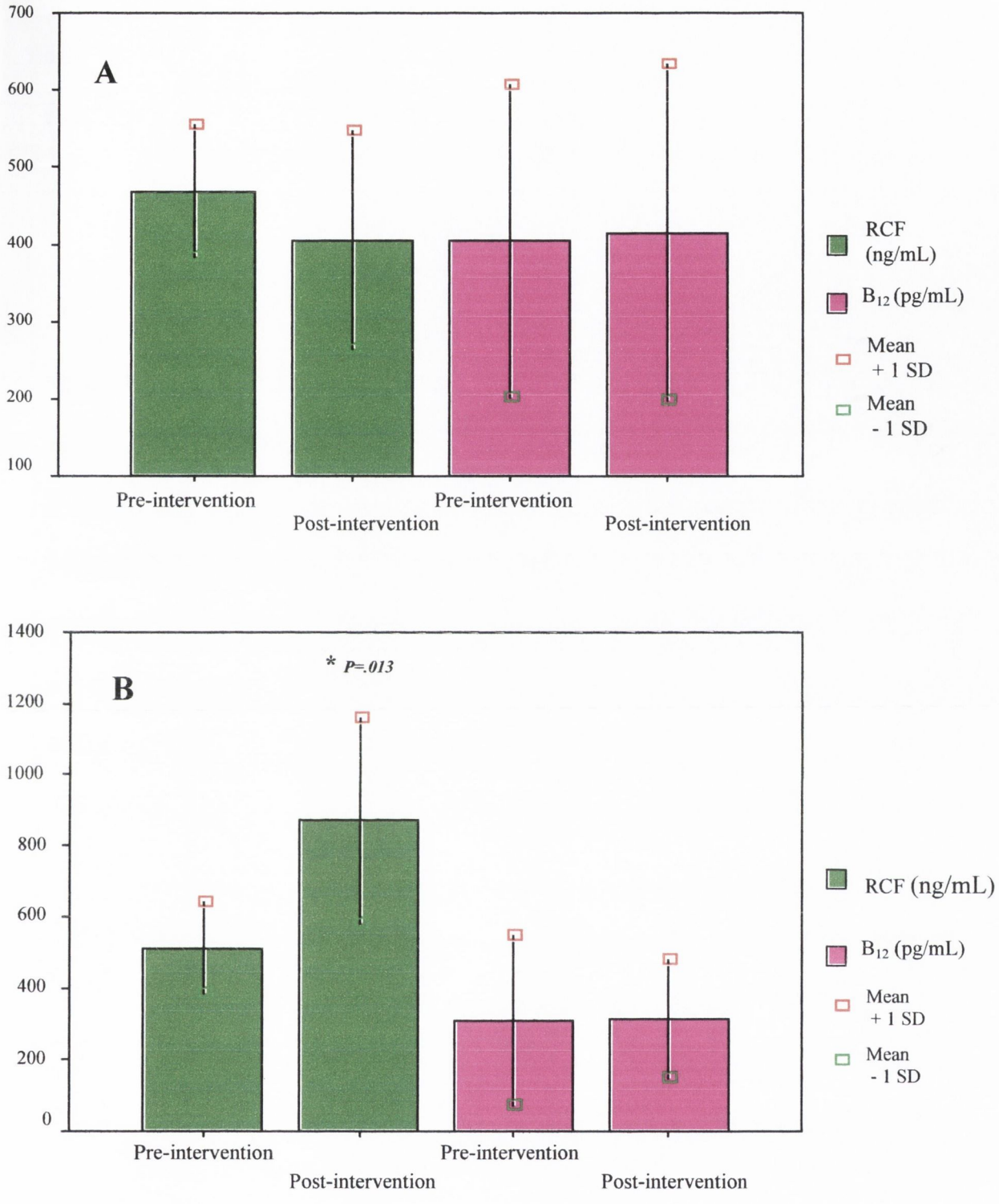


Figure 6.v Red Cell Folate and Vitamin B_{12} pre- and post-intervention represented as histographs with error bars.

A. placebo group B. folic acid supplement group

Table 6.iv Levels of significance following analysis of pre- and post-intervention data using a paired Student's t-test.

	Placebo Significance (2-tailed)	*Folic Acid* Significance (2-tailed)
Serum Folate	0.098	<0.001**
Red Cell Folate	0.571	0.013*
Homocysteine	0.348	0.032*
Vitamin B_{12}	0.759	0.899
Polyp Site Cell Folate	0.389	0.011*
Polyp Site UG Ratio	0.017*	<0.001**
Polyp Site HH Ratio	0.229	0.179
Ajacent Site Cell Folate[1]	0.406	0.186
Ajacent Site UG Ratio[1]	0.093	0.043*
Ajacent Site HH Ratio[1]	0.049*	0.015*
Normal Site Cell Folate	0.973	0.564
Normal Site UG Ratio	0.846	0.113
Normal Site HH Ratio	0.835	0.433

iii. Discussion

A small number of human intervention studies have been carried out that examine the influence of folic acid on various surrogate end-point biomarkers for CRC. The vast majority of trials have had small numbers of participants (n~20) and large, supraphysiological/pharmacological doses of folic acid (5-10mg/day) were used over an average of six months (Cravo *et al.* 1998;Paspatis *et al.* 1995;Kim *et al.* 1998). Though this intervention trial reported consisted of nine participants, it is part of an ongoing study where larger numbers shall be recruited. Also, having small numbers means it is usually difficult to obtain significant results, fortunately in this study that is untrue. The dose of folic acid used in the study was a physiological dose of 600μg per day, which allows the investigation of folate supplementation to occur under more "normal" conditions.

It was important that pill-taking compliance was high in such a small group. Close follow-up and pill counts were chosen and over the course of the intervention, they have proved to be effective in this group with an overall 100% compliance figure reported. The compliance can also be monitored through red cell folate status, as those not complying would not have a significant increase in red cell folate as the turnover of these cells is on a four monthly basis. All patients on the folic acid supplements had a significant increase in their red cell folate concentrations (mean increase = 359.11ng/mL, P= 0.013), while those on the placebo had no significant increase and a mean decrease of 30ng/ml was found at follow-up, as shown in table 6.iv and figure 6.v. Serum folate was, also, significantly increased in the supplementation group (mean increase = 17.1ng/mL, P<0.001), while plasma Hcy was significantly decreased (mean decrease = 1.8μmol/L, P = 0.032). Both these measures support patient compliance also, as shown in table 6.iv and figure 6.iv.

Analysis of the pre- and post-intervention biomarkers was done blinded. The values obtained from the repeat colonoscopy were compared to the relevant site, in the case of the adjacent to polyp site, it was compared to the anatomical site that matched the polyp location on repeat colonoscopy, as this was the original site for both tissue samples. The epithelial cell folate concentrations were not significantly altered in the placebo group, while in the supplemented group, there was a significant increase in the polyp site and a trend towards significance in the adjacent to polyp site at the end of the intervention period (figure 6.i). There was no significant increase in the normal tissue site during the intervention period, indicating that while the adenoma group tend to have significantly lower epithelial cell folate compared to normal individuals, this does not rule out localised areas of greater folate depletion at the site of adenomatous polyps as proposed previously (Meenan *et al.* 1997). It is these areas that appear to respond to the folic acid supplementation.

The modified comet biomarkers evaluation pre- and post-intervention produced some interesting results. For the UG modified comet as seen in figure 6.ii, both the placebo and supplemented group showed a significant decrease in UG ratio; the supplemented group had a highly significant decrease compared to the placebo group. The decrease in the placebo group is most likely due to the small number of participants but there is also a possibility that the individuals in the placebo group may have improved the quality of their diet over the six-month period as they were aware that the trial was related to folic acid and nutrition. It is feasible that other nutritional factors may have changed during the course of the trial and they may be able to exert change on DNA stability while also being involved in folate metabolism. Nutrients such as vitamin B_6, methionine or choline were not analysed for this study and could be involved in DNA stability. Other dietary factors have been implicated in CRC and some (total calorie and fat intake) have even been shown to modulate the degree of genomic DNA methylation (Cravo *et al.* 1998). Another other possibility is that the removal of the polyp at

the baseline time point removed a wide-ranging field effect associated with the adenoma, thereby leading to progressive changes with time (Einspahr *et al.* 1997).

For the HH modified comet as shown in figure 6.iii, the results were not in complete agreement with the UG comet ratios. The polyp site in the supplemented group resulted in a trend towards significance, while the adjacent site showed a significant decrease in the level of hypomethylation post-intervention versus pre-intervention and there was no significant change in the HH comet ratio for the normal site. The area of hypomethylation analysis and surrogate end point biomarkers has provided inconsistent results (Kim *et al.* 2001a;Pufulete *et al.* 2003c), this may be due to lack of total understanding of the full role of methylation in early carcinogenesis and the methylation paradox found in carcinoma of regions of hypermethylation and global hypomethylation (James *et al.* 2003).

At present, there are several large, randomised, double-blind, placebo-controlled multicentre folate chemoprevention trials ongoing in the US (National Cancer Institute communication 2003) and these may help elucidate the role of folic acid supplementation in the prevention of CRC. The current study, though limited by numbers, does point towards folic acid supplementation reducing localised folate deficiency of the epithelium and improving related biomarkers associated with carcinogenesis of the CRC.

CHAPTER 7

DISCUSSION

1. DISCUSSION

Worldwide, an estimated 875,000 cases of CRC occurred in 1996, accounting for 8.5% of all new cases of cancer (World Health Organisation 1997). Incidence rates vary approximately 20-fold around the world, with the highest rates seen in the developed world and the lowest in India. Colon cancer is the only cancer that occurs with approximately equal frequency in men and women (National Cancer Registry Ireland 2001). However, in high-incidence countries such as America, Australia, Italy and Ireland rates in men can exceed those in women by as much as 20%. Rectal cancer is up to twice as common in men as in women. The five-year survival rate following diagnosis is around 55% in the US, this figure compares to Irish rates (National Cancer Registry Ireland 1998). Rectal cancer may have a better overall survival rate where routine screening is more common. Cancer in the proximal colon is more common in women, those over 70 years of age and frequently displays MSI. Distal colon cancer appears to be more diet related and has a poorer prognosis compared to proximal colon cancer.

There are a number of establish and proposed risk factors for CRC (Potter 1999). These factors include physical activity, non-steroidal anti-inflammatory drugs, a diet rich in vegetables and, in women, hormone replacement therapy. The role of diet in the prevention of CRC is complex; in particular vegetables have been cited as having many potentially beneficial agents such as anti-oxidants, other bioactive compounds, dietary fibre and folate. The interest in folate has extended beyond the natural folate content of foods to include the use of supplementary folic acid.

The role of folate in the pathogenesis of several disorders in humans, such as macrocytic anaemia, cardiovascular disease (The Homocysteine Studies Collaboration 2002), neural tube defects and other congenital defects (MRC Vitamin Study Research Group 1991), adverse pregnancy outcomes (Vollset *et al.* 2000) and neuopsychiatric disorders (Seshadri *et*

al. 2002) has emerged in recent times. The expanding role of nutrition in public health and disease prevention has brought folate to the forefront of public attention. The decision of the Food and Drug Administration (U.S.Food and Drug Administration 1996) to issue a regulation stating the all flour and uncooked cereal grain within the US to be fortified with folic acid (140μg/100g) from January 1998 was a result of the substantial evidence for a protective effect of folate in prevention of NTD's. Mandatory fortification has also been instigated in Canada, Chile and other countries; however, Europe to date remains resistant to the idea.

An accumulating body of evidence has been gathered over the past decade suggesting that folate has a role in the development and prevention of certain cancers including CRC, pulmonary, pancreatic, cervical, breast, oesophageal, gastric and leukaemia (Kim 1999). These studies have suggested an inverse relationship between folate status and the risk of these cancers. However, neither the precise pathways through which folate acts nor the strength of the relationship have been clearly established in these diseases. The potential role of folate in CRC has been the most widely studied and appears to be the most promising.

It was folate's role in DNA synthesis and methylation that provided the mechanistic basis for the observed increase in cancer risk associated with inadequate folate intake (Choi and Mason 2002). Folate, as 5,10-methylene THF, donates a methyl group to uracil converting it to thymine, which is used for DNA synthesis and repair. However, if folate is limited, uracil misincorporation into DNA may occur. This is in itself mutagenic but DNA instability may also occur via a different mechanism. As the cell attempts to repair itself, it breaks the DNA molecule to excise the uracil. If folate is continually limited, imbalances in deoxynucleotide triphosphates in the precursor pool occur. Uracil is misincorporated and repaired in a "catastrophic repair cycle", which may lead to double-strand breaks, chromosomal damage and cancer (Reidy 1988). Folate in the form of 5-methyl THF serves as methyl donor in the remethylation of Hcy to methionine, which in turn is converted to SAM. SAM methylates

specific cytosines in DNA, this regulates gene transcription. As a consequence of folate deficiency, cellular SAM becomes depleted , which in turn induces DNA hypomethylation and potentially induces proto-oncogene expression leading to cancer (Counts and Goodman 1995b). Other nutrients, including methionine, vitamin B_{12}, choline and vitamin B_6, interact metabolically with folate in these processes and they, too, may influence cancer risk. The positive association between alcohol consumption and cancer risk may relate to alcohol's role as a folate antagonist and its suppressive effects on methyl group metabolism (Halsted *et al.* 2002).

The risk of developing colorectal adenomas may be significantly modified by an individual's folate status (Ulrich *et al.* 1999b). The association between folate intake and risk of developing colorectal adenomas was evaluated in the Nurses' Health Study and the Health Professionals' Follow-Up Study (Giovannucci *et al.* 1993). They found that risk was 30-40% lower in individuals whose median folate intake were 711μg/day for women and 847μg/day for men compared to those with the lowest intakes. Most individuals in the upper group were multivitamin users that contained folic acid. These results have been found to be similar in various other studies (Benito *et al.* 1991;Bird *et al.* 1995;Tseng *et al.* 1996) and the protective effect of a higher folate intake has also been shown to protective against CRC in a large number of case-control and cohort studies. The long-term use of multivitamins containing folic acid on CRC risk was examined in the Nurses' Health Study also and found that an individuals risk of developing CRC was significantly reduced after >15 years of multivitamin use and that the group that benefited the most was those with the lowest level of dietary folate (Giovannucci *et al.* 1998). Similar findings have been found in other studies looking at multivitamin usage and CRC (Ma *et al.* 1997;Kato *et al.* 1999;Fuchs *et al.* 2002).

Recently, molecular epidemiological studies have shown that the common polymorphism MTHFR C677T may modulate CRC risk. MTHFR is a critical enzyme in folate

metabolism that catalyses the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate; required for the production of methionine and SAM, the methyl donor for DNA. The prevalence of homozygous individuals is up to 20% in certain populations (Frosst *et al.* 1995b). By determining plasma tHcy levels, a strong gene-nutrient interaction was demonstrated in the phenotypic expression of this polymorphism (Jacques *et al.* 1996). Homozygotes with inadequate folate status show elevated tHcy concentrations. MTHFR plays an important role in DNA synthesis, maintenance of nucleotide pool balance and DNA methylation (Ma *et al.* 1997;Ulrich *et al.* 1999c). When folate status is low in those homozygous for the polymorphism, it is associated with an increase in tHcy and DNA hypomethylation (Friso *et al.* 2002). Homozygosity for the MTHFR polymorphism has been associated with a significant reduction in risk for CRC (Ulrich *et al.* 1999d;Giovannucci 2002). Data from the studies examined indicate that individuals homozygous for the MTHFR polymorphism were especially sensitive to the carcinogenic effect of alcohol (Chen *et al.* 1999). Individuals homozygous for the MTHFR genotype appear to be at relatively low risk if they have a high folate and low alcohol diet (Levine *et al.* 2000). Results from the whole biopsy method versus the epithelial cell folate method suggested that the concentration of epithelial cell folate in replete TT individuals was higher than CC's, though the results were not significant.

The relationship between blood levels of folate and the risk of CRC and adenoma is less well defined than that of dietary folate intake and the risk of colorectal neoplasia (Giovannucci 2002). Collectively, the retrospective studies suggest that a moderate reduction in folate status without overt clinical deficiency was sufficient to enhance the risk of developing CRC (Kim 1999).

Several small randomised, placebo-controlled intervention studies have examined the effect of folate supplementation on CRC risk. The vast majority of trials have had small

numbers of participants (n~20) and large, supraphysiological / pharmacological doses of folic acid (5-10mg/day) were used over an average of six months (Cravo *et al.* 1998;Paspatis *et al.* 1995;Kim *et al.* 1998). The results from these studies have been inconclusive as different endpoints have been used such as adenoma recurrence, genomic DNA hypomethylation, p53 strand breaks, rectal mucosal cell proliferation. These various endpoints prevent true comparision of results.

The comet assay in its modified forms provided a novel method of examining DNA damage in the context of misincorporation of BrdUrd in newly replicating DNA, misincorporation of uracil and global hypomethylation of DNA.

In the case of the BrdUrd comet assay, an increase in the comet tail length was indicative of a delay in DNA maturation, because of stalling of the replication fork at DNA lesions or defects in post-replicative repair. The BrdUrd comet assay proved to be suitable for the measurement of DNA maturation and integrity in small numbers of primary epithelial cells derived from colonic biopsies. The results of the small study group showed a low level of variability within the normal colonic tissue samples, while it was possible to detect elevated BrdUrd comets from histologically abnormal tissue when compared to that of normal tissue and this indicated stalled maturation. This may be due to a high level of intrinisic DNA damage or defective post-replication repair machinery in the tumour cells (Johnson *et al.* 1994). The pulse-chase experiments supported the ability of the BrdUrd comet assay to assess DNA maturation and determine the replicative integrity of human primary epithelial cells.

Uracil misincorporation is proposed to be one of the main pathways through which folate may be involved in CRC development. By using the bacterial enzyme uracil DNA glycosylase, it has become possible to detect uracil misincorporation in human DN; the method has been previously validated in human lymphocytes and human immortalised colon epithelial cells (Duthie and Hawdon 1998;Duthie *et al.* 2000b). No previous study has been performed

on the modified comet assay looking at hypomethylation using the combination of *HpaI* and *HhaII* DNA methyltransferases; though there is a previous publication using the *Sss1* methylase enzyme (Duthie *et al.* 2000b) but the results were not conclusive for that comet assay.

The FISH-comet assay used a fluorescently labeled DNA probe that hybridised to a specific gene sequence. The fluorescently labeled DNA probe used in this study was specific for a 200kb region of chromosome 17; this area includes the human *p53* gene. This was chosen as *p53* is the single gene most frequently altered in human malignancies and in about 70% CRC (Goh *et al.* 1994). The protein has a key role in the cellular defense against DNA damage and appears to function as a "guardian of the genome"(Lane 1992). Observations in folate-deficient rats have confirmed that dietary deprivation of folate is a sufficient force to produce hypomethylation within the "hypermutable region" of the *p53* gene (Kim *et al.* 1997). Subsequent studies have demonstrated that this depletion also decreases steady-state concentrations of *p53* mRNA (Kim *et al.* 2000). Consistent with the concept that these changes are involved in carcinogenesis are observations, which indicate that hypomethylation within exon 8 of the colonic *p53* gene, was also observed in animal models of carcinogenesis. The probe was used in combination with the methylation-specific modified comet assay, which meant that detection of *p53*-specific areas of hypomethylation could be analysed. This novel assay improves the specificity of the hypomethylation comet; it permitted the visualization of one of the key genes in CRC and to see in individuals whether it was hypomethylated or not.

At a cellular level, little is known about the mechanisms that regulate intracellular folate concentrations. There is evidence that cellular folate concentrations are tightly regulated folate degradation, and animal studies have demonstrated that increased rates of folate catabolism occur during states of rapid cell proliferation including cancer and pregnancy (Suh *et al.* 2001). Interestingly, cells are highly susceptible to folate deficiency during states of

increased folate turnover, because they do not accumulate excess folate relative to their binding capacity. Therefore it is apparent that physiological mechanisms exist that can trigger localised functional deficiency in the absence of a "generalised" dietary or systemic deficiency. The epithelial cell folate analysis supports this theory; a stepwise decrease was observed in hyperplastic and adenomatous polyp patients. The results for the intervention trial epithelial cell folate concentrations were significant for the supplement group only in the polyp site, with a trend towards significance in the adjacent to polyp area but no significant change in the normal site. This also supports a localised deficiency in the affected area. The mean decrease found in total cellular folate agreed with a previous publication showing a global reduction in tissue folate in colonic tissue of patients with adenomatous polyps, in that publication the alternative method of tissue folate estimation was used (Kim *et al.* 1998), suggesting that the reduced folate concentration in the mucosa of patients with adenomatous polyps may coincide with a metabolic defect in folate metabolism.

The hyperplastic polyps sampled in the case-control study were predominantly large in size and a large percentage came from the right side of the colon and it is these polyps that appear to have malignant potential (Rashid *et al.* 2000b;Hawkins and Ward 2001a;Morimoto *et al.* 2002a). The genetic alterations of the type seen in neoplasms have been reported in hyperplastic polyps (Rashid *et al.* 2000b) and epidemiological evidence shows that the risk factors for hyperplastic polyps were also risk factors for adenomas and carcinomas (Martinez *et al.* 1997a). It may be possible that the relationship between epithelial cell folate and systemic markers of folate status was stronger in this group owing to the importance of folate in the very early stages of neoplasia. A positive correlation was found between the UG comet ratio and the serum folate concentrations as well as strong trends towards significance with red cell folate and Hcy in the hyperplastic polyp group. A previous study using a folate- and methyl-deficient rat model was able to demonstrate that increased uracil misincorporation was preceded by an

increase in DNA strand breaks and abasic sites in preneoplastic liver (Pogribny *et al.* 1997b). Uracil misincorporation is associated with increased intracellular dUTP/dTTP (Goulian *et al.* 1980). An increase in dUTP/dTTP in vitro has been shown to promote folate fragile site expression, DNA strand breakage, error-prone DNA repair and mutagenesis (Kunz 1988). It is possible that UG comets in hyperplastic polyps as a biomarker were measuring a premalignant process that is of biological relevance in early DNA damage, especially as the polyp site drove the negative correlation.

The data from the case-control study illustrated a higher level of DNA damage was seen in the abnormal compared to the normal tissue as has been found in other comet work (Duthie and Hawdon 1998). Further analysis of variation of modified comet ratios per site displayed a stepwise increase in uracil misincorporation or hypomethylation in the case group; this was not seen in the control group. Uracil misincorporation has been previously shown to be higher in the leukocytes of folate-deficient individuals (Blount *et al.* 1997) as has hypomethylation (Jacob *et al.* 1998); though the evidence for hypomethylation is less convincing (Cravo *et al.* 1994;Fenech *et al.* 1998) as methylation status is affected by more factors than uracil misincorporation. HH modified comet ratios showed near significance when the variation between sites in the case group was examined, this was driven by the adenoma group. This would suggest that the alterations in methylation status occur later in the adenoma-to-carcinoma sequence than misincorporation of uracil. This has been examined in a recent publication (James *et al.* 2003) that hypothesised due to DNA methyltransferase's ancestral role as a DNA repair enzyme, it will bind with higher affinity to DNA strand breaks, gaps, abasic sites and uracil than it does to its hemimethylated CpG sites (Kim *et al.* 1997). Experiments have been performed that found lesion-containing DNA was less efficiently methylated than lesion-free DNA from folate/methyl-deficient lymphocytes and that an increase in DNA strand breaks preceded DNA hypomethylation (Pogribna *et al.* 1999). The

area of hypomethylation analysis and surrogate end point biomarkers has provided inconsistent results (Kim *et al.* 2001a;Pufulete *et al.* 2003d), this may be due to lack of total understanding of the full role of methylation in early carcinogenesis and the methylation paradox found in carcinoma of regions of hypermethylation and global hypomethylation (James *et al.* 2003).

The effect of folic acid supplementation on UG and HH modified comet ratios was significant in the small group examined. There was a significant improvement in supplement group for UG and HH comets; there was a significant improvement in the UG comet ratio for the polyp site in the control group also. The most likely explanation for this was that the removal of the polyp at the baseline time point removed a wide-ranging field effect associated with the adenoma, thereby leading to progressive changes with time (Einspahr *et al.* 1997). The improvement in uracil misincorporation and hypomethylation supports other studies that have found an improvement in these biomarkers following supplementation (Jacob *et al.* 1998;Kim *et al.* 2001a;Duthie *et al.* 2000b).

In conclusion, this thesis reports a modified ion-chelation method that permits the isolation of large numbers of viable colonic epithelial cells from standard endoscopic biopsies, free of lamina propria contamination. This technique was compared with the whole biopsy analysis technique in a group of normal individuals. The epithelial cell isolation achieved the same correlation with systemic markers as the whole biopsy technique, indicating that it is a valid methodology.

Applying the ion-chelation technique permitted addition of the modified comet assay; which enabled the examination of the extent of uracil misincorporation and hypomethylation present in both normal and abnormal tissue. A case-control study was performed that showed a significant decrease in the epithelial cell folate concentration and significant correlations between systemic markers and localised epithelial cell folate in cases. Although there was no significant difference between groups for the comet biomarkers; there was a significant

increase in UG and HH ratios between the polyp and the normal tissue. This indicates a higher level of misincorporation of uracil and hypomethylation in the abnormal polyp tissue in conjuction with a lower cellular folate.

The intervention trial examined the potential role of a physiological dose of folic acid over a six-month period in subjects diagnosed with having adenomatous polyps at the initial colonoscopy. The trial was placebo-controlled, double-blind and randomised. The placebo group showed no significant change in either epithelial cell folate concentrations, systemic markers or most modified comet biomarkers for any site. The supplemented group displayed a significant increase in serum, red cell folate and epithelial cell folate for the polyp site; while there was a significant decrease in plasma tHcy and UG/HH ratios for the abnormal site. This data together strengthens the argument that folate deficiency plays a role in the development of CRC and that folic acid may serve as a chemopreventative agent in the future.

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