

Effects of Synbiotics on Cancer Risk Biomarkers

A Thesis Presented to the National University of Ireland for the
Degree of Doctor of Philosophy

by

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Publications and Presentations Associated with this Thesis

Antitumorigenic activity of the prebiotic inulin enriched with oligofructose in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* on azoxymethane-induced colon carcinogenesis in rats.

Femia A, Luceri C, Dolara P, Giannini A, Biggeri A, Salvadori M, Clune Y, Collins J K, Paglierania M, Caderni G.
Carcinogenesis, 2002

Synbiotics and Colon Cancer

Bennett M.F. & Clune Y.E., Shanahan F., O'Sullivan G., Collins J.K
Functional foods; ageing and degenerative diseases. Edited by C. Remacle and B. Reusens. Woodhead Publishing, Cambridge, England, 2004.

The SYNCAN project: goals, set-up, first results and settings of the human intervention study

Van Loo, Jan; Clune, Yvonne; Bennett, Mary; Collins, John Kevin

British Journal of Nutrition Volume 93, Supplement 1, April 2005

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Week, New Orleans, May 2004

The influence of synbiotic consumption on proliferation in the mucosa of polypectomised human subjects

Yvonne Clune, Maddalena Salvadori, Michael Bennett, Michael O'Riordan, Colum Dunne, Gerald O'Sullivan, John Kevin Collins, Giovanna Caderni

Synbiotic consumption modulates the genotoxic potential of faecal water (FW) and genotoxic damage in the gut mucosa.

Yvonne Clune, Annett Klinder, Michael Bennett, Michael O'Riordan, Colum Dunne, Gerald O'Sullivan, John Kevin Collins, Beatrice L. Pool-Zobel

Presented at the European Association for Cancer Research, Innsbruck,

Austria, July 2004

Colorectal gene expression in cancer and polyp subjects and changes associated with synbiotic consumption.

Mary Bennett & Yvonne Clune, John Mac Sharry, Michael Bennett, Michael O’Riordan, Colum Dunne, Gerald O’Sullivan, Fergus Shanahan, John Kevin Collins

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Czech Republic, September 2004

The influence of synbiotic consumption on cancer risk biomarkers in previously resected colon cancer subjects

Yvonne Clune, Karen Meaney, Maurice O’Donoghue, Annett Klinder, Monika Roller, Pernilla Karlsson, Michael Bennett, Michael O’Riordan, Colum Dunne, Gerald O’Sullivan, Joseph Rafter, Bernhard Watzl, Gerhard Rechkemmer, Beatrice L Pool-Zobel, John Kevin Collins

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Seattle, USA, October, 2004

Synbiotic consumption modulates cancer risk biomarkers in colon cancer and polypectomised human subjects

Yvonne Clune, Karen Meaney, Maurice O’Donoghue, Annett Klinder, Monika Roller, Pernilla Karlsson, Michael Bennett, Michael O’Riordan, Colum Dunne, Gerald O’Sullivan, Joseph Rafter, Bernhard Watzl, Gerhard Rechkemmer, Beatrice L Pool-Zobel, John Kevin Collins

Abstract

Colorectal cancer (CRC) is the fourth most common cause of death from cancer in the world and second most common (behind lung cancer) in developed countries. In recent years there has been much interest in the potential use of prebiotics, probiotics and synbiotics in the prevention and treatment of CRC. We have previously shown that synbiotic consumption in Azoxymethane treated rats modulates the immune system, influences the genotoxic potential of caecal contents and reduces the number of colonic tumours compared to control rats who did not receive the synbiotic. The aim of the current study was to identify biomarkers suitable for use as cancer risk markers and as intervention markers. A second aim was to determine the influence of synbiotic consumption on cancer risk biomarkers such as *in vivo* colonic mucosal proliferation and genotoxic damage along with examining the genotoxic, cytotoxic and tumour promoting potential of faecal water (FW).

Synbiotic consumption altered the composition of the gastrointestinal flora and reduced *in vivo* genotoxic damage and the genotoxic potential of FW in cancer and polyp subjects. Synbiotic consumption also reduced the proliferative activity in the colonic mucosa in polyp subjects. In both cancer and polyp subjects gene expression in the colonic mucosa was modulated in synbiotic consuming subjects.

In this and other studies the activity of natural killer cells, the level of PGE₂ in FW, IL-12 production by PBMCs, genotoxic damage in the colonic mucosa and the tumour promoting activities of FW have been identified as possible biomarkers of cancer risk. Future large scale studies investigating these parameters in healthy and diseased individuals are needed to confirm the suitability of these markers in assessing cancer risk and the role of synbiotics in modulating them.

Chapter 1

Literature Review

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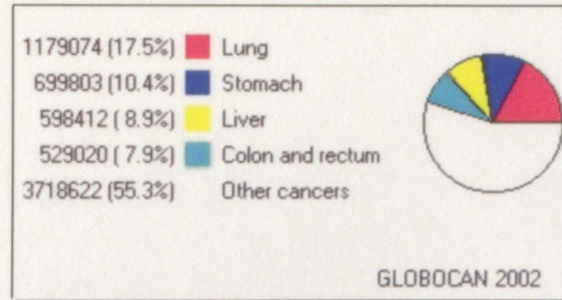


Figure 1 Cancer deaths worldwide (male & female)

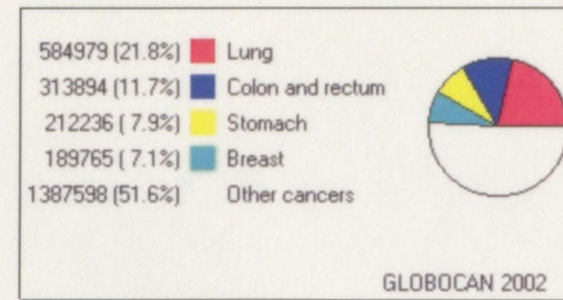


Figure 2 Cancer deaths in developed countries (male & female)

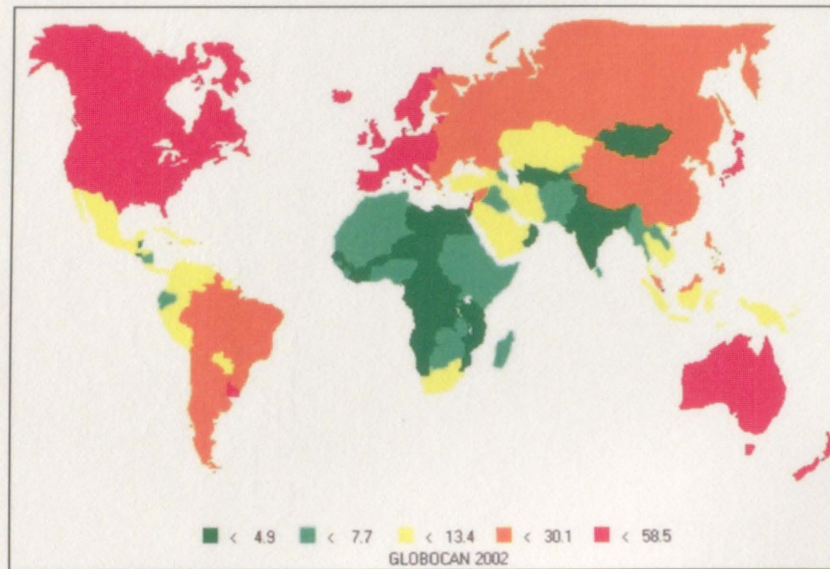


Figure 3 Colorectal cancer incidence worldwide (male)

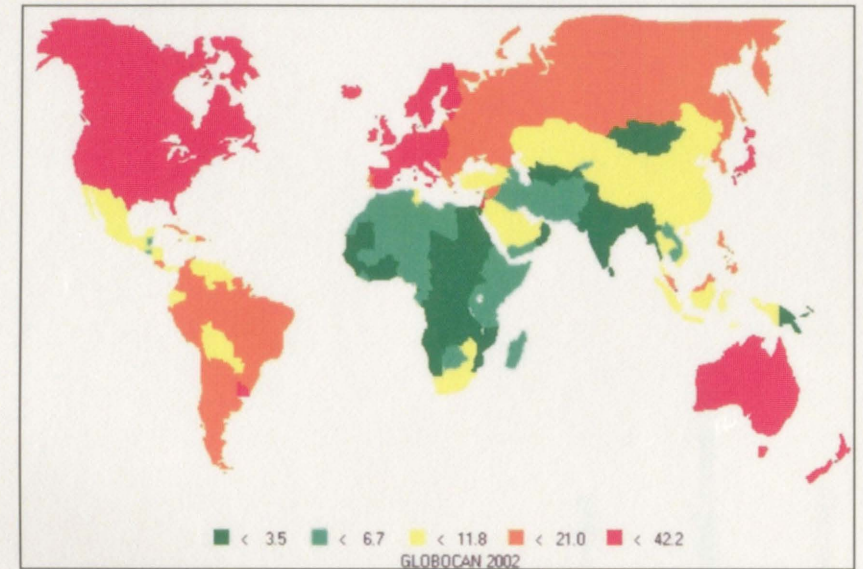


Figure 4 Colorectal cancer incidence worldwide (female)

1 Introduction

Colorectal cancer (CRC) is the fourth most common cause of death from cancer in the world and second most common (behind lung cancer) in developed countries (*Figures 1 & 2*). The incidence of colorectal cancer increases steeply after the age of 40 and exponentially thereafter. An individual's lifetime risk of developing colorectal cancer is estimated at five percent but this figure rises rapidly with age: by the age of 70, approximately half of the Western population will have developed an adenoma (Fodde *et al.* 2001). Individuals with first-degree relatives diagnosed with colon cancer or adenomatous polyps, have a risk two to three times greater than this.

Colorectal cancer is much more common in developed countries than in developing countries (*Figures 3 & 4*). Most recently available statistics show that the incidence / mortality of CRC in developed countries is 40.0 / 17.7 (males) and 36.6 / 12.3 (females) per 100,000 people compared to rates in developing countries of 10.2 / 6.2 (males) and 7.7 / 4.7 (females), (Ferlay *et al.* 2004). Several hypotheses exist to explain this dramatic difference, including dietary intake and infectious challenges in childhood. The interaction of bacterial endotoxins and enterotoxins with the intestinal epithelium in early life is thought to influence colon carcinogenesis, reducing the risk in later life and this may explain the differences between these populations, (Pitari *et al.* 2003).

EUCAN statistics from 1998 show the European Union age standardised (standardised to world figures) incidence of colorectal cancer at 29.36 cases per 100,000 and mortality at 13.63 per 100,000 cases. UK rates were 27.61 (incidence) and 13.52 (mortality) per 100,000 cases while in Ireland rates were above the EU

Table 1: Genetic syndromes carrying an increased risk for colorectal cancer

Syndrome	Characteristics	Reference
Familial adenomatous polyposis (FAP)	An autosomal dominant syndrome caused by germline mutation of APC (adenomatous polyposis coli) gene. Multiple colonic adenomas occur at an early age, occasionally during the preteen years, and proliferate throughout the colon, with malignant degeneration in most patients by the age of 40 to 50 years.	Nishisho <i>et al.</i> (1991) Kinzler <i>et al.</i> (1991) Groden <i>et al.</i> (1991)
Hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch Syndrome	An autosomal dominant disorder accounts for about 3-5% of all CRC cases, caused by mutation of one of the DNA mismatch repair genes. Colorectal cancer usually arises from a single colorectal lesion in the absence of polyposis. Individuals with a HNPCC gene mutation have approx. a 70-80% lifetime risk of developing colon or rectal cancer. Accelerated carcinogenesis occurs where colonic adenomas may emerge as carcinomas within 2-3 yrs, compared to 8-10 yrs.	Mecklin (1987); Jass & Stewart (1992); Lynch <i>et al.</i> (1993); Scapoli <i>et al.</i> (1994); Vasen <i>et al.</i> (1995, 1996) Rodriguez-Bigas <i>et al.</i> (1997); Boland (1998); Aaltonen <i>et al.</i> (1998); Lynch & de la Chapelle (1999)
Li-Fraumeni Syndrome (LFS)	Familial dominantly inherited cancer syndrome characterised by a wide spectrum of neoplasms occurring in children and young adults. Cancer may develop at a very young age, individuals often develop multiple primary tumours, and there is a high frequency of cancer within the family. Germline mutations in the p53 gene are the major cause of the predisposition to cancer in LFS. It is rare that colon cancers are the first tumour to develop, however, they may occur as second or third cancers	Srivastava <i>et al.</i> (1990) Li & Fraumeni (1969) Li <i>et al.</i> (1988)
Hamartomatous polyposis syndromes	Peutz-Jeghers syndrome consists of perioral pigmentation, upper and lower gastrointestinal hamartomatous lesions, small bowel and pancreatic cancers, colorectal cancer and sex-cord tumours of the ovary. The mutant gene is LKB1. Other syndromes of this type are familial juvenile polyposis, Cowden's disease, and Bannayan-Ruvalcaba-Riley syndrome.	Hemminki <i>et al.</i> (1998)

average at 34.12 (incidence) and 15.22 (mortality) per 100,000 cases (Ferlay *et al.* 1999).

Despite progress in chemotherapy, radiation therapy, and surgery, little change has occurred in the survival of patients with colorectal cancer. Currently the 5-year survival rate in Europe is approximately 50%, (Campo *et al.* 2004). This has lead to increased research into the aetiology of colorectal cancer with the aim of improving and developing early detection and prevention strategies.

2 Aetiology of Colorectal Cancer (CRC)

Genetic, experimental and epidemiological studies suggest that colorectal cancer results from a complex interaction between inherited susceptibility and environmental factors. Epidemiological studies have shown that changes in cancer incidence occur in migrant populations moving from environments of low cancer incidence to high cancer incidence, clearly demonstrating that environmental factors play an extremely important role in the aetiology of colorectal cancer, (Grandics 2003). The influence of genetic factors is equally well documented as colorectal cancers occur more frequently in certain families and there are several rare genetic syndromes that carry a markedly increased risk for CRC (*Table 1*), (Potter 1999).

In the 1980's the adenoma-carcinoma sequence was first postulated by Vogelstein, in which the steps required for the development of cancer involve the mutational activation of an oncogene or oncogenes coupled with the loss of several genes that normally suppress tumourigenesis. This process was thought to account for up to

Figure 5: The adenoma-carcinoma sequence for the development of colorectal cancer. Adapted from Vogelstein & Kinzler (1993).

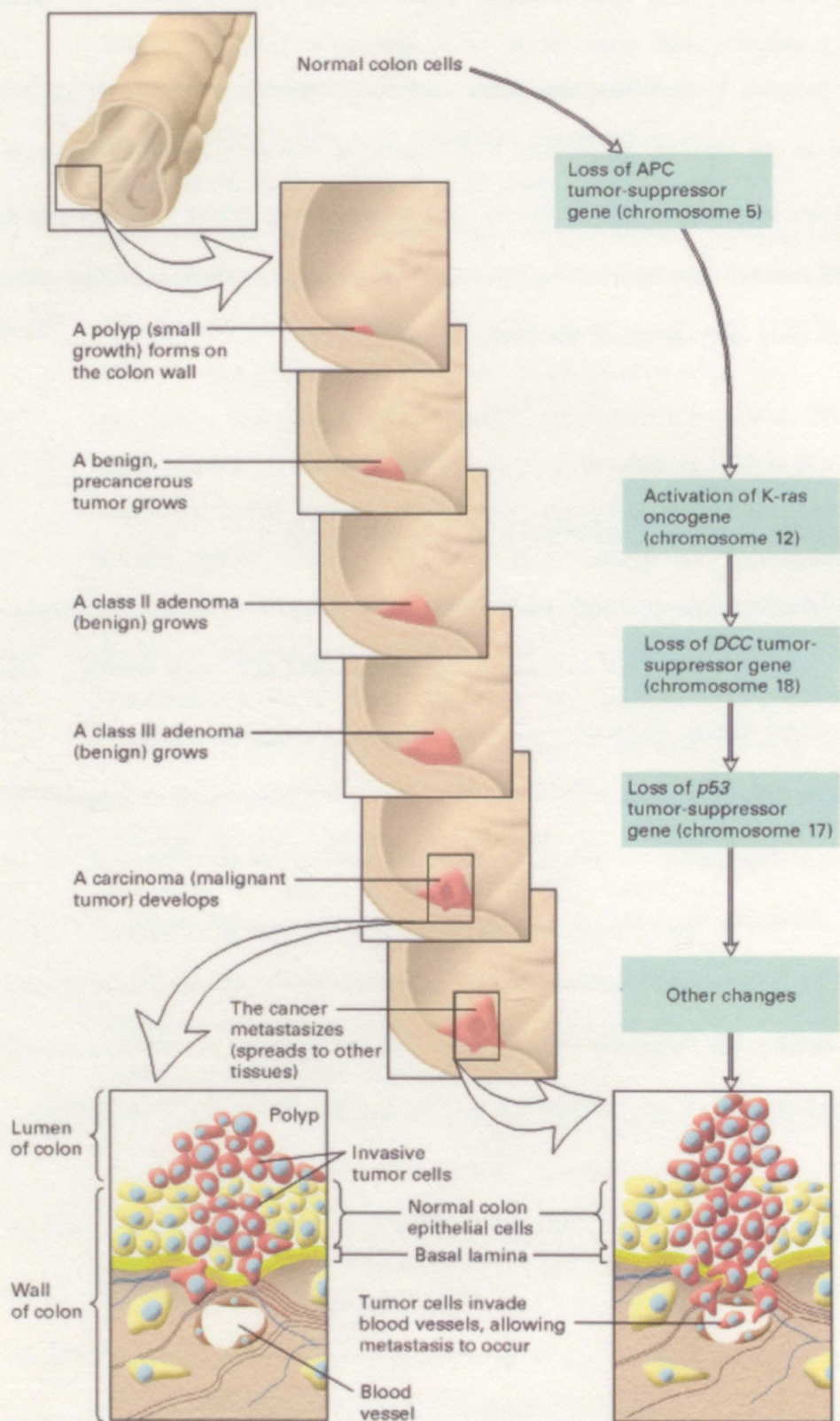
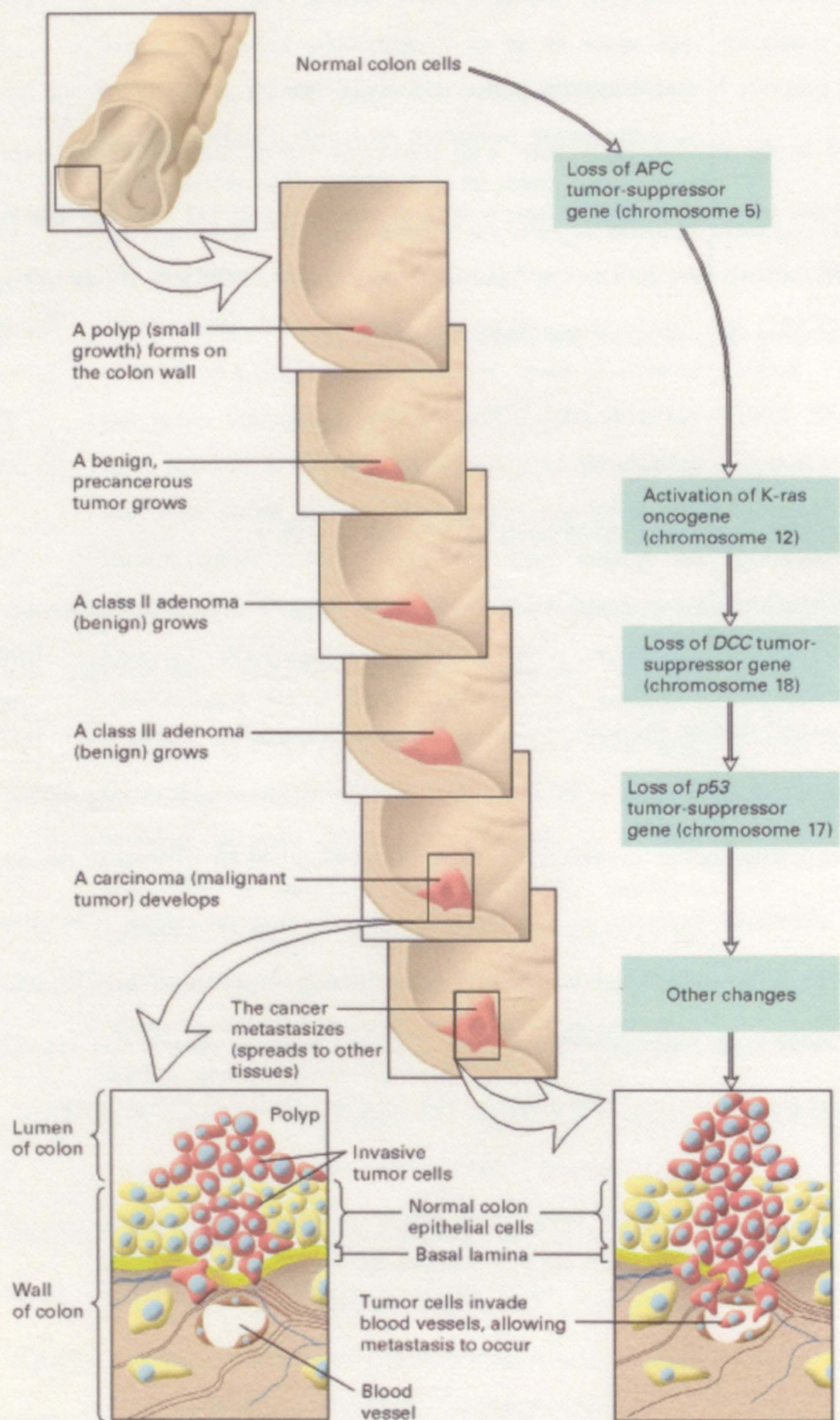


Figure 5: The adenoma-carcinoma sequence for the development of colorectal cancer. Adapted from Vogelstein & Kinzler (1993).



90% of cases of colorectal cancer, (Vogelstein *et al.* 1988). This cascade which describes the multi-step progression from normal epithelium to hyperproliferative epithelium, to focally dysplastic crypts, to tubular adenoma, to dysplastic or villous adenoma and finally onto invasive carcinoma and metastasis gave credence to the commonly held theory that most if not all malignant carcinomas arise from pre-existing benign tumours. The US National Polyp Study provided evidence supporting the Vogelstein sequence. This evidence included; the common finding of foci of cancer in adenomatous polyps, the similar colonic distribution of adenomas and cancer, the high correlation between the incidence of cancer and the prevalence of adenomas, and the reduction in the incidence of colon cancer seen following adenoma removal, (Winawer *et al.* 1993).

Following on from their initial work on the adenoma-carcinoma sequence, Fearon & Vogelstein (1990) identified the adenomatous polyposis coli (APC) gene, other genes on chromosome 18q, and the p53 gene as the genes in which mutations or epigenetic dysregulation contribute to evolution of colon cancer. Based on these findings they proposed the multi-gene clonal evolution and selection model for the initiation and progression of colon cancer. This multi-step model involves biallelic inactivation mutations in the APC tumour suppressor gene as an early event leading to the development of polyps, followed by inactivating mutations in the oncogene K-ras in the advancing adenoma, and biallelic inactivating mutations of p53 during the transition to malignancy in most colorectal cancers (*Figure 5*), (Fearon & Vogelstein 1990; Vogelstein *et al.* 1988). The types of genomic alterations included point mutations, and allelic loss events known as loss of heterozygosity (LOH). This cascade is the classical sequence of events, however, not all mutations are necessary

for tumour formation and although genetic alterations often occur according to a preferred sequence it is the total accumulation of these changes, rather than their order with respect to one other, which determines the biological properties of the tumour.

As mentioned earlier the aetiology of colorectal cancer is heterogeneous, with environmental or genetic factors playing varying key roles in different patients, (Potter 1999). Using the Swedish Family-Cancer Database containing 9.6 million individuals, Czene *et al.* (2002) estimated the genetic and environmental components in fifteen common cancers. They estimated that 13% of colon cancer could be associated with genetic effects. Thyroid cancer was by far the most associated with genetics at 53%. The results of this study showed that environment plays a principal causative role in cancer in all studied sites except for thyroid.

2.1 Microsatellite instability (MSI)

Exogenous and endogenous reagents constantly modify the genomic DNA of all organisms, (Friedberg *et al.* 1995). This modification often occurs during the process of DNA metabolism, commonly during DNA replication where errors may be introduced into newly synthesized DNA strands. Over time numerous processes aimed at maintaining the integrity of the genome have evolved including base excision repair, nucleotide excision repair, mismatch repair or recombination repair. Aaltonen *et al.* (1993) described a malfunction of the mismatch repair mechanism in tumours of the colon, endometrium, ovary and other organs targeted by the hereditary non-polyposis syndrome in humans. Faults in the mismatch repair process leads to microsatellite instability (MSI). A microsatellite is a repeated DNA

sequence in which the length of the core sequence that is repeated is small (one to five nucleotides). During DNA replication repetitive subunits may be misaligned leading to elongation or contraction and these abnormalities are usually repaired by the mismatch repair mechanism. When errors occur in the mismatch repair process new microsatellite alleles emerge which have lengths that differ from the host's two parent (i.e. normal tissue) alleles for that microsatellite. MSI is found in virtually all hereditary nonpolyposis colorectal tumours, (Aaltonen *et al.* 1994). Most microsatellites occur in non-coding DNA; therefore, contractions or elongations are believed to have little or no effect on protein function. However, there are genes that have microsatellites in their coding regions, and MSI will thus lead to altered proteins.

Studies have shown that in patients with sporadic cancer, high-frequency microsatellite instability in CRC is independently predictive of a relatively favourable outcome and, in addition, reduces the likelihood of metastases, (Gryfe *et al.* 2000; Halling *et al.* 1999). An interesting study by Elsaleh *et al.* (2000) found that adjuvant chemotherapy lead to a significantly greater survival rate in patients with MSI-positive tumours.

2.2 Sporadic cancer

Although the genetic process of sporadic cancer is not understood, genes described in the Fearon and Vogelstein cascade (APC, genes on 18q, ras, and p53), have been confirmed as affected in sporadic cancers. But mismatch repair genes have also been implicated, (Lynch & de la Chapelle 2003). The average age of onset of sporadic cancer is 63 years whereas for hereditary nonpolyposis colorectal cancer (HNPCC),

a cancer syndrome characterised by microsatellite instability it is 45 years. It may well be that in these cancers the accumulation of frame shift mutations in coding microsatellites leads to a loss of function, further affecting, and perhaps speeding up the evolution of cancer.

Genetics also seems to play a role in determining the site at which a tumour will develop. Proximal colorectal cancers are more likely to have diploid DNA, possess microsatellite instability; harbour mutations in mismatch repair (MMR) genes, and behave less aggressively, as in HNPCC. Colorectal cancers involving the distal colon are more likely to have aneuploid DNA, harbour mutations in the APC, p53 or K-ras genes and behave more aggressively as in sporadic cancer and also FAP, (Lynch & de la Chapelle 1999).

2.3 p53

p53 was first identified in 1979 as a protein that complexes with viral oncogenes such as SV40 large T-antigen, adenovirus E1B and papilloma virus E6, (Levine 1990). p53, or part of it's regulatory circuit, is functionally inactivated in almost all cancers highlighting it's importance in preventing tumourigenesis (Jin & Levine 2001), and p53 is referred to as the 'guardian of the genome' because of it's role in checkpoint control following DNA damage (Lane 1992).

DNA may be damaged due to UV radiation, (sunlight) or chemical carcinogens (e.g. diet). Such damage causes p53 induction and the levels of p53 protein in the cell increases. Increased p53 halts cell division in the late G1 phase, allowing the cell time to repair its DNA, and to ensure that a correct copy of each gene is present

before allowing the cell cycle to continue, (Kuerbitz *et al.* 1992). Increased p53 has been shown to cause apoptosis in some cells however the reason for this is not yet understood, (Yonish-Rouach *et al.* 1991). p53 therefore ensures that the correct genetic information is present in the cell before allowing the cell to replicate.

The role of p53 in tumourigenesis is highlighted in the Li-Fraumeni Syndrome (LFS), where the major cause of the predisposition to cancer in LFS families has been identified as germline mutations in the p53 gene, (Srivastava *et al.* 1990). p53 itself is not mutated in all cancers; however mutations may be present in genes involved in the p53 regulatory circuit. p53 regulates its own expression through feedback loops. One such regulatory mechanism works through a gene called mdm2. Following DNA repair p53 activates mdm2, and the mdm2 protein product binds to and inhibits p53 allowing the cell cycle to continue, (Wu *et al.* 1993). To induce apoptosis p53 transactivates the bax gene. There are many genes involved in the p53 functional circuit, the impairment of whose function may play a part in tumourigenesis, (Miyashita & Reed 1995).

2.4 Multiple Drug Resistance (MDR)

Efflux mechanisms have become the subject of considerable interest in recent studies of gut mucosal defence and the pharmacokinetics of drugs. The multi-drug resistance pump (MDR1), also known as P- glycoprotein 170 mediates the efflux mechanism in the intestinal epithelium. The MDR1 gene may have an important role in the tumourigenesis of colorectal cancer. It is hypothesised that over-expression of MDR1 may influence apoptotic pathways by secreting endogenous or xenobiotic toxic substances into the lumen or by mediating a more generic anti-apoptotic

function. The MDR1 gene product, P-glycoprotein, protects the cell against a wide variety of caspase-dependent death stimuli including fas ligand, tumour necrosis factor, and ultraviolet radiation, (Smith 1998; Smyth 1999). In colorectal tumours the expression of P- glycoprotein correlates with pathological grading of tumours, being most intense in the well-differentiated tumours and low in the poorly differentiated ones, (Potocnik *et al.* 2002).

2.5 Colonic crypt homeostasis

Homeostasis of the normal colonic mucosa relies on a balance between proliferation at the base of the crypt and apoptosis at the surface epithelium. Normal colonic epithelium is organised in crypts where cell proliferation, migration, differentiation and apoptosis are topographically organised in a linear fashion along the crypt axis. Normal colonic crypts are composed of stem cells at the base, a proliferation and a differentiation zone in the lower third of the crypt, a migration zone in the upper two thirds, and the surface epithelium where senescent cells are eliminated by apoptosis. Colonic epithelial cells migrate from the base of the crypt to the surface epithelium in six to seven days.

The stem cells at the base of the crypt are susceptible to somatic mutations and these mutations persist during migration along the crypt axis but will be eliminated by apoptosis at the surface epithelium. However, genes directly involved in the normal apoptotic process can themselves be mutated causing a malfunction in the apoptotic pathways, (Abdel-Rahman *et al.* 1999). Dysregulation of apoptosis in colorectal adenomas and carcinomas has been correlated with abnormal Bak expression, (Partik *et al.* 1998). Studies have also shown dysregulation and mutations in the caspase

family in colon cancer, especially caspases 3 and 5, (Leonardos *et al.* 1999; Schwartz *et al.* 1999). As a result, cells with mutations in the apoptotic machinery will accumulate at the top of the crypt and surface epithelium, be unable to detach and die, and contribute to neoplastic transformation.

Alternatively genes encoding for cell-cell and cell matrix adhesion proteins can also be over- or under expressed, or undergo mutations causing resistance to anoikis (detachment of epithelial cells from the basement membrane). As a result of these alterations, cells will be unable to undergo the timely detachment and apoptotic process normally found at the surface colonic epithelium, (Kim *et al.* 1994).

2.6 Mucins

Mucins are high molecular weight glycoproteins that represent the major secreted products of the gastrointestinal tract. They are a major component of the mucus layer, which guards the epithelium of the gastrointestinal tract from mechanical, chemical, enzymatic, and microbial damage. Mucins are secreted in large quantities by colon cancer cells, and alterations in mucin expression and glycosylation occur during tumourigenesis and tumour progression in the colon, (Bresalier *et al.* 1991, 1996, 1998; Chang *et al.* 1994; Hanski *et al.* 1997).

MUC 2 mucin is the major product of the goblet cells in the small and large intestine in humans and is the principle mucin produced by most colon cancers. Previous studies have shown that mucins are frequently altered in colon cancers. Such alterations include differences in mucin-associated antigen expression between normal colon, preneoplastic adenomas and dysplasia, primary colon cancers, and

Figure 3 Colorectal cancer incidence worldwide (male)

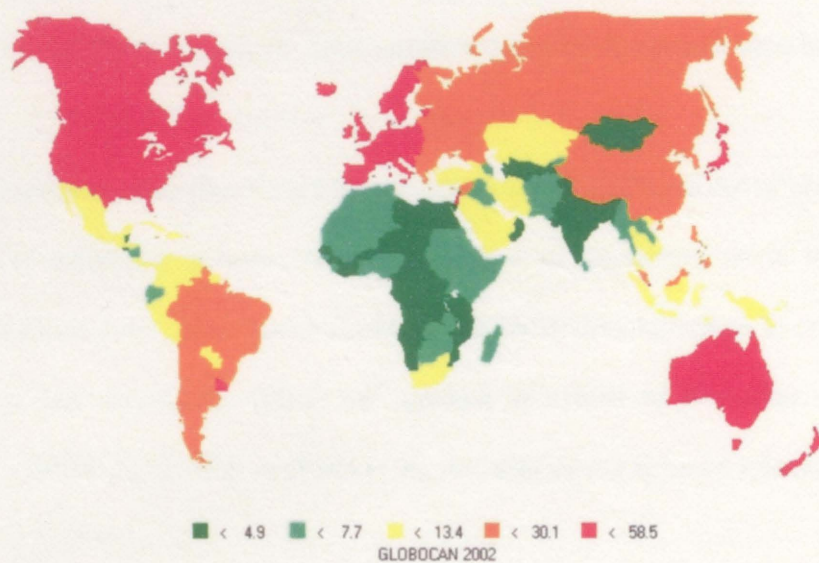
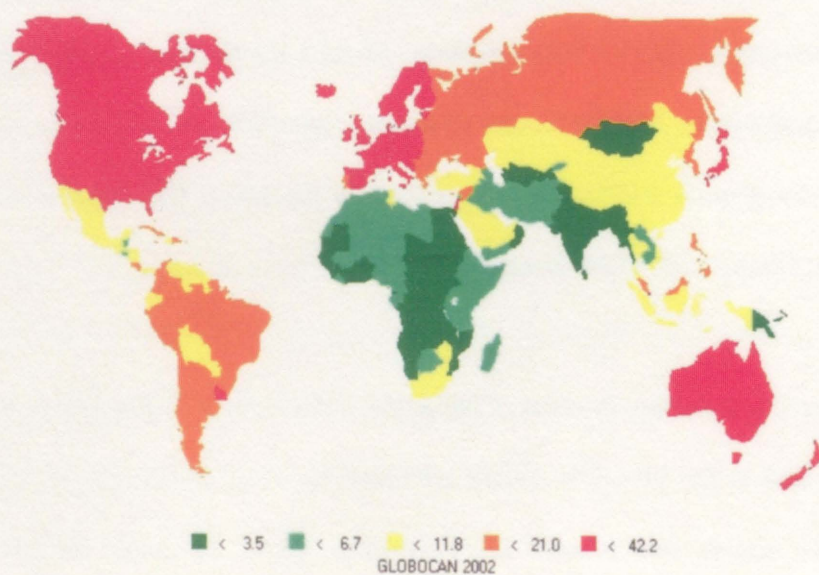


Figure 4 Colorectal cancer incidence worldwide (female)



metastatic disease, (Bresalier, 1996; Hanisch, 1992; Itzkowitz, 1990; Nakamori, 1993). Studies in athymic mice have shown that mucin production by human colon cancer cells is correlated with their metastatic potential, (Bresalier *et al.* 1991) and specific inhibition of MUC 2 mucin synthesis by antisense technology in cultured colon cancer cells inhibited colon cancer metastasis and liver colonisation, (Sternberg *et al.* 1999). Also, alterations in cellular behaviour such as proliferation, apoptosis, and cell migration occur with loss of MUC2 mucin and this could play a part in the early stages of carcinogenesis in the intestine, (Velcich *et al.* 2002).

3 Risk Factors & Prevention of Colorectal Cancer

Despite advances in surgical techniques and adjuvant therapy, there has only been a modest improvement in survival for patients who present with advanced neoplasms. Most colorectal cancers arise from precursor adenomatous polyps that develop into invasive carcinoma, typically over a five to ten year period or longer. Hence, effective primary and secondary preventive approaches must be developed to reduce the morbidity and mortality from CRC. Central to this is the identification of at risk subjects, who can be targeted for prevention strategies such as chemoprevention coupled with modification of lifestyle.

- **Diet and excess weight**

In the case of colorectal cancer diet has long been regarded as the most important environmental influence and this is reflected in the volume of studies testing the dietary hypothesis. International variation in the incidence and mortality of colorectal cancer (*Figures 3 & 4*), rapid increases in colorectal cancer in several countries and data on migration patterns and colon cancer support a role for diet in

the genesis of colon cancer. Many studies of various dietary components have yielded some evidence for the association of certain dietary habits with CRC while the evidence on other types of diet is conflicting.

A comprehensive epidemiological meta-analysis by Bergstrom *et al.* (2001) found consistent evidence of a positive association with obesity. A 2002 report from the World Health Organisation's International Agency for Research on Cancer documented that 20-33% of all breast, kidney, colon and endometrial cancers are related to obesity, (Vainio & Bianchini 2002). A high total fat intake is associated with increased colon cancer rates whereas rates are lower in populations consuming less fat, (Rose *et al.* 1986).

A possible explanation for the association of obesity with cancer has been generated by epidemiological studies, which demonstrate a close association between colon cancer risk and insulin resistance. Consumption of high-risk diets provides excess dietary energy, in response to which intravascular levels of insulin and energy substrates accumulate leading to increased cell proliferation, (Bruce *et al.* 2000). If cells were under normal cell cycle control this proliferative stimulus would have little effect, however, cells with defective cell cycle control will be stimulated to proliferate possibly giving rise to tumours. It is not known whether increased insulin resistance alone leads to increased proliferation or whether other factors are also involved. Higginbotham *et al.* (2004) found that diets with high glycemic quantities (i.e. processed foods such as white bread, white rice, cakes etc.) were associated with an increased risk of colorectal cancer.

Many studies have shown an inverse association between vegetable intake and colon cancer, the most convincing evidence being for cruciferous vegetables, (Potter 1997). However there are some cohort studies where the frequent consumption of vegetables did not appear to confer protection from colon or rectal cancer, (Michels *et al.* 2000). The role of dietary fibre in colon cancer was first proposed by Burkitt (1969) and has been widely studied since then. Some studies have shown a protective effect against colon cancer, (Trock *et al.* 1990; Giovannucci *et al.* 1992; Howe *et al.* 1992) while other more recent large scale studies, such as the Polyp Prevention Trial have found no effect of dietary fibre on adenoma recurrence, (Alberts *et al.* 2000; Schatzkin *et al.* 2000). However authors point out that the lack of effect may be attributed to the age of the study population, the type of fibre, compliance, and the duration of follow up. So, although the evidence is inconsistent we cannot definitively say that dietary fibre is not protective against colorectal cancer and as there are many other health benefits, diets rich in fibre should be encouraged in subjects considered at risk of colon cancer.

Total meat consumption has been studied in two meta-analyses that showed no statistically significant overall association (Norat *et al.* 2002; Sandhu *et al.* 2001). The same studies found a positive association of red meat and processed meat with the risk of colorectal cancer. The Nurses' Health Study found that women who consumed red meat frequently had a 2.5 fold increase in colon cancer risk compared with women who consumed red meat rarely, (Willett *et al.* 1990). Overall, the evidence suggests a strong association of red meat and colorectal cancer.

- Physical activity

The relationship between physical activity and reduced risk of colon cancer is among the most consistent findings in the literature. Studies indicate a consistent inverse relationship, with a 50% reduction in risk among those with the highest level of exercise, (Araya *et al.* 2002; Kiningham, 1998; Colditz *et al.* 1997; Friedenreich, 2001).

- Smoking

Although earlier studies of smoking and colorectal cancer showed no association, more recent studies have shown long-term smokers to be at elevated risk, with relative risks typically in the range of 1.5 to 3.0, (Giovannucci *et al.* 2001). Most recently interest has focused on the possibility that risk associated with smoking is modified by polymorphisms in metabolising enzymes (Welfare *et al.* 1997).

- Alcohol

The association between colorectal cancer and alcohol is not clear. Although the majority of studies suggest a positive association between alcohol consumption and colorectal cancer, a substantial portion of studies show no association, (Potter 1997).

- Hormone therapy

Protective effects against CRC of both hormone replacement therapy (HRT) and oral contraceptives (OC) have been postulated. The majority of evidence shows an inverse relationship between postmenopausal HRT and colorectal cancer (Crandall 1999; Potter 1997; Rossouw *et al.* 2002).

- Chemoprevention e.g. nonsteroidal anti-inflammatory drugs (NSAIDs)

The sequential process of tumourigenesis provides opportunities for the development and testing of both primary and secondary prevention strategies. Chemoprevention is defined as the use of natural or synthetic agents to reverse the process of carcinogenesis.

Epidemiological studies have consistently shown that chronic intake of nonsteroidal anti-inflammatory drugs (NSAIDs), principally aspirin, can reduce the incidence of colorectal adenomas and carcinomas, (Giovannucci *et al.* 1995; Thun *et al.* 2002). In a randomised trial of 1121 subjects with a previous history of adenoma, Baron *et al.* (2003) reported a reduction in adenoma recurrence in subjects taking aspirin compared to subjects in the placebo group. Another randomized trial of 635 CRC patients who had undergone curative resection, showed that taking 325mg of aspirin daily led to fewer adenomas and delayed the time of adenoma development compared to placebo (Sandler *et al.* 2003).

The cyclooxygenase (COX) family of enzymes which are involved in the conversion of arachadonic acid to prostaglandins are believed to be one of the most important enzyme classes associated with colon cancer. COX-2 has been found to be over expressed in the hamartomatous polyps of patients with Peutz-Jeghers Syndrome, a genetic syndrome that carries a markedly increased risk for CRC (McGarrity *et al.* 2003). Inhibition of COX-2 increases arachadonic acid, which, in turn stimulates the conversion of sphingomyelin to ceramide, a mediator of apoptosis (Sheng *et al.* 1998). COX-2 inhibition may also cause apoptosis by altering prostaglandin

production and by decreasing angiogenic factors, (Chan *et al.* 1998; Tsujii *et al.* 1998).

Waddell & Loughry, (1983) reported that sulindac, an indene acetic analogue of the NSAID indomethacin, caused the regression of adenomatous polyps in three of four members of a Gardner's syndrome family. This observation was confirmed in uncontrolled studies, case reports, and controlled trials. In a placebo-controlled, randomised study of 22 patients with familial adenomatous polyposis and established adenomatous polyps, the effect of 150 mg of sulindac, orally twice a day, was greater after six months of treatment than after nine months, suggesting the emergence of resistance to sulindac, (Giardiello *et al.* 1993). This effect may have been mediated by a decrease in the expression of COX-2 or other molecular events such as a mutation in the K- *ras* gene, (Keller *et al.* 2001).

More recently, the selective Cox-2 inhibitor Celebrex was also shown to be effective in familial adenomatous polyposis (Steinbach *et al.* 2000) and was approved by the Food and Drug Administration as an adjunct to usual care in these patients. In addition to demonstrating efficacy, chemopreventive agents must be safe and well tolerated for chronic administration and should be relatively cost-effective. In September 2004 the Adenomatous Polyp Prevention on Vioxx (APPROVe) randomised, placebo-controlled clinical trial, designed to evaluate the efficacy of a Cox-2 inhibitor marketed as Vioxx in preventing recurrence of colorectal polyps in patients with a history of colorectal adenomas was stopped. In this study, there was an increased relative risk for cardiovascular events, such as heart attack and stroke,

beginning after 18 months of treatment in the patients taking Vioxx compared to those taking placebo.

Other chemopreventive agents which have been assessed include folate, calcium, and oestrogens. In the Nurses' Health Study, supplementation with folate was protective against colorectal cancer, with the greatest risk reduction among women taking high daily doses of folate; this reduction became significant only after 15 years of use, (Giovannucci *et al.* 1998). The long time period required for clinical benefit suggests that folate acts early in colon carcinogenesis.

Some, but not all, studies of humans consuming high calcium diets or receiving calcium supplements have shown protective effects against colon carcinogenesis due to decreased proliferation of colorectal epithelial cells, changes in bile acid composition, or decreased cytotoxicity of faecal water, (Baron *et al.* 1995; Bostick *et al.* 1995; Holt *et al.* 1998; Lapre *et al.* 1993; Lipkin & Newmark 1985).

4 Screening & Diagnosis

Early diagnosis of colorectal cancer is often difficult clinically, as symptoms and signs only present late in the disease. The presenting features of colon cancer include rectal bleeding, changes in bowel habit, abdominal pain, tenesmus, iron deficiency anaemia, emergency presentation with large bowel obstruction or perforation and symptoms of malignancy such as weight loss and anorexia. However, screening of asymptomatic individuals can lead to detection of the disease at an early stage when it is more amenable to treatment.

4.1 Screening

The most widely investigated screening modality has been faecal occult blood testing (FOBT), a meta-analysis of around 443,000 people aged 40 or over in five countries concluded that FOBT resulted in a 16% reduction in colorectal cancer mortality, and when adjusted for attendance for screening, this improved to a 23% reduction (Towler *et al.* 1998). Sigmoidoscopy, colonoscopy or double contrast barium enema are also commonly used for screening.

The age at which screening is begun and the technique employed is dependant on the patients' history. The American Gastroenterological Association guidelines for screening recommends beginning screening in average risk people who are >50 years old, to stratify patients by risk, to offer various options for screening, to follow up a positive screening test with a colonoscopy, and appropriate and timely surgery for detected cancers, and follow up surveillance is required after polypectomy and surgery. Screening begins at a younger age and occurs at more regular intervals in HNPCC, familial adenomatous polyposis (FAP), inflammatory bowel disease (IBD) and patients with a family history of colon cancer, (Winawer, *et al.* 2003).

It is obvious that screening leads to earlier diagnosis and reduces the mortality of colorectal cancer, however, there are many issues regarding the relative cost effectiveness of the various tests, and specifically whether routine colonoscopic screening should be the test of choice. Further studies are required in this area.

4.2 Diagnosis

Colonoscopy is an extremely sensitive diagnostic test for colorectal cancer and has the major advantages of allowing both biopsy and polypectomy and does not involve exposure to ionising radiation. There are some disadvantages however: failure to reach the caecum in 5-30% of cases, (Theoni & Laufer 1994), intravenous sedation is nearly always required, tumour localisation can be inaccurate, and there is a small but significant risk of complications (Atkin 1999; Waye *et al.* 1996). The procedure related mortality is approximately 1 in 5,000 for colonoscopy and 1 in 50,000 for sigmoidoscopy and for double contrast barium enema.

In 1981, an Israeli physician, Dr. Gavriel Iddan, began development of a video camera that would fit inside a pill and now this wireless capsule endoscopy is being used in clinical settings. The capsule is about the size of a large vitamin capsule and is resistant to stomach acid and digestive enzymes. It relays video pictures of the gastrointestinal (GI) tract, via a wireless radiofrequency transmitter, as it moves naturally via peristalsis. Its main role is in the diagnosis of small bowel disorders and it is not useful in large bowel lesions as the folds of the lower intestine make visualisation of the entire colon difficult.

Double contrast barium enema is a radiological procedure using air and barium as contrast to highlight the lining of the large bowel. It may be employed as a sensitive, safe alternative to colonoscopy. Combining double contrast barium enema and sigmoidoscopy results in a sensitivity of 94% and specificity of 99%, (Jensen *et al.* 1990).

Computed tomography (CT) colonography is a sensitive method for detection of colorectal cancer, but not for polyps less than 10mm. Thin section, helical, CT is followed by off line processing (“virtual colonoscopy”) which yields high resolution, three-dimensional images of the colon, (Miao *et al.* 2000).

4.3 Tumour markers

Biochemical markers for colorectal cancer are potentially useful in screening for early disease, aiding diagnosis, predicting likely responses to specific therapies, surveillance of patients undergoing curative resection, and monitoring the treatment of advanced disease. Although multiple markers have been described, confusion remains about how best to use these factors.

Carcinoembryonic antigen (CEA) has been in use for approximately 30 years and is one of the most widely used tumour markers worldwide. It is a high molecular weight glycoprotein belonging to the immunoglobulin superfamily of molecules and is thought to play a role in a number of biological processes including cell adhesion, immunity and apoptosis, (Duffy, 2001; Hammarstrom 1999). Inadequate sensitivity severely limits the value of CEA for the diagnosis of early or low stage colorectal cancer and the specificity of CEA is also impaired as it is elevated in the absence of malignancy (e.g. patients with benign liver disease and in cigarette smokers).

A study by Harrison *et al.* (1997) in 572 node negative colon cancer patients found that preoperative serum CEA levels may be useful in identifying a subgroup of node-negative or Dukes’ B colon cancer patients that have a poor prognosis, and might therefore benefit from adjuvant chemotherapy. However there is no current evidence

that selecting patients for adjuvant chemotherapy based on an elevated preoperative CEA level is associated with a better prognosis.

An elevated preoperative CEA concentration should return to normal within approximately 6 weeks following complete surgical resection of a colorectal cancer, (Filella *et al.* 1994). Failure to do so suggests residual or metastatic disease. Serial monitoring with CEA has been shown to detect recurrent or metastatic disease with a sensitivity of approximately 80% and specificity of approximately 70%, (Duffy, 2001; Fletcher 1986). CEA is most useful for the early detection of liver metastasis in patients with diagnosed colorectal cancer.

Although the oldest, CEA is still the best available serum marker for CRC. One of its major limitations in monitoring, however, is that 20-30% of patients fail to produce elevated serum levels, despite the presence of advanced disease. For follow up of these patients, other markers are therefore necessary.

After CEA, Carbohydrate antigen 19.9 (Ca 19.9) is the most widely used tumour marker. It is the best available marker for pancreatic cancer but it is less sensitive than CEA for detecting colorectal carcinoma (Duffy, 1998). Elevated preoperative levels of CA 19.9 have been found to correlate with adverse patient outcome, (Lindmark *et al.* 1995; Nakayama *et al.* 1997; Reiter *et al.* 1997, 2000) despite being of little value in early diagnosis of the disease, (Moertel *et al.* 1993).

Other newer markers have been identified, but there is insufficient data available to recommend their use in the early detection of colorectal cancer. Studies suggest that

they may provide independent prognostic information. These markers include the following:

Carbohydrate antigen 242 (CA242) does not detect early stage colorectal cancer. However this marker may be useful when employed in combination with CEA in the surveillance of patients diagnosed with cancer, (Hall *et al.* 1994). TPA (tissue polypeptide antigen) measures fragments of cytokeratin 8, 18, and 19 and TPS (tissue polypeptide-specific antigen) and detects fragments of cytokeratin 18. These have been subjected to limited evaluation in colon cancer. Tissue inhibitor of metalloproteinase 1 (TIMP-1) is a multifunctional glycoprotein that inhibits metalloproteinase activity, stimulates cell growth and inhibits apoptosis, (Chambers & Matrisian 1997). In a study of TIMP-1 levels in blood from healthy individuals, IBD patients, breast, colon and rectal cancer patients, TIMP-1 levels were elevated in the colon cancer patients only. The specificity / sensitivity of TIMP-1 for colon cancer were 95% / 65% and 95% / 42% for rectal cancer, (Holten-Andersen *et al.* 2002). Combining CEA and TIMP-1 measurements increased the sensitivities above those obtained for TIMP-1 measurements alone.

None of the biochemical tumour markers currently available have the ability to detect early stage colorectal cancers nor are they 100% specific or sensitive. Combining markers may provide stronger prognostic information but further work is needed to validate the use of such combinations. However it should be remembered that these markers may be elevated in benign conditions such as cirrhosis and pancreatitis, (Duffy. *et al.* 2000).

5 Treatment

Treatment depends mainly on the size, location, and extent of the tumour, and on the patients' general health. Several different types of treatment are used to treat colorectal cancer and sometimes the treatments are combined.

5.1 Surgery

Surgery remains the definitive treatment for apparently localised colorectal cancer, offering the only chance of cure. The colon and the rectum are anatomically distinct and present different difficulties to the surgeon. The principles of radical surgical resection for colonic tumours have generally remained unchanged in the past few decades. In patients with colon cancer there is no evidence that radicality of excision has an effect on outcomes. Rouffet *et al.* (1994), compared survival in 270 colon cancer patients. They found that there was no difference in survival in patients who had undergone segmental colectomy compared to patients who had undergone the more radical procedure hemicolectomy. Total mesorectal excision (TME) is recommended for most rectal cancers where the patient is fit for radical surgery, (Heald *et al.* 1998; Wiig *et al.* 1998). As with all surgical procedures there are complications associated with colorectal cancer surgery. Anastomotic leakage is an important and potentially fatal complication of CRC surgery, and measures to minimise it should be taken.

Certain rectal cancers are amenable to local excision. A randomised trial of local resection for early rectal carcinomas compared outcomes using the gold standard method of anterior resection (AR) and the newer, less invasive technique of transanal endoscopic microsurgery (TEM). Lower morbidity, shorter operation time and

hospital stay, less blood loss and less opiate analgesia were associated with TEM, while local recurrence and survival rates were similar to AR, (Winde *et al.* 1996).

Evidence from several randomised controlled trials, case control studies, and cohorts indicate that laparoscopic surgery for colorectal cancer is feasible, and can reduce postoperative pain, analgesia use, hospital stay and blood loss in the short term, (Maxwell-Armstrong *et al.* 2000). But these have the potential for bias as blinding is impractical. Reliable outcomes are also lacking.

5.2 Staging

Following surgery, pathological investigation of the resected tumour is performed to assess prognosis and the need for adjuvant therapy. Staging identifies patients who might benefit from adjuvant chemotherapy, and circumferential resection margin (CRM) helps select patients with rectal cancer that might benefit from postoperative radiotherapy, (Adam *et al.* 1994). Pathological investigation allows tumours to be classified according to two different staging systems Dukes' and TNM, based on the extent of local invasion and the presence of disease in the lymph nodes.

The American Joint Committee on Cancer (AJCC) has designated staging by the TNM classification (AJCC, 1997). T refers to the primary tumour and TX, T0, Tis, T1, T2, T3 and T4 refers to status of the tumour (invasive, metastatic etc.). N refers to lymph nodes, where NX, N0, N1 and N2 refers to the presence, absence and number of lymph node metastases. Similarly M refers to distant metastasis and MX, M0, M1 refer to the presence, absence and number. Staging is as follows; Stage 0 is

Tis, N0, M0; Stage 1 is T1, N0, M0, T2, N0, M0; Stage 2 is T3, N0, M0, T4, N0, M0; Stage 3 is any T, N1, M0 or any T, N2, M0; Stage 4 is any T, any N, M1.

Dukes' staging was created in 1929 by Sir Cuthbert E. Dukes, a pathologist in St. Mark's Hospital in London and originally described for rectal cancer but is now applied to all colorectal adenocarcinomas. Dukes' A is where the tumour is confined to the bowel wall, B is tumour invasion through the bowel wall into the surrounding tissue, C is lymph node involvement, with C1 where the tumour has not reached the apical (highest) regional lymph node, and C2 where the apical node is involved. Dukes himself did not describe a D, but this was later added to refer to distant metastases. Approximately 10% of patients will present with Dukes A, 20-30% with Dukes B, 30-40% with Dukes C and the remainder with Dukes D, (McLeod *et al.* 2000). Five year treated survival according to Dukes staging is 90% for A, 65% for B, 30% for C, with 5 year survival of only 5% if distant metastases are present (Dukes' D).

5.3 Radiotherapy & Chemotherapy

Both radiotherapy and chemotherapy can improve survival rates after potentially curative surgery, and chemotherapy prolongs overall survival of patients with advanced disease. The anatomical differences between colon and rectal cancer and the ease of surgical intervention determine the pattern of recurrence, which defines the most appropriate adjuvant and relapse treatments. About 50% of recurrences of rectal cancer occur in the pelvis and are, therefore, amenable to local radiotherapy. By contrast, relapse of colon cancer is generally at distant sites – liver, lungs and bone and systemic chemotherapy seems more appropriate. In fact the use of adjuvant

radiotherapy in colon cancer has not been shown to improve outcome, (Martenson *et al.* 1999).

Fluorouracil has remained the cornerstone chemotherapy for colorectal cancer for over 40 years. It is a pro-drug that is converted intracellularly to various metabolites that bind to the enzyme thymidylate synthase, inhibiting synthesis of thymidine, DNA, and RNA. The decision to use adjuvant chemotherapy is influenced by the tumour stage. The absolute survival benefit from adjuvant chemotherapy in Dukes' C colon cancer is 4% to 13% at five years and the evidence for Dukes' B tumours, shows no overall benefit from adjuvant chemotherapy (Figueredo *et al.* 1997; Marsoni 1995; O'Connell *et al.* 1997).

Efforts to improve the efficacy of 5-fluorouracil have confirmed the benefit of including folinic acid. Preliminary results of the SAFFA study (short course adjuvant 5-fluorouracil, 5-fluorouracil plus folinic acid) appear to at least support the reduced toxicity of this regimen in the adjuvant setting (Saini *et al.* 2000).

There is evidence from two systematic reviews that chemotherapy for metastatic colorectal cancer can improve survival, and should be considered in all cases (Jonker *et al.* 2000; Simmonds 2000). This form of treatment is given with palliative intent, and a major aim should be to alleviate symptoms or delay their onset.

Up to 20% of patients who relapse after apparently curative resection of colorectal cancer present with disease macroscopically confined to the liver. Studies have shown that response rates are higher with hepatic artery infusion (HAI) of

fluorouracil but improvements in survival are meagre (Harmantas *et al.* 1996; Kemeny *et al.* 1999; Lorenz & Muller 2000). Any advantages achieved through the use of HAI are offset by technical difficulties associated with the technique.

The Swedish Rectal Cancer Trial found that a short-term regimen of high-dose preoperative radiotherapy reduced the rates of local recurrence and improved survival among patients with resectable rectal cancer. Patients received either radiotherapy prior to surgery or surgery alone. After five years of follow-up, the rate of local recurrence was significantly lower in the radiotherapy group (11%) than in the group treated with surgery alone (27%). The overall five-year survival rate was improved in the radiotherapy group (58%) compared to the surgery group (48%) and the cancer-specific survival rates at nine years were 74% (radiotherapy) and 65% (surgery), (Renehan *et al.* 2002; 1997). In cases of advanced disease, radiotherapy has the potential to improve operability of unresectable disease, in the curative treatment of inoperable disease, (Habr-Gama *et al.* 1998), and in palliative management of symptoms in patients with persistent or locally recurrent disease, (Spanos *et al.* 1993).

5.4 Follow –up

Patients who have undergone curative resection for colorectal cancer should undergo formal follow up in order to facilitate the early detection of metastatic disease. Interval CT scanning and CEA estimation may be of value in follow up of patients who have undergone curative resection for colorectal cancer but further studies are required to define an optimum approach, (Jeffery *et al.* 2002; Renehan *et al.* 2002; Rosen *et al.* 1998). Colonoscopy after curative resection for colorectal cancer should

be carried out as for adenomatous polyps. Clinicians should investigate promptly any symptoms or signs of recurrence.

6 Need for New Markers

Recent years have seen much research into the aetiology and management of colorectal cancer and some progress has been made in the areas of therapy, surgery and follow-up. However the long time period between exposure to a carcinogen and tumour formation poses a difficult problem when studying the relationship between suspected aetiological agents of colon cancer and the end point of tumour formation. In identification of at risk subjects, early detection methods, and methods to monitor patients during and after treatment, only modest improvements have occurred. The currently available biochemical markers for CRC discussed earlier are not markers of cancer risk and therefore are not particularly useful in cancer risk lowering studies or in evaluating the effectiveness of prevention strategies. There is a real need for the development of new, non-invasive, reliable markers predictive of tumour formation, which are altered by changing risk.

The expression of a biomarker, which is to be used in surveillance or early diagnosis of cancer should correlate with early stage of the disease, i.e. it should be differentially expressed in normal, pre-malignant and malignant stages. Such biomarkers have been proposed and developed from an understanding of the chain of events leading to colon cancer and an understanding of the changes, which occur in the colon before the manifestation of cancer. With regard to colon cancer there are

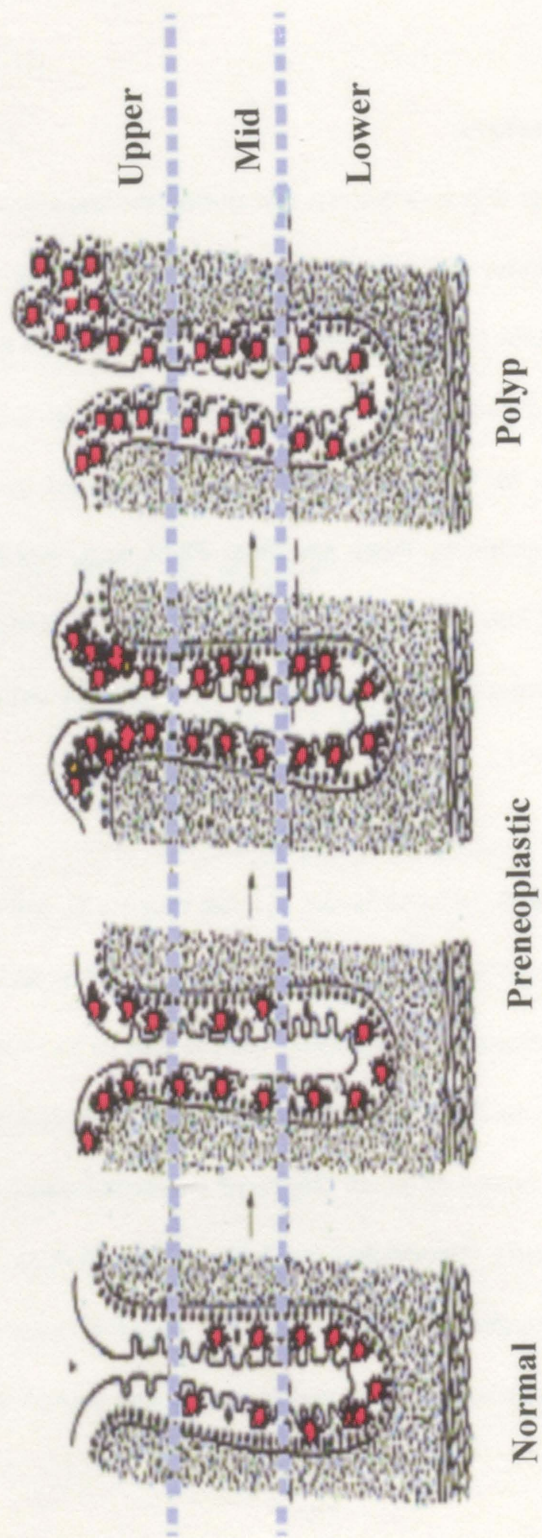
two main types of biomarkers (1) cell and tissue biomarkers and (2) biochemical biomarkers.

6.1 Cell Biomarkers

As discussed earlier it is now known that during the transition from normal mucosa to invasive carcinoma multiple molecular alterations occur at the genome level, (Fearon & Vogelstein 1990). It seems logical to hypothesise that these genes, which undergo mutation during the carcinogenesis process, might provide markers that are relatively specific to malignant and premalignant disease and also aid early diagnosis. Cells containing these abnormal DNA (e.g. mutant p53 or ras genes) sequences are shed into the lumen by exfoliation and can be detected in the stools of colorectal cancer patients, urine in bladder cancer patients and sputum in lung cancer patients, (Duffy, 1995).

The presence of DNA adducts in the colonic mucosa is associated with increased risk of colon cancer, (Pfohl-Leszkowicz *et al.* 1995). DNA adducts are formed when carcinogenic or mutagenic substances (from the diet or environment e.g. polycyclic aromatic hydrocarbons PCA) covalently bind to DNA. Oxidative DNA adducts have been shown to be increased in all stages of colorectal carcinogenesis and also in polyps of FAP patients, (Hendrickse *et al.* 1994; Schmid *et al.* 2000). Mucosal cells, which have been sloughed off in the colon, are excreted in the faeces. DNA adducts can be measured in faecal samples and as such seem a promising choice as a non-invasive biomarker.

Figure 6: Distribution of the proliferative activity along the colonic crypt



6.2 Tissue Biomarkers

There are many different types of tissue parameters, which have been proposed as possible biomarkers. Tissue biomarkers are analysed in tissue biopsies, which may be obtained by a number of techniques of varying levels of invasiveness.

6.2.1 Proliferation

As discussed earlier dysregulation of colonic crypt homeostasis is associated with increased risk of CRC. In the normal colonic mucosa proliferation occurs in the lower third of the crypt, and as cells mature they move to the top of the crypt where they are eliminated by apoptosis and eventually sloughed off. However in the abnormal situation, e.g. polyp or tumour formation, proliferation occurs throughout the entire crypt often with the zone of maximum activity occurring at the top of the crypt (*Figure 6*).

Proliferative activity is one of the most commonly used biomarkers to assess cancer risk and to monitor the effectiveness of risk lowering strategies. Studies have shown that subjects at high risk of colon cancer, such as subjects with familial adenomatous polyposis (Mills *et al.* 2001), ulcerative colitis (Shinozaki *et al.* 2000) or sporadic colon cancer (Roy *et al.* 1999) have a high mucosal proliferative activity as compared to controls. de Leon *et al.* (1988) reported that proliferation in the upper crypt was significantly increased in patients with polyps and colorectal cancer compared to healthy controls. In animal models of carcinogenesis, the carcinogen often increases cell proliferation and causes a shift in the zone of proliferation.

In human populations different geographic zones with variable colon cancer incidence show different proliferation patterns in the colorectal mucosa e.g., in Trieste, northern Italy, which has a colon cancer incidence of 30.9 / 100,000 proliferation is higher than in Florence in central Italy where colon cancer incidence (21.9 / 100,000) is lower, (Dolara *et al.* 2000).

Caderni *et al.* (1999) investigated the influence of different diets in subjects who had previously had at least two sporadic colonic adenomas removed. They found that subjects reporting a diet rich in fish, starch, and β -carotene and low in red meat had lower colorectal mucosa proliferation and a normal pattern of proliferation along the crypt. Also patients who had had villous adenomas (considered to have higher malignant potential) tended to have a higher proliferative activity than patients who had had tubular adenomas and that proliferation was higher in the upper compartment in subjects who ate the most red meat.

Mucosal cell proliferation is easily measured in biopsy samples. A commonly employed method to measure the proliferative activity in tissue sections is immunostaining for proliferating cell nuclear antigen (PCNA). Another commonly used method involves incubating a biopsy sample with $^3\text{[H]}$ -thymidine and the level of thymidine incorporation being measured by autoradiography where tissue sections are dipped in photographic emulsion containing a dense suspension of very small grains. Slides are then developed by standard photographic procedures, stained with haematoxylin-eosin and radiolabelled cells are visualized due to an accumulation of dark grains. An advantage of this method is that it determines the rate as well as the zone of proliferation. To date studies assessing the association of proliferation and

cancer risk have produced variable results and the usefulness of proliferation as a biomarker is still under consideration, (Mills *et al.* 2001; Sandler *et al.* 2000). However, inclusion of apoptosis and differentiation markers in studies assessing proliferative activity may strengthen this parameter as a biomarker.

6.2.2 Genotoxic Damage

The human colon is continually exposed to a complex mixture of compounds, which are either of direct dietary origin or the result of digestive, microbial and excretory processes. It is therefore useful to develop methods to analyse the mutagenic burden to which the colonic mucosa is exposed, and develop strategies to reduce this burden. The mutagenic potential of a compound has traditionally been measured using the Ames test. Mutagenicity is determined based on the ability of the test substance to induce mutations in *Salmonella typhimurium*. Bruce *et al.* (1977) first reported on the mutagenicity of organic faecal extracts using this assay. However the usefulness of this method is limited as the mutagenic potential of a substance is evaluated using prokaryotic cells, limiting the ability of this method to identify human carcinogens. In recent years, the use of methods, which measure genotoxic damage in eukaryotic cells, have proven to be more promising as an indicator of cancer risk.

One class of mutagenic agent of particular interest in the area of CRC are genotoxic agents. Genotoxic agents cause cellular DNA damage but are not cytotoxic to the cell. One of the most commonly used methods to assess genotoxic damage is the single cell microgelelectrophoresis assay or comet assay, which was developed by (Ostling & Johanson 1984), and modified by (Singh *et al.* 1988), as the alkaline

comet assay. Briefly, a single cell suspension is prepared either from a cell line or cells isolated from the target organ and treated with the agent under investigation. Cell numbers and viabilities are assessed. The cells are then immobilised in agarose on a glass slide and treated to lyse the cell membrane. This is followed by a period of alkaline treatment to allow unwinding of DNA. An electric current is then applied to the DNA and broken DNA fragments (damaged DNA) migrate away from the nucleus producing tail lengths, which vary based on the amount of DNA damage.

This method has been used to study the genotoxic potential of substances (e.g. heterocyclic amines) associated with cancer risk in freshly derived colon cells from healthy subjects (Pool-Zobel & Leucht 1997). Hambly *et al.* (1997) investigated the influence of diets containing high and low risk factors for colon cancer on 1,2-dimethylhydrazine (DMH) induced carcinogenesis in human flora-associated (HFA) rats using DNA damage as one of the end point markers. DNA damage was significantly higher in colon cells from animals on the high-risk diet.

(Schaeferhenrich *et al.* 2003) described a novel in vitro method in genetic toxicology using the comet assay to detect DNA damage connected with altered migration of the tumour protein p53 in primary human colonocytes. Further refinement of this method may perhaps generate another biomarker for genotoxicity and cancer risk.

6.2.3 Gene expression

Studies of the gene expression profiles of tumours, polyps and normal tissues help to identify genes involved in the carcinogenic process and to determine their potential usefulness as risk / diagnostic markers or as intervention biomarkers. As previously

discussed the expression of oncogenes, tumour suppressor genes and DNA repair genes are often altered in cancer. Using molecular techniques such as gene array analysis, the expression of these genes can be investigated and alterations in normal gene expression levels can be identified. Polymorphisms of certain genes have been associated with CRC risk. Some polymorphisms in genes, which encode the cytochrome P450 enzyme family lead to altered expression of the active enzyme and their increased expression is found in individuals with a history of CRC, (Kiss *et al.* 2000; Martinez *et al.* 2001).

The Glutathione S-transferase (GST) enzyme family are important detoxification enzymes, which catalyse the conjugation of xenobiotics with glutathione. GST μ enzymes are more effective at detoxifying potential cytotoxic and genotoxic epoxides than other GSTs. Polymorphisms in the GST μ gene leading to loss of or decreased enzyme function have been characterised in healthy individuals who later went on to develop colorectal and gastric cancers, (Chenevix-Trench *et al.* 1995).

It must be remembered that cancer arises from an accumulation of mutations in a single cell. Therefore genotyping of neoplasms has proven useful in detecting mutated genes whose loss or altered function may contribute to the carcinogenic process and which may prove useful as diagnostic / intervention biomarkers.

6.3 Biochemical markers

Biochemical markers can be measured in blood, urine and faeces and samples are obtained by less invasive methods than for tissue biomarkers. There are two types of biochemical markers (1) gut flora associated biomarkers which include bacterial

enzymes and faecal metabolites and (2) mammalian enzymes for example GSTs and the Cytochrome P450 family.

6.3.1 Gut flora associated markers

The use of gut flora associated biomarkers is not a new concept. The normal colonic microflora produces enzymes such as β -glucuronidase, β -glucosidase, nitrate reductase, nitroreductase and azoreductase, the activities of which have been implicated in colon carcinogenesis.

The liver is the site where metabolism of many toxic and carcinogenic compounds occurs. These compounds are conjugated with glucuronic acid and are then excreted via the bile into the small intestine. However bacteria in the colon produce β -glucuronidases which hydrolyse the glucuronide conjugates and release potential carcinogens and mutagens into the lumen thereby increasing the risk of tumour formation.

Many studies have confirmed the role of bacterial derived colonic β -glucuronidases in colorectal cancer. Reddy *et al.* (1974) found that germ free rats treated with the carcinogen 1,2-dimethylhydrazine (DMH) or Methylazoxymethanol (MAM)-glucuronic acid, which is formed following bacterial deconjugation of DMH, had fewer colonic tumours than rats with a normal flora, confirming the role of this enzyme in the promotion of carcinogenesis. The activity of β -glucuronidases in the gastrointestinal tract is influenced by diet. Diets considered high risk for CRC have been associated with increased β -glucuronidase activity compared to low risk diets, (Eriyamremu & Adamson 1995; Hambly *et al.* 1997b). Kim & Jin (2001) reported

that faecal β -glucuronidase activity was 1.7 fold higher in patients with colon cancer than in controls. However when these faecal samples were sonicated the enzyme activity of patients with colon cancer was 12.1 fold higher than that of healthy controls.

Diets high in fruit and vegetables are considered low risk for colon cancer. However fruit, vegetables and drinks produced from plants (e.g. wine), contain plant glycosides, which consist of low-molecular weight substances linked to sugar moieties. Plant glycosides are poorly digested in the upper GI tract and reach the colon in their safe glycosidic form. β -glucosidases produced by the colonic flora cleave the sugar moieties releasing toxic, mutagenic or carcinogenic aglycones, (Hughes & Rowland 2000). The breakdown of plant glycosides to aglycones is influenced by the composition and metabolic activity of the gut flora.

Nitroreductases reduce aromatic and heterocyclic nitrocompounds, which are found in cigarette smoke and diesel exhaust, to potentially toxic N-nitroso and N-hydroxy compounds. Bacterial nitrate reductases produced in the colon convert nitrate ingested in food and water to nitrite. Oxides of nitrogen derived from nitrite may further react with amines and amides to produce N-nitroso compounds many of which are highly carcinogenic. Red meat increases colonic N-nitrosation, which may explain the positive association between red meat intake and CRC risk. Hughes *et al.* (2002) reported that excretion of N-nitroso compounds was reduced by consumption of soy in seven male volunteers consuming a high red meat diet.

Another bacterial derived enzyme associated with cancer is azoreductase, which promotes the reduction of azo food dyes to substituted phenyl and naphthyl amines, which are potent carcinogens.

6.3.2 Mammalian enzymes

One of the most important phase II detoxifying enzymes are the glutathione-S-transferase (GST) family (alpha [α], pi [π] and mu [μ]) which are known to inactivate a number of genotoxic and carcinogenic risk factors by catalysing their conjugation with Glutathione and a reduction in their activity is associated with an increased risk of colon cancer (Szarka *et al.* 1995). Thus the inducibility of GSTs in normal tissues is a marker of chemoprevention. In contrast, human colonic tumours express high levels of GST, and especially GST placental enzyme pi type (GST-P); this characteristic is associated with failure of cancer chemotherapy and poor patient survival, (Sutoh *et al.* 2000).

Szarka *et al.* (1995) measured the GST activity of blood lymphocytes of healthy control individuals and individuals at high risk for colon cancer. They found that the GST activity of blood lymphocytes from high-risk individuals was significantly lower than that of blood lymphocytes from control individuals. They also measured GST activity in colon tissue from the high-risk individuals and paired analysis indicated a strong correlation between the GST activity of blood lymphocytes and colon tissue. Further work is needed, but the use of GST activity as a biomarker for cancer risk and to monitor responsiveness to treatment is a promising prospect. (Hengstler *et al.* 1998) reported that the expression of GST α and π was significantly increased in tumour tissue compared to adjacent normal tissue in the colon.

6.4 Faecal water activity

The colonic mucosa is continually exposed to the end products of diet, digestive and excretory processes as well as the metabolic activities of the gut flora many of which are carcinogenic and mutagenic. Colonic tumours arise from damage to the colonic mucosa, which is as a result of interactions with damaging components in the gut luminal contents. The aqueous phase of faeces called faecal water (FW) contains most of the free reactive and soluble factors, which interact with the mucosa in vivo, Rafter *et al.* (1987). The genotoxic and cytotoxic potentials of FW are considered as measures of the potential of the dissolved factors to induce damage in the colonic mucosa and have become popular intervention biomarkers in recent years.

6.4.1 FW Genotoxicity

Venturi *et al.* (1997) were the first to use the comet assay to assess the ability of FW from healthy individuals to induce genotoxic damage in Caco-2 cells. More recent studies have shown that there is an inter- and intra-individual variation of faecal water genotoxicity even in subjects consuming the same diet (Oßwald *et al.* 2000; Woods *et al.* 2002). Despite this variation, Oßwald *et al.* (2000), have suggested that FW genotoxicity is suitable as a biomarker for application in intervention studies when each subject is analysed as his or her own control.

A study by Rieger *et al.* (1999) in which healthy volunteers consumed a diet high in fat and meat, but low in fibre for twelve days (high CRC risk) followed by a high vegetable and wholemeal, low fat and meat diet (low CRC risk) for another twelve days found that the genotoxic damage induced by FWs from the high risk diet was approximately twice as high as damage induced by the low risk diet.

6.4.2 FW Cytotoxicity

Many of the substances to which the colonic mucosa is exposed induce cytotoxic damage leading to cell death. Stimulation of proliferation in the colon is, in part, to compensate for the loss of cells due to cytotoxic mechanisms, causing an increase in mitotic activity, increasing the risk of colon cancer. The cytotoxic potential of faecal water is now accepted as a risk marker for colon cancer, (Lapre & Van der Meer 1992; Lipkin 1991) and in vitro faecal water cytotoxicity towards colon cells is often employed as a biomarker in intervention studies.

van Muster *et al.* (1994) demonstrated that the consumption of resistant starch (a prebiotic) significantly decreased proliferation in rectal biopsies and the cytotoxicity of faecal water in healthy volunteers. Glinghammar *et al.* (1997) demonstrated that a shift from a dairy-product rich diet (low cancer risk) to a dairy product-free diet (high risk) increased cytotoxicity of FW from healthy volunteers; however the genotoxic potential of FW was unaltered. The exact mechanism by which dairy products lower cancer risk is unknown however it may be speculated that, in this study, the increase in cytotoxicity may have been due to the reduction of calcium in the diet.

6.4.3 Gene modulation by FW

Zeng & Davis (2003) reported that incubation of HT-29 cells with human FW induced apoptosis, reduced cell growth, decreased the mRNA levels of cyclin A and PCNA, and increased the levels of cyclin-dependent kinase inhibitor gene, p21^{waf1}. Further investigation of the influence of FW on these parameters may lead to the development of a novel approach for assessing the effect of diet on colon cancer risk

7. Gut Microflora

As discussed previously diets high in fat and low in fibre are associated with a high risk of colon cancer whereas diets high in fibre, fruit and vegetables and low in red meat are considered low risk for colon cancer. It is not certain how diet affects cancer risk but it is likely that a combination of diet and host genetic factors influences the composition and metabolic activity of the gut flora therefore influencing the carcinogenesis process.

7.1 The Gastrointestinal (GI) Tract

The GI tract harbours a rich flora of greater than 500 different bacterial species; the human body contains 10 times more bacteria than it does eukaryotic cells. After the respiratory tract the GI tract constitutes the second largest body surface area. The human gastrointestinal tract consists of the oral cavity, oesophagus, stomach, small intestine (duodenum, jejunum, ileum), large intestine (caecum, colon) and the rectum.

The mucosal surface of the adult human gastrointestinal tract measures up to 300 m², making it the largest body area in contact with the external environment. In comparison the skin surface measures only 2m². Only a single cell layer thick, the large surface area in the gastrointestinal tract is achieved by different degrees of folding (villi and microvilli) of the mucosal surface, Holzapfel *et al.* (1998). This large surface area provides the required space for the interactions of the digestive process and allows bacterial adhesion to the mucosal wall and colonisation. During a normal lifetime 60 tonnes of food pass through this canal, which is essential for

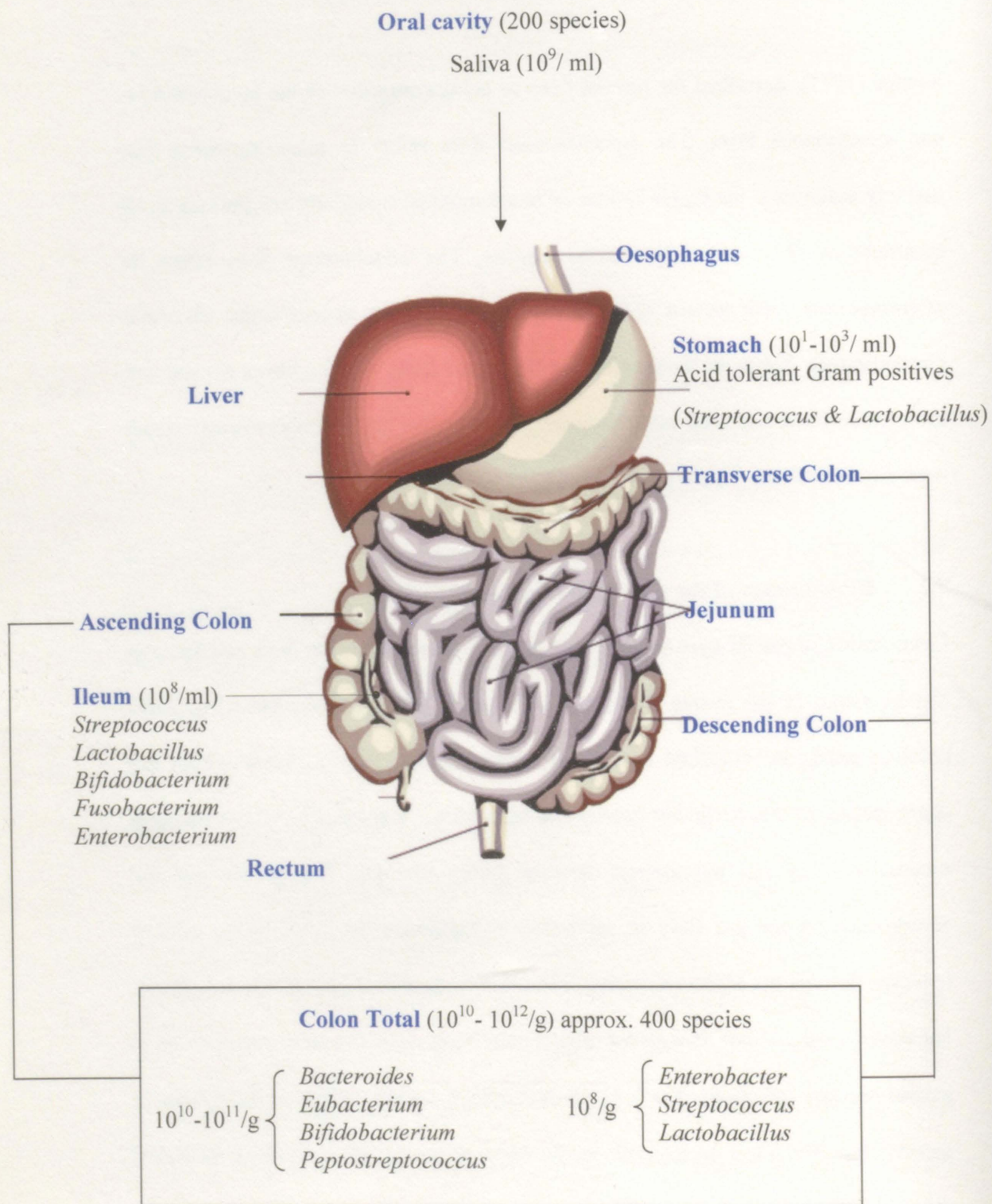
health, but also poses a significant threat to the integrity of the digestive tract and the body as a whole, (Bengmark 1998).

Savage (1977), described the normal flora as being composed of the autochthonous and allochthonous flora. The autochthonous flora refers to microorganisms that natively colonize a particular habitat or environmental niche and are present in all communities of a particular animal species. The allochthonous flora refers to microorganisms that cannot colonize a particular habitat, except under abnormal circumstances. However these terms are little used today and the terms normal and indigenous flora are used interchangeably to describe the microorganisms, which normally inhabit the gastrointestinal tract, (Berg 1996).

7.2 Development of the gastrointestinal flora

Colonisation of the GI tract of newborns begins immediately after birth and bacteria can be found in the faeces within a few hours of birth. Certain microorganisms colonize particular intestinal habitats at various times following birth, which are characteristic of that particular habitat and host. This is a process known as bacterial succession. Bacterial populations develop during the first day of life and the composition of the gut flora in newborns is influenced by the delivery method (passage through the birth canal versus caesarean section) and also by the diet (breast fed versus formula fed). Breast-fed infants have been shown to have a lower risk of gastrointestinal infections than formula-fed infants. Infants delivered by caesarean section have far fewer lactobacilli in the early stages of life than those delivered vaginally (Collins & Gibson 1999).

Figure 7: Microbial distribution in the gastrointestinal tract



Cooperstock & Zedd (1983) divided the development of the intestinal microflora in infants into four phases, 1) weeks 1-2, initial acquisition, 2) breast-feeding alone, 3) introduction of supplementation and cessation of breast-feeding and 4) cessation of breast-feeding and conversion to adult like flora.

Initially the facultative anaerobes such as *E. coli* and streptococci are dominant, and produce a reduced environment allowing the establishment of anaerobic bacteria such as *Bacteroides*, bifidobacteria and clostridia, generally by day 4-7, (Stark & Lee 1982). In breast fed infants bifidobacterial numbers increase sharply, while *E. coli* and streptococci decrease, and clostridia and *Bacteroides* are low or absent. This change does not occur in formula fed infants and the composition of the gastrointestinal flora becomes rather complex with high numbers of *Bacteroides*, clostridium and streptococci, and bifidobacteria, although present, are in much lower numbers than in breast fed infants. During phases 3 and 4, following the introduction of solid food and weaning, the flora becomes similar to the normal adult flora, and the composition of the flora while being distinct for each individual, remains relatively stable throughout life, (Holzapfel *et al.* 1998).

7.3 Microbial distribution in the gastrointestinal tract

Mackie *et al.* (1999) stated that “indigenous bacteria are not distributed randomly throughout the gastrointestinal tract but instead are found at population levels and in species distribution that are characteristic of specific regions of the tract”. The distribution of the normal flora of an adult human is shown in *Figure 7*. The oral cavity contains an indigenous flora of about 200 species, however, most of these are washed into the stomach with saliva and most research of GI tract flora centres

around the stomach, small and large intestines and faecal matter.

After the relatively neutral environment of the oral cavity the low pH of the stomach (pH 2.5 to pH 3.5) is destructive to most microorganisms and bacterial numbers are low (10^1 and 10^3 cfu/ml of contents). Following a meal bacterial numbers in this region may increase temporarily 100 to 1000-fold due to increases in pH, (Lambert & Hull 1996). Acid tolerant Gram-positive species such as streptococci and lactobacilli predominate in the stomach and duodenum. *Helicobacter pylori* a significant causative agent of gastritis in adults, thrives on the mucosa of the stomach (Berg 1996). Bacterial numbers are also low in the duodenum due to the detrimental effects of biliary and pancreatic secretions and also the short transit times.

The concentration and variety of microorganisms increase significantly in the distal small intestine (Ileum). Here bacterial numbers of 10^8 cfu/ml of intestinal contents are found, consisting mainly of species of streptococci, lactobacilli, bifidobacteria, bacteroides, fusobacteria and enterobacteriaceae, (Nielsen *et al.* 1994).

The primary site of microbial colonisation in humans and animals is the large intestine, possibly due to the low oxidation-reduction potentials and the slow intestinal motility in this area (transit times up to 60hr), (Tannock 1995). Bacterial numbers exceed 10^{10} - 10^{12} cfu/g and represent 40-55% of luminal contents. It is thought that greater than 400 different species inhabit the colon, however a considerable number of these remain unculturable using existing culture techniques. It is also important to note that 99.9% of the indigenous flora of the large intestine

are obligate anaerobes. Genera such as *Bacteroides*, *Eubacterium*, *Bifidobacterium* and *Peptostreptococcus* dominate at levels of approximately 10^{10} - 10^{11} cfu/g. Enterobacteriaceae, streptococci and lactobacilli are also present at levels of 10^8 cfu/g, (Naidu *et al.* 1999).

Faecal samples are most often used to study the colonic flora. However, the bacteria found in faecal samples represent the luminal environment and may differ greatly from the flora of the mucosa. The faecal flora may indicate the bacteria present in the distal colon but is not a true indication of the flora of the transverse and ascending colon and certainly not of the flora of the small intestine. Investigation of faecal samples alone may underestimate true bacterial numbers, and many researchers suggest that microbiological investigation of biopsy samples may give a truer indication of the diversity of the colonic flora, however this approach is much more difficult.

Within the GI tract bacteria are found in different microhabitats stretching from the gut lumen to the mucosal epithelium, (Freter 1992). These microhabitats are;

1. Surface of the cells of the epithelium
2. Crypts of the ileum, caecum and colon
3. Mucus gel that overlays the epithelium
4. Lumen of the intestine

Certain bacteria reside freely in the gut lumen possibly associated with the surface of particles. Others colonize the crypts or adhere to the mucus layer or to the surface of

epithelial cells. The obligate anaerobes associate intimately with the gut wall to form layers on the mucosal epithelium (Berg 1996).

7.4 Unculturables

Of the 400 different species of bacteria inhabiting the colon, a considerable number are unculturable using existing culture techniques. Traditional culture techniques depend on the use of selective media for the enumeration of bacterial genera or species of interest and are limited in their potential to reproduce the complex mixture of gases and nutrients present in the colon. Therefore such techniques provide a limited view of the diversity of the flora and comparison of results from electron microscopic investigation of the gastrointestinal flora and traditional culture counts has demonstrated that culture methods yield only between 50 to 80% of the total microscopic counts, (Tannock 2001).

Recent years have produced major advances in the area of molecular biology and the development of the tools required to study the diversity of complex bacterial environments without the use of traditional culture methods. Molecular methods have indicated that 60-80% of the organisms in the total human microflora have not been cultivated, (Langendijk *et al.* 1995). These molecular techniques are based on 16S ribosomal RNA (16S rRNA) gene sequences which contain regions that are conserved across all bacterial species and are interspersed with regions in which sequences are variable among bacterial types, (Stackebrandt & Rainey 1995). Microbial DNA is extracted from faecal samples and from the GI tract and the 16S rRNA gene sequences are amplified by polymerase chain reaction (PCR). Two methods for analysing the PCR products are denaturing gradient gel electrophoresis

(DGGE) and temperature gradient gel electrophoresis (TGGE). DGGE is based on electrophoresis of PCR-amplified 16S rDNA fragments in polyacrylamide gels containing linearly increasing gradients of denaturants allowing DNA fragments of the same length but with different base-pair sequences to separate, (Muyzer *et al.* 1993). TGGE separates rDNA fragments in polyacrylamide gels with a temperature gradient. With both DGGE and TGGE specific DNA fragments can be excised from the gel and sequenced to identify the bacterial species represented by the particular band.

Fluorescent *in situ* hybridisation (FISH) is also based on the 16S rRNA region. With this method rRNA targeted oligonucleotide probes specific for phylogenetic groups, are used for the *in situ* identification of individual bacterial cells in a complex environment, (Amann *et al.* 1995).

Suau *et al.* (1999), investigated the bacterial diversity within an adult-male faecal sample using comparative analysis of cloned 16S rRNA gene sequences. They found that the majority of the generated rDNA sequences (76%) did not correspond to known organisms. The use of these molecular methods is greatly contributing to our understanding of the diversity of the gastrointestinal ecosystem and with further developments in methods the possibility of identifying all components of the gut microflora no longer seems an impossible dream.

7.5 Functions of the Gut Microflora

With more bacterial cells in the gut than eukaryotic cells in the entire body, the gut has often been described as the forgotten organ. The collective metabolic activity of

the normal flora would rival that of the activity of the liver, (Berg 1996; Bocci 1992). The functions of the intestinal microflora contribute to the health of the host in many ways.

The important effects that the gut microflora exerts on its host have been revealed through the comparative studies of animals bred under germ-free conditions and conventional animals with a normal indigenous flora. Such studies have shown that the microflora has an influence on the physiological and immunological characteristics of the host. Germ-free animals have been found to have:

- No intestinal plasma cells
- Smaller Peyer's patches
- A slower intestinal passage
- A lower intestinal motility
- A retarded gastric emptying
- An accelerated carbohydrate absorption
- An enlarged caecum

(Schaafsma 1997)

The beneficial activities of the normal gut flora include vitamin synthesis, and improved absorption of minerals by the host e.g. calcium, magnesium and iron, (Younes *et al.* 2001). The flora is also involved in the conversion of pro-drugs to their active form and the metabolism of carcinogens ingested in the diet to both active and inactive compounds. It is thought that the composition of the flora influences whether ingested carcinogens are converted to active or inactive forms.

7.5.1 Metabolism

A major metabolic activity of the colonic microflora is the fermentation of non-digestible dietary residues and endogenous mucus produced by epithelial cells, which results in the production of absorbable substrates and energy for the host, and a supply of energy and nutrients for bacterial growth, (Guarner & Malageldá 2003). The major source of energy within the colon is the fermentation of carbohydrates by the colonic flora, the endpoint of which is the production of short-chain fatty acids (SCFA). SCFAs provide between 10-30% of basal metabolic requirements (including energy for liver cells, colonocytes and peripheral tissues) and only 5% of SCFA is excreted in the faeces, (Dunne 2001). Acetate, propionate and butyrate are the principal SCFA produced in the colon.

Acetate traverses the liver and enters the peripheral circulation. Propionate is extracted by the liver and effects lipid metabolism, (Bridges *et al.* 1992). Butyrate is a major source of energy for colonocytes and is almost completely consumed by the colonic epithelium. Only a portion of butyrate leaves the colon for extraction by the liver. Butyrate has been associated with many biological properties in the colon and is thought to directly enhance cell proliferation in normal cells and suppress proliferation in transformed cells. In addition, apoptosis may be increased in transformed cells but inhibited in normal cells when butyrate is present, (Hague *et al.* 1995; Hass *et al.* 1997; Marchetti *et al.* 1997). Butyrate is known to reduce risk factors involved in the aetiology of colon cancer and adenoma development, (Smith *et al.* 1998).

7.5.2 Barrier to infection

The normal flora plays a major role as a physical barrier against pathogenic and opportunistic infection. Germ-free animals, which lack a gut flora, are very susceptible to infections. In humans the importance of this barrier effect or colonisation resistance is best demonstrated when the composition of the normal flora is altered by broad-spectrum antibiotic therapy. In such cases the use of antibiotics disturbs the normal balance allowing the overgrowth of opportunistic bacteria, which are normally resident in the gut but have restricted growth, leading to diarrhoea and other symptoms. *Clostridium difficile* overgrowth is often an unwanted side effect of antibiotic therapy.

7.5.3 Immune education

The gut-associated lymphoid tissue (GALT) transforms the gastrointestinal tract into the largest immune organ in the human body. Approximately 10^{10} immunoglobulin producing cells per meter of small bowel are suggested to be present, accounting for approximately 80% of all immunoglobulin producing cells in the body, (Targan & Shanahan 1994). The antigens of the gut are derived from the diet and the resident microbiota. Without the microflora, mucosal associated lymphoid tissue is underdeveloped and cell mediated immunity is defective. In order to prevent unnecessary and potentially harmful reactions, the gut immune system must be able to distinguish the normal microflora and dietary antigens from pathogens (oral tolerance). However the immune system must be ready for an immediate response in the presence of a pathogen. One of the mechanisms for the rapid discrimination between potential pathogens, commensal bacteria, and food antigens is via a restricted number of preformed receptors. A newly discovered class of cell surface

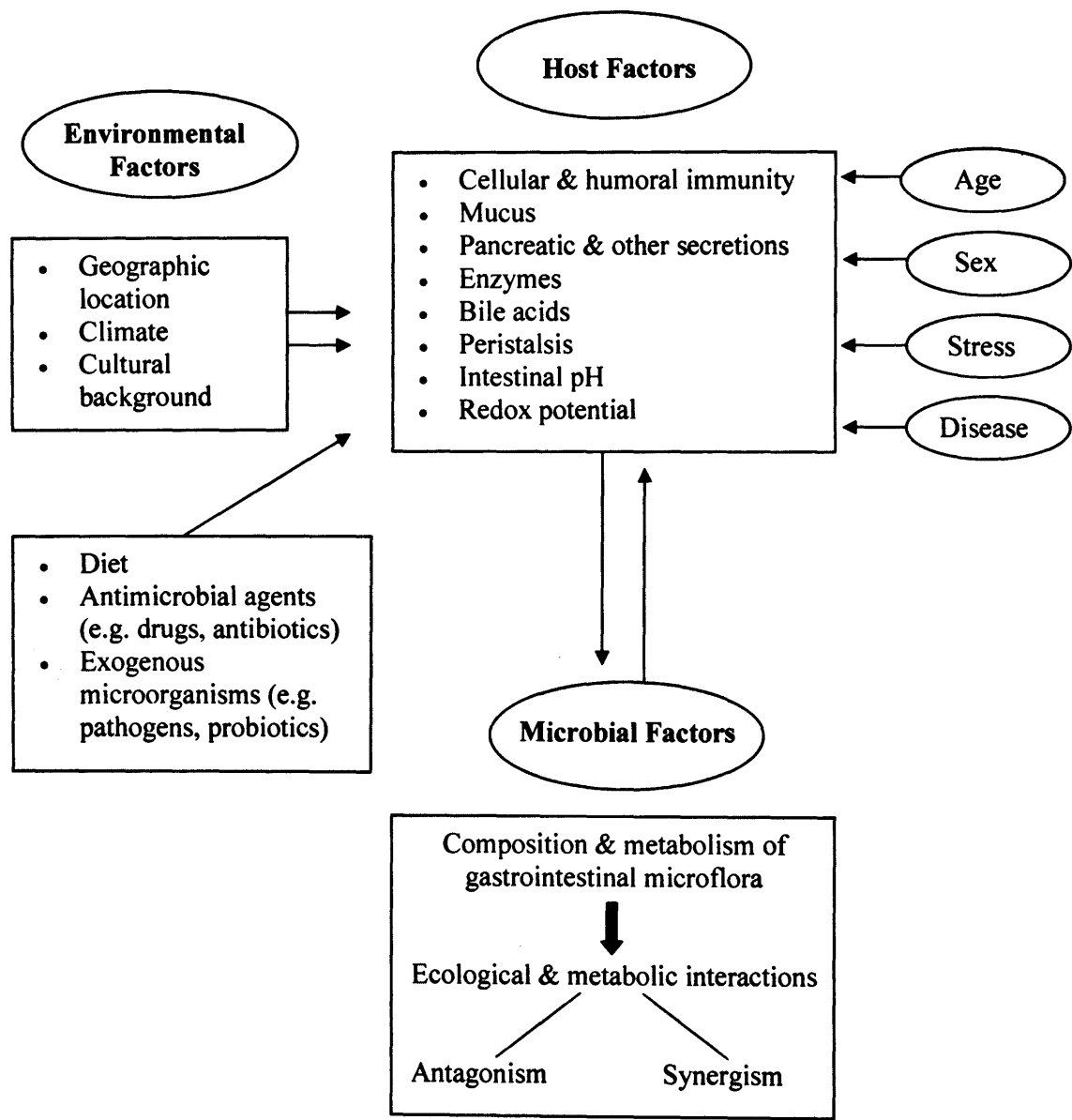
receptors, the Toll-like receptors, specifically interact with bacterial walls or antigens, (Krutzik *et al.* 2001).

Dendritic cells are crucial in determining the local development of an adaptive immune response. Two subsets of monocyte-derived dendritic cells (MDC) exist; MDC1, which responds to lipopolysaccharide, lipotechoic acid or CpG DNA by means of the Toll mannose receptor, which leads to NF- κ B activation and potentiates Th1 responses, whereas MDC2 responds to immune complexes and cellular factors and leads to a Th2 response, (Hemmi *et al.* 2000). Hence, according to the nature of the antigen a Th1/Th2 polarisation will occur.

Th1 type responses are characterised by production of pro-inflammatory cytokines; interferon-gamma (IFN- γ), tumour necrosis factor- α (TNF- α), Interleukin-12 (IL-12), and IL-2. Th2 cells mediate humoral immunity through the action of IL-4, IL-5, and IL-6. It is important to maintain a balance between Th1 and Th2 responses in relation to oral tolerance to ingested antigens. Inappropriate polarisation can eventually lead to disease, for example, allergy (Th2) or autoimmunity (Th1).

The involvement of regulatory T cells which produce anti-inflammatory cytokines like tumour growth factor- β (TGF- β) and IL-10 is an area of increased research interest. The Th3 cell subset is generated in the gut-associated lymphoid tissue (GALT) and suppresses the function of effector cells by releasing the inhibitory cytokine TGF- β . Th3 cells are also implicated in IgA production and in the regulation of oral tolerance by blocking the Th1 or Th2 response.

Figure 8: Factors affecting the stability of the gastrointestinal flora.
Adapted from (Kleessen *et al.* 2000).



7.6 Stability of the Gut Microflora

Studies of the faecal flora have shown that individuals tend to maintain their own distinctive flora compositions and although many bacterial species are shared, individuals generally have their own distinct predominant species combinations, even after drastic changes in diet, (Kleessen *et al.* 2000). McCartney *et al.* (1996) investigated the bifidobacterial and lactobacillus populations of faecal samples from two human subjects over a 12-month period. They discovered that while stability in species composition may be a feature of the normal microflora, stability at the level of bacterial strains was less common.

Although the composition of the GI flora is rather stable in healthy individuals it can be altered by many endogenous and exogenous factors such as antimicrobial agents, radiation therapy, emotional stress, GI surgical procedures, peristalsis disorders, inflammatory bowel diseases, cancer, immune disorders and age. The variety of factors relating to the host, the bacteria and the environment, which affect the composition, and function of the flora in the GI tract is shown in *Figure 8*.

7.7 The Role of the Microflora in Colon Cancer

Imbalance in the composition of the GI flora has been associated with increased risk of colon cancer, (Gorbach & Goldin, 1990). A study of the composition of the faecal flora of human subjects at varying risks of colon cancer found that the presence of two *Bacteroides* species, *Bacteroides vulgatus* and *Bacteroides stercoris* were associated with high risk. Low risk was associated with the presence of *Lactobacillus acidophilus*, *Lactobacillus S06* and *Eubacterium aerofaciens*, (Moore & Moore 1995). Contrary to popular opinion, this study found that *Bifidobacterium*

longum and *Bifidobacterium angulatum* were associated with a high risk of colon cancer. Bifidobacterial counts are low and clostridia counts high in elderly subjects with colon cancer (Benno *et al.* 1991). Although not conclusive, the evidence does show that the colonic flora plays a major role in modulating cancer risk in humans.

Reddy *et al.* (1974) showed that treatment with the carcinogen DMH or MAM-glucuronic acid, (formed following bacterial deconjugation of DMH), produced less colonic tumours in germ-free rats than in rats with a normal flora. Treptow-van Lishaut *et al.* (1999) reported that the level of GST expression in colon cells from germ-free rats is much lower than that observed in rats with a conventional flora, indicating the importance of the gut flora in inducing the expression of enzymes protective against colon cancer.

Hirayama *et al.* (2000), compared the effect of three mutagens associated with colon cancer on DNA adduct formation in the colons of germ-free and human flora associated rats. They found that the microbial flora played an essential role in DNA adduct formation and that the role played by the flora was dependent on the type of mutagen. The formation of DNA adducts is a possible mechanism by which the gut flora influences the carcinogenic process.

It is now accepted that the normal microflora plays a role in the onset and progression of colon cancer. Therefore it is logical to propose that altering the intestinal microflora may influence tumour development. The consumption of functional foods in an effort to alter the gastrointestinal flora is considered an attractive alternative to the use of antibiotic / pharmaceutical based treatments.

8. Probiotics, prebiotics and synbiotics

A food can be said to be functional if it contains a component (which may or may not be a nutrient) that affects one or a limited number of functions in the body in a targeted way so as to have positive effects on health, or it has a physiologic or psychologic effect beyond the traditional nutritional effect. As such pre-, pro- and synbiotics are considered functional foods as they have been shown to exert positive health effects, (Roberfroid 2000).

8.1 Probiotics

It was at the beginning of the 20th century that Metchnikoff first suggested the concept of what we now know as probiotics (Metchnikoff 1907). His hypothesis was that the long, healthy life of Bulgarian peasants resulted from their consumption of fermented milk products, which he suggested modified the activity of the colonic microflora suppressing the putrefactive-type fermentation of the intestinal flora in a health promoting way.

In the early 1900's there was much interest in the health benefits of consumption of fermented dairy products. In fact, it was during this period that some of the companies still associated with the production of yoghurt and fermented milk products (Danone & Yakult) were founded (www.yakult.co.uk, www.danoneinstitute.org).

8.1.1 Definition

The word probiotic is derived from the Greek meaning 'for life'. The term was first used in 1965 by Lilly & Stillwell (1965), to describe substances secreted by one

protozoan to stimulate the growth of another. Over the years the definition of a probiotic has evolved with the advancement of research in the area. In 1989 Fuller defined a probiotic as a 'live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance', (Fuller 1989). The definition was again revised in 1992 by Havenaar & Huis In't Veld (1992), who defined a probiotic as 'a mono- or mixed-culture of live microorganisms which when applied to man or animal affects beneficially the host by improving the properties of the indigenous microflora'. However, the most accurate definition of a probiotic was proposed by Salminen *et al.* (1999): 'Probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host.' This definition was based on research, which showed that probiotics need not be viable in order to exert an effect and also that some beneficial probiotic effects were non-microflora mediated.

8.1.2 Selection criteria

Fermented dairy products have traditionally been produced using combinations of microorganisms often *Lactobacillus* and *Streptococcus* species because of their supposed positive health effects. However many people, especially some members of the scientific community have remained sceptical of these products and their associated probiotic claims. In order to overcome this problem much effort has been expended in recent years to remove the myth from probiotic research and to replace it with confirmed facts. Participants involved in the Lactic Acid Bacteria Industrial Platform (LABIP) workshop on probiotics concluded that 'probiotics may be consumed either as a food component or as a non-food preparation'. Criteria, outlined by others, for use in the assessment of potential probiotics were summarised

Table 2: Microorganisms considered as probiotics adapted from Holzapfel et al. (2001)

Lactobacillus species	Bifidobacterium species	Other lactic acid bacteria	Nonlactic acid bacteria
<i>L. acidophilus</i>	<i>B. adolescentis</i>	<i>Enterococcus faecalis</i> ¹	<i>Bacillus cereus</i> var. <i>toyoi</i> ^{1,2}
<i>L. amylovorus</i>	<i>B. animalis</i>	<i>Enterococcus faecium</i>	<i>Escherichia coli</i> strain nissle
<i>L. casei</i>	<i>B. bifidum</i>	<i>Lactococcus lactis</i> ³	<i>Propionibacterium freudenreichii</i> ^{1,2}
<i>L. crispatus</i>	<i>B. breve</i>	<i>Leuconstoc mesenteroides</i>	<i>Saccharomyces cerevisiae</i> ²
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> ³	<i>B. infantis</i>	<i>Pediococcus acidilactici</i> ³	<i>Saccharomyces boulardii</i> ²
<i>L. gallinarum</i> ¹	<i>B. lactis</i> ⁴	<i>Sporolactobacillus inulinus</i> ¹	
<i>L. gasseri</i>	<i>B. longum</i>	<i>Streptococcus thermophilus</i> ³	
<i>L. johnsonii</i>			
<i>L. paracasei</i>			
<i>L. plantarum</i>			
<i>L. reuteri</i>			
<i>L. rhamnosus</i>			

¹ Main application for animals.

² Applied mainly as pharmaceutical preparations.

³ There is either little known about the probiotic properties or the microorganism is nonprobiotic.

⁴ Probably synonymous with *B. animalis*

by Dunne *et al.* (1999), and a definitive set of selection criteria for probiotics was put forward.

Probiotic microorganisms should:

- Be of human origin
- Demonstrate non-pathogenic behaviour
- Exhibit resistance to technological processes (i.e., viability and activity in delivery vehicles)
- Prove resistant to gastric acid and bile
- Adhere to gut epithelial tissue
- Be able to persist, albeit for short periods, in the gastrointestinal tract
- Produce antimicrobial substances
- Modulate immune responses
- Have the ability to influence metabolic activities (e.g. cholesterol assimilation, lactase activity, vitamin production).

Most recently the joint Food and Agriculture Organization (FAO) / World Health Organization (WHO) working group on drafting guidelines for the evaluation of probiotics in food recommended that probiotics be defined as ‘live microorganisms which when administered in adequate amounts confer a health benefit on the host’ (Araya *et al.* 2002).

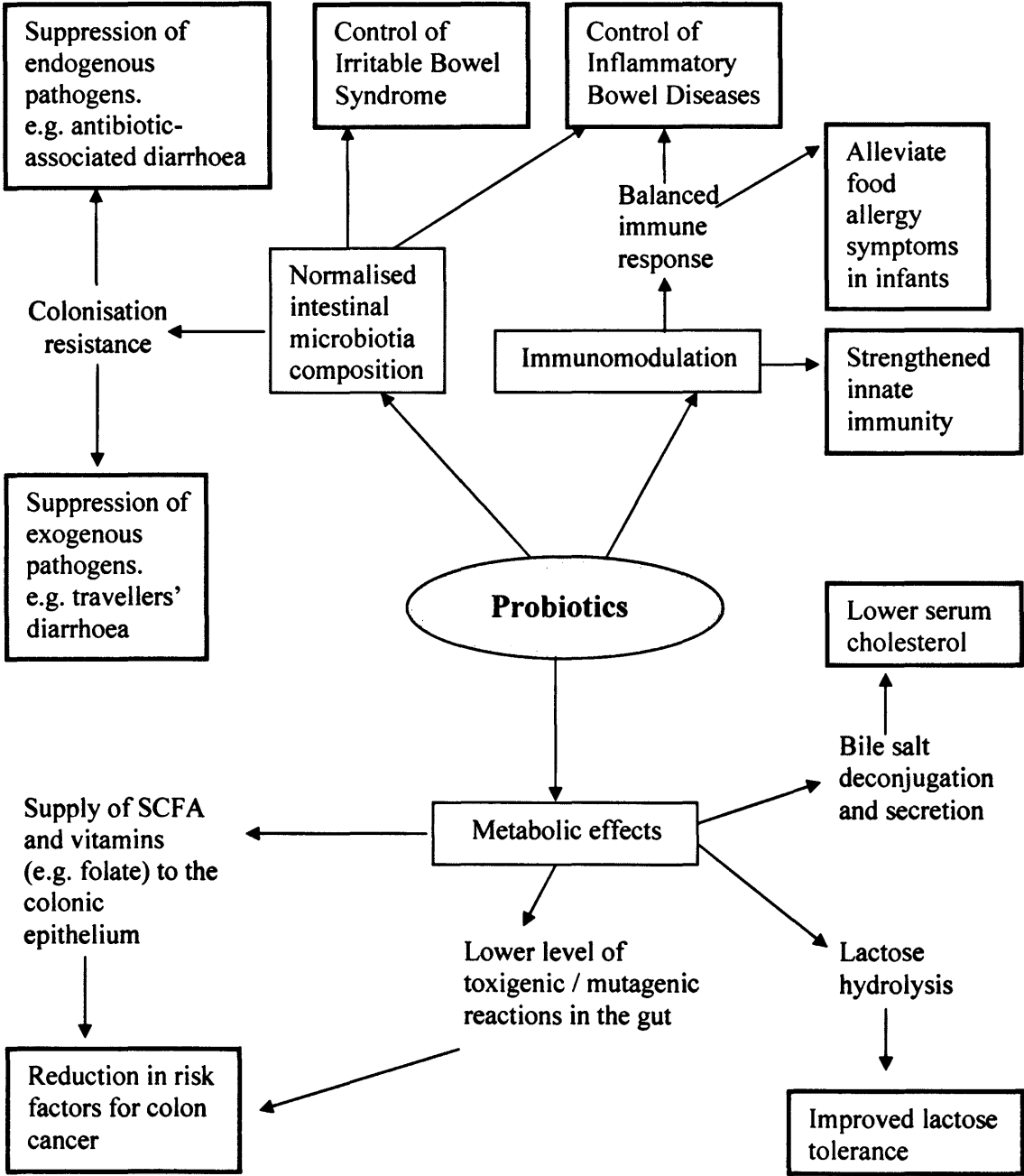
Probiotics are generally lactic acid producing bacteria (LAB), and are primarily of the genus *Lactobacillus* and *Bifidobacterium*. However some non-LAB bacteria and yeasts are considered as having probiotic properties (*Table 2*).

8.1.3 Beneficial health effects

Numerous health-promoting properties have been associated with probiotics over the years. Probiotics have been shown to lower the frequency and duration of diarrhoea associated with antibiotic therapy, (D'Souza *et al.* 2002; Pochapin 2000), rotavirus infection, (Vanderhoof & Young, 1998; Guandalini *et al.* 2000), chemotherapy, and, to a lesser extent, traveller's diarrhoea, (Hilton *et al.* 1997; Katelaris *et al.* 1995). Probiotics have been shown to have a positive effect on urogenital health. Reid *et al.* (2001) demonstrated a reduced recurrence rate of urinary tract infections with oral consumption of lactobacilli. Lactobacilli have also proven useful in the treatment and prevention of bacterial vaginitis, (Sieber & Dietz 1998). Di Benedetto *et al.* (1998) reported a reduction in infections and abdominal pain and an increase in weight in children with cystic fibrosis when *Lactobacillus rhamnosus* GG was administered as an adjunct to standard treatment.

Probiotics have also been extensively investigated as possible treatments of inflammatory bowel disease. VSL#3 which is a combination of eight strains (4 lactobacilli, 3 bifidobacteria, 1 streptococcus) has been shown to successfully maintain remission in pouchitis, ulcerative colitis and Crohn's disease, (Friedman & George 2000; Gionchetti *et al.* 2000; Gupta *et al.* 2000). *Escherichia coli* Nissle 1917 has been found to be as effective as mesalazine (standard treatment) for maintaining remission in ulcerative colitis, (Kruis *et al.* 2004).

Figure 9: Health benefits associated with probiotic consumption



Probiotics when administered subcutaneously or orally in animal models of colitis and arthritis show efficacy in attenuating disease. This suggests a role for probiotic bacteria in treating gastrointestinal and systemic autoimmune diseases. Probiotics have also demonstrated the ability to stimulate the host immune system, (Schiffrin *et al.* 1997; Erickson & Hubbard, 2000) to improve gut barrier function, (Isolauri *et al.* 1993; Madsen *et al.* 2001), alleviate symptoms of lactose intolerance (de Vrese *et al.* 2001; Montes *et al.* 1995; Shermak *et al.* 1995) reduce blood pressure in hypertensive subjects (Hata *et al.* 1996; Takano 1998), reduce cholesterol and triacylglycerol plasma concentrations, (Kiessling *et al.* 2002; Taranto *et al.* 1998) and play a part in cancer prevention, (Wollowski *et al.* 2001; Rafter, 2002; Gallaher & Khil, 1999). **Figure 9** shows the many health promoting activities associated with probiotics.

8.1.4 *Lactobacillus rhamnosus* GG

Lactobacillus rhamnosus GG (LGG) is one of the most thoroughly studied probiotic strains, (Salminen *et al.* 1993). LGG has been shown to adhere to the human Caco-2 cell line and the adherence was significantly better than that of other dairy strains of lactobacilli and bifidobacteria, (Elo *et al.* 1991), to adhere *in vitro* to both human colonic mucosa, (Sarem-Damerdjji *et al.* 1995) and human intestinal mucus, (Kirjavainen *et al.* 1998). LGG also survives and colonises the gastrointestinal tract of adults and children, (Goldin *et al.* 1992; Millar *et al.* 1993). A study in which 20 healthy volunteers consumed LGG for 7 days at 1.2×10^{10} cfu/day in gelatine capsules resulted in faecal recovery of 1.2×10^5 cfu/ gram of faeces, (Saxelin *et al.* 1995).

As all previous studies had investigated the numbers of LGG recoverable from faeces as an indication of strain survival and transit in the GI tract, Alander *et al.* (1997) fed five human subjects with a whey drink containing LGG for 12 days and investigated the presence of LGG in both faeces and colonic biopsies. In all subjects LGG was the dominant lactic acid bacterium recovered from faeces and was also detected in biopsies in four of the five subjects (one subject was diagnosed with ulcerative colitis during study), demonstrating the ability of LGG to adhere in vivo to the colon.

In another study by the same group, subjects were again fed the whey drink containing LGG and at the cessation of feeding were split into 3 groups, 1) subjects biopsied immediately after cessation, 2) subjects biopsied 7 days after cessation 3) subjects biopsied 14 days after cessation, (Alander *et al.* 1999). The presence of LGG in faeces was also investigated at these time points. Results show that LGG can persist on the colonic mucosa after cessation of feeding and even after the strain's disappearance from faecal samples, and that the study of probiotic numbers in faecal samples may actually underestimate colonisation by probiotic strains.

Consumption of LGG has been shown to reduce the activities of bacterial enzymes implicated in carcinogenesis, (Ling *et al.* 1994), reduce the accumulation of aflatoxins in the intestine, (Kankaapaa *et al.* 2000) improve the immunogenicity of an oral rotavirus vaccine, (Isolauri *et al.* 1995), treat and prevent diarrhoea associated with antibiotic use, (Vanderhoof *et al.* 1999), rotavirus infection, (Majamaa *et al.* 1995), *Clostridium difficile* overgrowth, (Pochapin 2000), and traveller's diarrhoea, (Hilton *et al.* 1997). LGG consumption has also proven

effective in the treatment and management of food allergy in infants, (Majamaa & Isolauri 1997).

8.1.5 *Bifidobacterium lactis* Bb12

The most commonly studied probiotic *Bifidobacterium* strain is *Bifidobacterium lactis* Bb12 (Bb12). Bb12 has the ability to survive in the human GI tract, (Alander *et al.* 2001), to adhere to human intestinal mucus, (Kirjavainen *et al.* 1998) and to human ileostomy glycoproteins, (Ouwehand *et al.* 2000). The adherence of Bb12 to ileostomy glycoproteins and to the intestinal mucus of infants was enhanced in the presence of LGG, indicating that simultaneous use of these strains may be more efficacious than individual use, (Juntunen *et al.* 2001; Ouwehand *et al.* 2000). The ability of Bb12 to adhere to the intestinal mucus of infants was unaltered during or after rotavirus diarrhoea compared to healthy infants, (Juntunen *et al.* 2001).

An *in vitro* study by Fooks & Gibson (2002) on the ability of probiotic strains to inhibit pathogen growth found that Bb12 inhibited the growth of pathogens, especially *E. coli*, to a greater extent than that observed for the other probiotic strains tested. They also found that this inhibition was influenced by the type of carbohydrate which was provided *in vitro*, the most significant results were from inulin and fructo-oligosaccharide containing carbohydrates. Bb12 has been shown to exert an inhibitory effect against *Helicobacter pylori* (*H. pylori*) *in vitro* and in a human intervention study when administered as a yoghurt which also contained *Lactobacillus acidophilus* LA5, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, (Wang *et al.* 2004).

Consumption of Bb12 in a fermented milk product by healthy volunteers for three weeks enhanced the phagocytic activity of peripheral blood leukocytes as well as increasing the numbers of faecal bifidobacteria, (Schiffrin *et al.* 1997). In a six-month placebo controlled trial Bb12 consumption resulted in a significant reduction in atopic eczema in infants with severe food allergy compared to the control group, (Mattila-Sandholm *et al.* 1999).

A double-blind placebo controlled trial to evaluate the efficacy of a formula containing Bb12 and *S. thermophilus* for the prevention of acute diarrhoea in infants admitted to hospital was conducted by (Saavedra *et al.* 1994). Significantly fewer infants receiving probiotic formula than receiving control formula developed acute diarrhoea during their hospital stay. The prevalence and the rate of rotavirus shedding was significantly less in the probiotic group compared to control.

8.2 Prebiotics

Oligosaccharides of various types are found as natural components in many common foods including fruit, vegetables, milk and honey. The use of oligosaccharides as food ingredients has risen rapidly over the past 20 years especially in Europe and Japan, (Crittenden & Playne 1996). In 1991 the Japanese government legislated for 'foods for specified health use' (FOSHU): fructo-, galacto-, soybean and palatinose oligosaccharides are among the foods currently listed. Most dietary oligosaccharides are hydrolysed in the upper part of the gastrointestinal tract, however some oligosaccharides resist the digestive process and reach the colon in an intact form where they are hydrolysed to small oligomers and monomers, which are further metabolised by the microflora. One such class of dietary oligosaccharides are the

non-digestible oligosaccharides (NDOs), which are of short (degree of polymerisation (DP) >10) or medium length (DP >60).

8.2.1 Definition

Delzenne & Roberfroid (1994) proposed the term prebiotics for certain types of non-digestible oligosaccharides (NDOs) used as food ingredients to modify the composition of endogenous gut flora. The definition was further developed by Gibson & Roberfroid (1995) who defined a prebiotic 'as a non-digestible food ingredient that benefits the host by selectively stimulating the growth or activity of one or a limited number of bacteria in the colon'. Therefore any food ingredient that enters the large intestine may be considered a candidate prebiotic; however it is the selectivity of the fermentation in the presence of the diverse microflora that is the defining feature.

Collins & Gibson (1999), stated that in order to classify a food ingredient as a prebiotic, it must be

- 1) neither hydrolysed nor absorbed in the upper part of the gastrointestinal tract;
- 2) a selective substrate for one or a limited number of potentially beneficial commensal bacteria in the colon, thus stimulating the bacteria to grow, become metabolically activated, or both
- 3) able as a consequence to alter the colonic microflora toward a healthier composition.

Examples of prebiotics are specific oligosaccharides, starch fractions (resistant starch), pectins, lactose, lactulose, lactitol, sorbitol and xylitol. In the European

market some of the most extensively researched prebiotics are the fructo-oligosaccharides (FOS) especially those which are derived from the chicory root, (inulin and its enzymatic hydrolysate oligofructose). The average daily consumption of inulin and oligofructose has been estimated to be one to four grams in the USA and three to eleven grams in Europe, (Van Loo *et al.* 1995).

8.2.2 Health benefits

Prebiotics exhibit a dietary fibre-like action. Fermentation of prebiotics lowers the intestinal pH. This leads to a decreased activity of the enzyme 7 α -hydroxylase responsible for the formation of secondary bile acids. Secondary bile acids comprise over 80% of faecal bile acids and are thought to play an important role in colon carcinogenesis by acting as promoters in tumour formation, (Nagengast *et al.* 1995).

One of the most interesting aspects of prebiotics is that they enhance the growth of bifidobacteria and other Gram-positive bacteria but are not fermented by the majority of Gram-negative bacteria in the colon. Effective bifidogenic doses appear to vary among the different oligosaccharide types. However, most oligosaccharides have been demonstrated to increase bifidobacteria numbers in the colon at doses of <15grams/day, (Crittenden & Playne 1996) although some prebiotics have demonstrated an effective bifidogenic effect at lower doses. Most *in vivo* human studies suggest that the log increase in the number of bifidobacteria depends on the initial number of bifidobacteria, irrespective of the dose of the FOS, (Rao 2001; Roberfroid *et al.* 1998), although a study by Bouhnik *et al.* (1999), found a linear relationship between the increase in bifidobacterial counts and FOS consumption and also found the optimal daily dose of FOS to be 10grams.

Prebiotic consumption is associated with many beneficial effects. van den Heuvel *et al.* (1999), demonstrated that oligofructose consumption (15grams/day) stimulated calcium absorption in male adolescents. In rats consumption of inulin or resistant starch or a combination of the two significantly increased the intestinal absorption and balance of calcium and magnesium compared to placebo, (Younes *et al.* 2001). Scheppach *et al.* (2001) described the influence of prebiotics on sugar digestion and absorption, glucose and lipid metabolism and protection against known risk factors of cardiovascular disease.

Prebiotics have laxative properties similar to carbohydrates. Carbohydrates that reach the large intestine, such as non-starchy polysaccharides and resistant starch, are known to have a laxative effect on bowel function. Stimulation of microbial growth leads to increased bacterial mass and bowel content, which in turn stimulates peristalsis, decreases transit times and prevents constipation. Consumption of chicory inulin was shown to relieve constipation in elderly subjects, (Kleessen *et al.* 1997) and in healthy volunteers, (Hond *et al.* 2000). Prebiotics may also influence the host's natural defence against pathogens by enhancing bacterial colonisation resistance. Hopkins & MacFarlane (2003), demonstrated that NDOs enhanced colonisation resistance against *Clostridium difficile* *in vitro*.

8.3 Synbiotics

In general if a probiotic is to exert a beneficial effect on the host, the bacterium must be metabolically active in the gut. Within the colon the supply of fermentable carbohydrate is a growth-limiting factor for bacteria. The bacterial species, which occur in the greatest numbers, are those which utilise the available nutrients most

effectively. As discussed previously, prebiotics resist digestion in the upper GI tract and reach the colon intact, where they are available as nutrients for the colonic flora. Current research indicates that consumption of prebiotics with probiotic strains enhances the growth of the introduced strain in the gut. This is known as the synbiotic effect and the term synbiotic is applied to a product, which contains both prebiotics and probiotics.

8.3.1 Definition

In defining a synbiotic, Schrezenmeir & de Vrese (2001) stated, 'because the word alludes to synergism, this term should be reserved for products in which the prebiotic compound selectively favours the probiotic compound'. In the strictest sense, therefore, a product containing oligofructose as a prebiotic and bifidobacteria as a probiotic would fit the definition of a synbiotic but a product containing oligofructose and a lactobacillus strain would not. However, it may be argued that promotion of the growth of indigenous bifidobacteria *in vivo*, combined with the introduction of a lactobacillus strain and subsequent beneficial effects on the health of the host fulfils the definition of synergism.

8.3.2 Health Benefits

One of the principal benefits of synbiotics is believed to be the increased persistence of the probiotic in the GI tract. Consumption of a prebiotic in a synbiotic compound provides a readily fermentable carbohydrate source for the introduced probiotic strain, giving the probiotic a competitive advantage over the indigenous flora. This advantage increases the persistence of the probiotic in the GI tract increasing the time period in which the probiotic strain can exert its influence.

This synbiotic effect has been proven in human studies. Morelli *et al.* (2003), investigated the effect of a synbiotic preparation of two *Lactobacillus paracasei* strains, a *Lactobacillus gasseri* strain and inulin, glutamin, zinc and vitamin B6 on the flora of twelve healthy individuals. After fifteen days *Lactobacillus paracasei* counts had increased by at least one log in all subjects, with a 2-3 log increase in subjects who had low counts of this group at baseline. *Lactobacillus paracasei* persisted after cessation of feeding with counts three days after cessation of feeding similar to or slightly lower than these counts at the end of treatment but higher than at baseline. Bifidobacterial numbers also increased but no consistent effects were seen on enterobacteria, enterococci or *Clostridium perfringens*.

Gallaher & Khil (1999) demonstrated that feeding rats, treated with the chemical carcinogen DMH, either bifidobacteria or oligofructose alone had no influence on the number of aberrant crypts compared to controls. However in five of six experiments, the combination of oligofructose and bifidobacteria led to a reduction in aberrant crypt numbers compared to controls indicating an additive or synbiotic effect of oligofructose and bifidobacteria for reduction of colon cancer risk in carcinogen-treated rats.

An interesting application of synbiotics as a supportive therapy for critically ill patients in intensive care units has been suggested by Kanamori *et al.* (2002). They fed a synbiotic combination of *B. breve*, *L. casei* and galacto-oligosaccharides to a 9-month old baby born with laryngotracheo-eosophageal cleft, who had undergone her initial surgery at 3 months, had suffered repetitive episodes of pneumonia and whose growth remained unsatisfactory. Abdominal x-ray had shown no gas in the large

bowel and lactobacilli and bifidobacteria were not detected in the faeces. Bowel movements were restored just 1 day after commencement of synbiotic treatment and a dramatic increase in faecal short-chain fatty acids (SCFAs) was achieved within 1 month. Pneumonia developed only once after the beginning of synbiotic treatment, body weight doubled after 11 months treatment and final curative surgery was performed at this time. The authors suggest that the dramatic effectiveness of synbiotic therapy in such cases may be due to improved nutrition, prevention of bacterial translocation due to the presence of the probiotics and also modulation of the immune system. Such therapies should be considered as an immunonutritional therapy for critically ill patients in intensive care units.

8.4 Influence of pre-pro and synbiotics on carcinogenesis

As discussed earlier it is an accepted fact that the normal microflora plays a role in the onset and / or promotion of colon cancer. Therefore it is not unexpected that there has been much interest in the development of methods to alter the composition or the metabolic activity of the intestinal microflora in an attempt to influence the carcinogenic process.

A protective role for pre-, pro- and synbiotics has been shown in animal studies, (Gallaher & Khil 1999; Wollowski *et al.* 2001). The exact mechanisms by which pre-, pro- and synbiotics may inhibit colon carcinogenesis are as yet uncertain. However, based on results from the vast amount of *in vitro* and *in vivo* studies performed to date, it is possible to speculate on some potential mechanisms.

(Rafter, 2002) suggested the following mechanisms by which lactic acid bacteria may inhibit colon cancer:

- Altering the physicochemical environment in the colon
- Enhancement of the host's immune response
- Inactivating potential carcinogens and mutagens
- Altering the composition of the intestinal flora to a more beneficial flora
- Anti-mutagenic or anti-tumourigenic activities
- Altering the metabolic activity of the flora
- Increase activities of protective enzymes and reduce activities of enzymes involved in carcinogenesis

8.4.1 Altering the physicochemical environment in the colon

Modler *et al.* (2001) suggested that large bowel cancer may be influenced directly by reducing the intestinal pH, causing the growth of putrefactive bacteria to be reduced. Silvi *et al.* (1999) reported that resistant starch selectively increased the levels of lactobacilli and bifidobacteria by ten to one hundred fold in faecal samples from rats associated with a human flora, while decreasing enterobacteria, caecal ammonia concentrations, caecal pH and β -glucuronidase. Rowland *et al.* (1998) demonstrated the co-administration of *Bif. longum* and inulin significantly reduced the pH of caecal contents and the number of aberrant crypt foci (ACF) in the colon of azoxymethane (AOM) induced rats compared to controls. Challa *et al.* (1997) found that administration of a synbiotic combination of *Bif. longum* and lactulose reduced the numbers of ACF in the colon and the caecal pH as well as inducing the activity of colonic glutathione S – transferase (a phase II detoxifying enzyme). However pH has not been consistently reduced in studies of probiotic and synbiotic consumption.

8.4.2 Enhancement of the host's immune response

The presence of a normal healthy microflora is essential for the proper functioning of the host's immune system. The gastrointestinal immune system (GALT etc.) is responsible for distinguishing between the normal flora and potential pathogens, which are ingested in the diet. As probiotic bacteria originate from the normal gut flora their consumption does not induce an immune response of the type, which occurs in the presence of a pathogen. However probiotics have been shown to exert various immune enhancing effects following oral consumption. These include enhanced macrophage phagocytosis, (Perdigon *et al.* 1988), promotion of IgA responses, (Isolauri *et al.* 1993; Link-Amster *et al.* 1994; Malin *et al.* 1996), and altered cytokine production, (Miettinen *et al.* 1998), leading to a shift in the balance between Th1 and Th2 immune responses.

Natural killer (NK) cells are a sub-population of lymphocytes important for the recognition and elimination of virally infected cells and tumour cells. NK cells are thought to play a role in the non-specific mechanism in immuno-surveillance for cancer. Studies have suggested that low NK cell activity is associated with an unfavourable prognosis, (Tartter *et al.* 1987). Imai *et al.* (2000) investigated NK cell activity in 3625 Japanese people mostly older than forty and carried out a follow-up survey of cancer incidence for eleven years. Their results indicate that medium and high NK cell activity of PBMCs is associated with reduced cancer risk, whereas low activity is associated with increased cancer risk. Probiotic supplementation has been shown to enhance NK cell activity, (Takagi *et al.* 2001; Gill *et al.* 2001). Matsuzaki & Chin (2000) demonstrated that in adult mice fed the probiotic *Lactobacillus casei*

strain Shirota for five days the NK activity of spleen cells was significantly higher than the NK activity of spleen cells from mice not fed the probiotic.

One of the most interesting aspects of probiotic modulation of the immune response is through its influence on cytokine production, and many studies have shown that probiotics can alter cytokine expression profiles in healthy and diseased states in both animals and humans. Miettinen *et al.* (1998) stimulated human peripheral blood mononuclear cells with three non-pathogenic *Lactobacillus* strains and one pathogenic *Streptococcus pyogenes* strain. All bacteria strongly induced IL-1 β , IL-6 and TNF- α mRNA and secretion of the cytokine protein. IL-12 and IFN- γ production was greatest following stimulation with *Strep. pyogenes* but was also induced by two *Lactobacillus* strains. All strains induced IL-18 protein secretion.

Schultz *et al.* (2003) investigated the effect of five-week oral consumption of *Lactobacillus rhamnosus* GG in ten healthy volunteers on the cytokine secretion profile of peripheral blood mononuclear cells (PBMCs) following stimulation with 'self' and 'non-self' intestinal organisms and whole stool preparations. Probiotic consumption increased the activational response of CD4⁺ T-lymphocytes towards heat-inactivated *E.coli* and *Bacteroides fragilis* group isolated from faecal samples. The secretion of TNF- α , IL-6 and IFN- γ by PBMCs was decreased, while the IL-10 and IL-4 secretion was increased following probiotic consumption irrespective of the origin of the antigen (self or non-self).

Sheih *et al.* (2001) conducted a three-stage intervention study where fifty-four healthy volunteers consumed reconstituted low fat milk (LFM) for a three-week run

in period (stage 1) followed by three-weeks consuming *Lactobacillus rhamnosus* HN001 in either LFM or reconstituted lactose-hydrolysed LFM (stage 2) and a follow up period of three weeks consuming reconstituted LFM alone (stage 3). They found that immune responses were unaltered in the run-in period (stage 1). Phagocytic activity of polymorphonuclear cells isolated from whole blood and the NK cell activity of mononuclear cells was significantly increased by HN001 consumption irrespective of the delivery method (stage 2). These immune responses declined three weeks after cessation of HN001 consumption (stage 3), but remained elevated compared to baseline. This study confirms that probiotic consumption can enhance natural cellular immunity in healthy individuals and this enhancement can persist after cessation of probiotic consumption. Delivery of the probiotic in lactose hydrolysed LFM results in immune enhancing effects comparable to those yielded by normal LFM indicating that this delivery method may prove useful in designing probiotic products targeted to lactose intolerant individuals.

The ability of the probiotics LGG and Bb12 (probiotic group), the inulin enriched oligofructose prebiotic called Synergy[®]1 (prebiotic group) and a synbiotic combination of Bb12, LGG and Synergy[®]1 (synbiotic group) to modulate the immune system of healthy rats consuming a high fat low fibre diet (western style diet) was investigated by Roller *et al.* (2004). In the prebiotic fed group IL-10 production in the Peyer's patches was enhanced and secretory IgA production was increased in the caecum compared to the control group receiving a standard, non-supplemented diet. The synbiotic combination increased secretory IgA production in the ileum and NK cell activity in peripheral blood, while it reduced the oxidative burst activity of blood neutrophils. Immune markers were unaltered in the group fed

probiotics (Bb12 & LGG) alone, however this may in part be due to the use of a high fat low fibre diet. This study indicates that combining probiotics and prebiotics in a synbiotic formulation does not simply result in an additive effect but in an enhanced effect greater than the combined effects of the individual components.

Probiotics have been shown to influence the balance between the arms of the T helper cell response. When fed probiotics *Lactobacillus salivarius* spp *salivarius* UCC118 and *Bifidobacterium infantis* 35624, Interleukin-10 (IL-10) knockout mice, had reduced levels of IL-12 and TNF- α both systemically and mucosally, and TGF- β levels were maintained, (McCarthy *et al.* 2003). IL-10 knockout mice develop colitis, which is similar to Crohn's disease in humans (Th1 type disease), and this study illustrates that probiotic consumption reduced production of Th1 type cytokines with an associated attenuation in colitis.

The modulation of cytokine production may also be mediated by constituents of probiotic bacteria and may not necessarily require whole bacteria. Lammers *et al.* (2003) showed that bifidobacterium genomic DNA induced secretion of IL-10 by PBMCs. Total bacterial DNA from faeces collected after probiotic administration modulated the immune response by a decrease of IL-1 β and an increase in IL-10.

It is postulated that the immune response to pre-, pro- and synbiotics depends on the host's immune status and for example, in atopic individuals, consumption may lead to an improved Th1 response. Pierre *et al.* (1997) reported that six week consumption of short chain fructo-oligosaccharides by multiple intestinal neoplasias (Min) mice significantly reduced the incidence of colon tumours and as well as

developing gut-associated lymphoid tissue compared to controls. Following on from this work this group reported that fructo-oligosaccharide fed to CD4⁺/CD8⁺ depleted Min mice resulted in twice as many colonic and caecal tumours as fructo-oligosaccharide fed immunocompetent Min mice, (Pierre *et al.* 1999), suggesting that prebiotic consumption stimulates a mechanism of tumour immuno-surveillance in which T cell status plays a primary role.

The immune response may also vary with different strains of probiotic, types of prebiotic and synbiotic combinations. Specific products with immuno-regulatory and/or anticancer effects need to be clearly identified and their mechanism of action characterised.

8.4.3 Inactivating potential carcinogens and mutagens

Mutagens and potential carcinogens are ingested every day as part of the normal diet. One possible mechanism by which pre-, pro- and synbiotics may protect against colon cancer is by binding and degrading potential mutagens and carcinogens. High meat consumption is considered a risk factor for colon cancer due to the production of mutagenic heterocyclic amines (HCA), which are formed during cooking, (Sinha & Rothman 1999). Common HCAs are 2-amino-3-methylimidazo [4,5-*f*] quinoline (IQ), 2-amino-3,4-dimethylimidazo [4,5-*f*] quinoline (MeIQ), 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP), the tryptophan pyrolysis products Trp-P-1 and Trp-P-2 and the glutamic acid pyrolysis products Glu-P-1 and Glu-P-2. HCAs themselves are not mutagenic but, like most chemical carcinogens, require metabolic activation. The metabolism of HCA is under genetic control, (Potter 1996).

Lactic acid bacteria have been shown to bind HCA *in vitro* dependent on the pH of the culture medium, (Morotomi & Mutai 1986; Orrhage *et al.* 1994; Zhang & Yoshiyuki 1991). *L.casei* YIT 9018 has been shown to bind *in vitro* to the mutagenic pyrolysate Trp-P-2 formed in cooked meat, and consequentially to prevent the mutagenic effect of Trp-P-2 in the Salmonella mutagenicity assay, (Morotomi & Mutai 1986). Consumption of lactobacilli in healthy human feeding studies has been shown to reduce the mutagenicity of faeces and urine associated with the ingestion of carcinogens in cooked meat, (Hayatsu & Hayatsu 1993; Lidbeck *et al.* 1992).

A lyophilised culture of *Bif. longum* has been shown to have a 100% inhibitory effect on IQ induced colon carcinogenesis in male colon cancer and to lesser extent (50% inhibition) in mammary carcinogenesis in female rats, (Reddy & Rivenson 1993). A possible explanation for the complete inhibition in colon carcinogenesis in this model may be that the *Bif. longum* binds the IQ in the lumen preventing interaction of the mutagen with the mucosa. Rowland & Grasso (1975) demonstrated that lactobacilli have the ability to degrade nitrosamines, mutagens associated with cooked meat. Wollowski *et al.* (2001) showed that *Lactobacillus bulgaris* 191R fed to rats reduced DMH induced DNA damage in colon cells as measured in the comet assay.

8.4.4 Altering the composition of the intestinal flora

Imbalance in the composition of the GI flora has been associated with increased risk of colon cancer, (Gorbach & Goldin, 1990). The consumption of pre-, pro- and synbiotics has been reported to shift the composition of the gut flora to a more beneficial composition. Most studies in this area have shown that pre-, pro or

synbiotic consumption increases bifidobacteria and lactobacilli numbers, (Kruse *et al.* 1999; Tuohy *et al.* 2001). However some studies have demonstrated that pre-, pro- or synbiotic consumption can affect other members of the gut flora.

Spanhaak *et al.* (1998), demonstrated that consumption of a milk fermented by *Lactobacillus casei* strain *shirota* significantly increased lactobacilli and bifidobacteria and decreased the numbers of clostridium although not significantly. Gibson *et al.* (1995) demonstrated that consumption of the prebiotics, oligofructose and inulin, significantly increased the numbers of faecal bifidobacteria, while decreasing the numbers of Bacteroides, clostridia, fusobacteria and Gram positive cocci.

Fujiwara *et al.* (2001) fed seven healthy volunteers a rifampicin-streptomycin-resistant variant of the probiotic *Bifidobacterium longum* SBT2928 for seven days with a run in period of three days and a follow up period of thirty days. They found that the introduced probiotic strain was recovered in high numbers from the faeces of all the volunteers during the seven-day administration period and was detectable in the faeces of two volunteers up to thirty days after cessation of administration. The numbers of Enterobacteriaceae, lecithinase-negative *Clostridium* spp and *C. perfringens* were significantly reduced after seven days administration of the probiotic, however numbers increased towards baseline levels thirty days after cessation of feeding.

In a study investigating the influence of the probiotic *Lactobacillus salivarius* spp *salivarius* UCC118 (UCC118) on inflammation and tumour development in IL-10

knockout mice, the numbers of coliforms and enterococci recovered from faeces were decreased in the mice fed UCC118 (n=10) for sixteen weeks compared to the control mice (n=10). Total lactobacilli, bifidobacteria and bacteroides levels were unchanged in both the control and probiotic fed groups. The prevalence of colon cancer and mucosal inflammatory activity was significantly reduced in the mice fed UCC118 compared to control mice, (O'Mahony *et al.* 2001).

8.4.5 Anti-mutagenic or anti-tumourigenic activities

The antimutagenic potential of pre-, pro- and synbiotics has been confirmed in numerous studies. Klinder *et al.* (2004b) carried out *in vitro* fermentations of various combinations of a prebiotic (Synergy[®]1), Bb12 and GG (probiotics) and the synbiotic combination (Bb12, GG and Synergy[®]1) either alone (pure culture fermentations) or in the presence of faecal slurries. Control fermentations where glucose replaced Synergy[®]1 as the sole carbon source were also performed. Supernatants from the fermentations containing Synergy[®]1 (prebiotic and synbiotic) were less cytotoxic to HT-29 and Caco-2 cells, compared to supernatants derived from control fermentations. The reduced cytotoxic potential of the supernatants seems to be associated with the increased butyrate concentrations in the prebiotic containing fermentations compared to controls and probiotics.

Zsivkovits *et al.* (2003) reported that *Lactobacillus bulgaricus* 291, *Streptococcus thermophilus* F4, *S. thermophilus* V3 and *Bifidobacterium longum* BB536, strains commonly used in yoghurt production, completely inhibited genotoxic damage in the colons of rats induced by hetero-cyclic amines of the type found in cooked beef. Other studies have shown that some lactic acid bacteria inhibit N-Methyl-N'-Nitro-

N-Nitrosoguanidine (MNNG) and DMH induced genotoxicity in colon cells isolated from rats, (Pool-Zobel *et al.* 1996; Wollowski *et al.* 1999).

Klinder *et al.* (2004a) reported that the genotoxic potential of FW from rats treated with AOM was significantly reduced in animals fed both Synergy[®]1 and a synbiotic (Synergy[®]1, Bb12 and GG). Burns & Rowland (2004) investigated the effect of incubating a genotoxic faecal water sample from a human subject with six different strains of lactic acid bacteria on the DNA damaging potential of the FW. They found that DNA damage in HT-29 cells was significantly reduced by all strains except *Strep. thermophilus* and that *Lactobacillus plantarum* and Bb12 showed the greatest protective effect. The bacterial strains were also fermented in the presence of prebiotics and the ability of the resultant supernatant to protect against FW induced DNA damage investigated. Incubations involving *Lactobacillus plantarum* and fructo-oligosaccharide based prebiotics were most effective in preventing genotoxic damage. Reduction in FW induced DNA damage was also seen with supernatants from fermentations of Bb12 and prebiotics.

In animal studies co-administration of pre-, pro- or synbiotics with a chemical carcinogen reduces the incidence of both pre-cancerous lesions (aberrant crypt foci) and colonic tumours. Aberrant crypt foci (ACF) are early preneoplastic lesions in the colon from which adenomas and carcinomas may develop. ACF have consistently been observed in experimentally induced colon carcinogenesis in laboratory animals and have also been shown in the colonic mucosa of patients with colon cancer, (McLellan *et al.* 1991; Pretlow *et al.* 1992; Wargovich *et al.* 1996). ACF express mutations in the *apc* and *ras* oncogenes, which are involved in cancer development,



(Vivona *et al.* 1993). ACF formation is often used in animal studies as an end point for investigating potential anti-tumourigenic agents and several inhibitors of ACF formation have been shown to reduce the incidence of colonic tumours in laboratory animals, (Wargovich *et al.* 1996). The multiplicity of aberrant crypts is also measured in many studies and ACF with four or more crypts is considered predictive of tumour incidence, (Pretlow *et al.* 1992).

Prebiotics have demonstrated an inhibitory effect on ACF and tumour formation. Reddy *et al.* (1997) demonstrated that consumption of inulin or oligofructose inhibits ACF formation and multiplicity in the colons of rats treated with the chemical carcinogen AOM. The degree of ACF inhibition was more pronounced in rats fed inulin than in those fed oligofructose. Taper *et al.* (1997) reported that the consumption of oligofructose or inulin significantly inhibited the growth of transplantable mouse tumours, EMT6 and TLT in mice.

Prebiotics have also been shown to stimulate apoptosis in the colons of rats, consuming a high fat western type diet and treated with the carcinogen DMH, (Hughes & Rowland 2001). Apoptosis was significantly higher in the colon of rats fed diets supplemented with either oligofructose or inulin compared to rats fed the basal diet. Induction of apoptosis and the resultant effect on proliferation may be one of the mechanisms by which prebiotics exert cancer protecting effects.

Probiotics have also been shown to inhibit colon cancer. *Lactobacillus acidophilus* feeding has been shown to decrease the incidence of colon tumours in rats challenged with the colon carcinogen DMH from 77% to 40%, (Goldin & Gorbach

1980). Reddy & Rivenson (1993) demonstrated that *Bif. longum* consumption inhibited the IQ-induced incidence of colon (100%) and liver (80%) tumours and multiplicity (tumours/animal) of colon, liver, and small intestinal tumours in male rats. In female rats, IQ-induced mammary carcinogenesis was inhibited (although not significantly) to 50% and liver carcinogenesis to 27% compared to control animals, however, the mammary tumour multiplicity (tumours/animal) was significantly inhibited in female rats fed *Bif. longum*. A possible explanation for the complete inhibition of colon carcinogenesis in this model may be that the *Bif. longum* binds the IQ in the lumen preventing interaction of the mutagen with the mucosa. Singh *et al.* (1997) demonstrated that consumption of lyophilised *Bif. longum* significantly inhibited tumour incidence and multiplicity in AOM treated animals compared to controls.

Consumption of *Lactobacillus salivarius* ssp. *salivarius* UCC118 in interleukin-10 knockout mice (colitis model) was associated with a trend towards reduced tumour development and attenuation of gastrointestinal inflammation. Gastrointestinal neoplastic transformation was evident in 50% of mice from the placebo group while only 10% of the probiotic group demonstrated any evidence of neoplastic change, (O'Mahony *et al.* 2001).

The anti-tumourigenic potential of probiotics has also been shown in animal studies although research in this area is limited. Biasco *et al.* (1991) reported that elevated cell proliferation in the colonic mucosa was decreased by probiotic consumption in some patients with benign tumours in the colon.

Synbiotics have demonstrated similar anti-tumourigenic properties. Rowland *et al.* (1998) investigated the effect of a derivative of inulin (Raftiline HP) alone and in combination with *Bif. longum* on ACF induction by AOM. *Bif. longum* consumption alone resulted in a 26% reduction in small ACF (one to three aberrant crypts per focus), and inulin alone inhibited ACF formation by 41% compared to control. However when administered together as a synbiotic, small ACF formation was inhibited by 80%. This synergistic effect was particularly evident as the synbiotic inhibited large ACF formation (>4 aberrant crypts/focus) by 59% while inulin and *Bif. longum* alone were ineffective.

Many animal studies designed to investigate the effect of pre-, pro- or synbiotics on colon carcinogenesis are short-term experiments where intervention is carried out for approximately 12 weeks and use the formation of ACF as a marker. There is very little data available for pre-, pro- or synbiotics in long term carcinogenesis experiments. Femia *et al.* (2002) investigated the effect of consumption of a prebiotic Synergy[®]1, the probiotics *Lactobacillus rhamnosus* GG (GG) and *Bifidobacterium lactis* Bb12 (Bb12), and a synbiotic containing the prebiotic and both probiotics for 31 weeks on AOM induced carcinogenesis in rats. The results show that the prebiotic and the synbiotic fed groups, but not the probiotic fed group, had significantly reduced tumour incidence in the colon compared to the control group. The synbiotic fed group also had fewer tumour bearing rats than the prebiotic, probiotic or control groups.

8.4.6 Altering the metabolic activity of the flora

The normal colonic microflora produces enzymes such as β -glucuronidase, β -glucosidase, nitrate reductase, nitroreductase and azoreductase, the activities of which have been implicated in colon carcinogenesis. Bifidobacteria and lactobacilli have lower activities of these xenobiotic-metabolising enzymes than do Bacteroides, clostridia and enterobacteriaceae. Decreasing the numbers of bacteria with high activities of these enzymes and increasing the numbers of bacteria with lower activities is a potential mechanism for cancer prevention. Pre-, pro- and synbiotics can alter the composition of the gut flora however the activities of bacterial enzymes are not always altered.

Consumption of pre- and probiotics has been shown to decrease the activity of β -glucuronidase. Ling *et al.* (1994) demonstrated that consumption of LGG alone or LGG with fibre for two or four weeks significantly reduced faecal β -glucuronidase and nitroreductase activities as well as glycocholic acid hydrolase activities in healthy volunteers. Activities of these enzymes returned to baseline levels following cessation of feeding. β -glucosidase activities were not significantly altered by intervention. Fujiwara *et al.* (2001) demonstrated that seven-day consumption of *Bif. longum* SBT2928 significantly decreased β -glucuronidase and β -glucosidase levels and was associated with decreases in the numbers of Enterobacteriaceae and *C. perfringens*.

Marteau *et al.* (1990) reported that three-week consumption of a dairy product fermented with *L. acidophilus*, *B. bifidum*, *S. lactis* and *S. cremoris* reduced faecal

nitroreductase and increased β -glucosidase activities. β -glucuronidase, β -galactosidase and azoreductase activities were unaltered.

Azoreductase promotes the reduction of azo food dyes to substituted phenyl and naphthyl amines, which are potent carcinogens. Laboratory rats, which have been experimentally, fed beef and lactobacilli have reduced faecal amounts of beta-glucuronidase, azoreductase, and nitroreductase, (Goldin & Gorbach 1984; Simon & Gorbach 1986).

Bouhnik *et al.* (1996) investigated the effect of bifidobacteria fermented milk with or without fructo-oligosaccharide (inulin) on the activity of pro-carcinogenic enzymes in twelve healthy subjects. The results showed that consumption of bifidobacteria fermented milk increased the number of total bifidobacteria and decreased β -glucuronidase, while nitrate reductase, nitroreductase and azoreductase were unaltered. Co-administration of inulin also increased the total number of bifidobacteria but had no influence on enzyme levels. Rowland *et al.* (1998) found that co-administration of *Bif. longum* and inulin significantly reduced the number of ACF in the colon, the concentration of ammonia in caecal samples as well as caecal β -glucuronidase activities. Caecal β -glucosidase activities however were increased.

However as mentioned by Rafter (2002), 'it is important to note that the studies to date do not always find reductions in the same enzymes, although findings with β -glucuronidase and nitroreductase are the most consistent'.

8.4.7 Modulating the activity of host enzymes

The activity of detoxifying enzymes plays an important role in the protection of the colonic mucosa from carcinogens and mutagens. The Glutathione-S-transferase (GST) and UDP-glucuronosyl transferase (UGT) superfamilies play an important role in the conversion of the reactive intermediate back to the parent amine therefore protecting epithelial DNA from adduction by activated HCA, (Airoldi *et al.* 2004).

Treptow-van Lishaut *et al.* (1999), demonstrated that resistant starch (a prebiotic), increased levels of GST π in the rat colon. Consumption of the prebiotic lactulose, either alone, or in conjunction with *Bif. longum* reduced the number of ACFs, and significantly increased GST levels in the rat colon compared to controls, (Challa *et al.* 1997). SCFAs, the main fermentation products of the microflora have been shown to induce GST π in a Caco-2 cancer cell line. It is also interesting to note an increase in GST in rectal biopsies following consumption of Brussels sprouts was mirrored by an increase in GST activity in blood lymphocytes. GST levels in lymphocytes may be used as a marker for cancer risk or to assess the effect of intervention on cancer progression.

The genes hepatic cytochrome P4501A2, (CYP1A2) and acetyltransferase-2 (NAT-2), which are polymorphic in humans have been implicated in the metabolic activation of heterocyclic amines to colon carcinogens which readily form DNA adducts, (Gooderham *et al.* 1996; Sawada & Kamataki 1998). NADPH-cytochrome P-450 reductase (CYC) is a phase I xenobiotic metabolising enzyme involved in the biotransformation of noxious xenobiotics before excretion. Lactic acid bacteria have been shown to increase CYC activity in the colon, (Pool-Zobel *et al.* 1996).

Ornithine decarboxylase (ODC) is the first and rate-limiting enzyme in the synthesis of polyamines. Polyamines are involved in cell proliferation and differentiation and in macromolecular synthesis. In humans ODC levels are elevated in neoplastic colonic mucosa compared to normal appearing mucosa, in dysplastic polyps compared to nondysplastic polyps and in colonic adenocarcinomas compared to adjacent non-involved mucosa, (Reddy 1999). Studies have shown that probiotic administration in animal models of chemically induced colon carcinogenesis reduce the levels of ODC, (Singh *et al.* 1997).

Ras activation is one of the earliest and most frequently occurring genetic alterations associated with human cancers, especially colon cancer, (Barbacid 1990). *Bif. longum* consumption has been shown to reduce ras-p21 expression and cell proliferation in AOM induced carcinogenesis in rats (Reddy 1998; Singh *et al.* 1997).

The evidence outlined suggests possible mechanisms by which pre-, pro- or synbiotics may protect against colon cancer. However it must be noted that there is no direct experimental evidence that pre-, pro- or synbiotics play a role in cancer suppression in man.

9. Future Trends

Many studies have shown that the co-administration of pre- and probiotics in a synbiotic combination leads to a greater effect than that achieved with either component alone. However, many of these studies did not screen candidate pre- and

probiotics in order to determine the optimal synbiotic combination. In general there is a lack of studies concentrating on the optimal formulation of synbiotics from thoroughly characterised prebiotics and probiotics followed by confirmation of their effectiveness both *in vitro* and most importantly *in vivo*. When selecting a synbiotic combination for clinical use the synbiotic formulation should be targeted towards the disease, as a synbiotic combination effective, for example, in ameliorating antibiotic associated diarrhoea may be ineffective in lowering cancer risk.

The design of effective synbiotics may further be improved by targeting prebiotics for a specific probiotic. Rabiou *et al.* (2001) isolated β -galactosidases from five bifidobacterium strains and used these enzymes in glycosyl transfer reactions to synthesize oligosaccharides from lactose. Fermentation tests showed an increased growth rate compared to oligomate 55, a commercial prebiotic galacto-oligosaccharide, for four out of five of the bifidobacterium strains on the product derived using their own enzyme. One of the novel prebiotics was further tested in the presence of mixed populations of intestinal bacteria and was seen to have an equivalent prebiotic activity to that of Oligomate 55. However, this novel prebiotic exhibited a lower stimulatory effect on *Bacteroides* and *Lactobacilli* than did Oligomate 55, suggesting that it is more selective. The potential of plant cell wall polysaccharides as sources of novel prebiotics has recently been investigated. Specific glycanases can be used to produce novel oligosaccharides from plant cell wall polysaccharides, (Van Laere *et al.* 2000).

The tolerance of the immune system to pre-, pro- and synbiotics has led to much research in their use as delivery methods for vaccines and treatments. Attenuated

pathogens are effective vaccines against the pathogen itself, however these organisms are often strongly immunogenic and are therefore not suitable for use in immunocompromised individuals for whom they may be potentially pathogenic. As lactic acid bacteria have been consumed by large numbers of people for many years without causing health problems, they are an attractive vehicle for the delivery of vaccines.

Isolauri *et al.* (1995) administered LGG concomitantly with the D x RRV reassortant rotavirus vaccine to young infants and evaluated the immune response following vaccination. Results suggested that the immunogenicity of an oral live rotavirus vaccine may be improved by concomitant and subsequent administration of LGG. Kruisselbrink *et al.* (2001) used a recombinant lactobacillus to induce tolerance in mice against a component of the house dust mite, demonstrating the usefulness of probiotic bacteria in the treatment of allergic and autoimmune diseases. Steidler *et al.* (2000) engineered a *Lactococcus lactis* strain to secrete an anti-inflammatory cytokine (IL-10). The therapeutic effect of the engineered strain was comparable to conventional steroid treatment in murine models of inflammatory bowel disease. The potential of probiotic bacteria to be used as delivery vehicles seems very promising.

The multistage carcinogenesis process occurs over a long time period and makes it difficult to assess the effectiveness of pre-, pro- and synbiotic intervention without a lengthy follow-up period. An attractive alternative to time consuming, labour intensive follow-up studies is the use of validated cancer risk biomarkers as endpoints in intervention studies. Information on the ability of pre-, pro- and synbiotics to modulate many different biological parameters has been generated

from studies investigating the efficacy of pre-, pro- and synbiotics in the treatment or prevention of many different disease processes. This information has and will continue to prove invaluable in the development of a strategy for their application in colorectal cancer.

Analysis of the gene expression profiles of normal tissue, polyps, tumour tissue and normal tissue adjacent to tumours has identified many genes which may be involved in the promotion or progression of colon cancer. Gene array analysis has already led to the development of several new methods for use in diagnosis, patient stratification and therapy. All factors of the genetic pathway in colorectal cancer are potential targets for therapy and further work is needed to determine if pre-, pro- or synbiotic intervention can effectively target specific genes with an associated beneficial effect.

10. Concluding Remarks

Symptoms of colorectal cancer present at a late stage in the disease process and therefore, early prevention and detection of preneoplastic changes should be the main area of focus in the coming years. Non-invasive cancer risk biomarkers, similar to those discussed earlier, which herald early events in the cancer process, can be used to identify individuals at risk of colon cancer and also to assess efficacy of preventative measures.

Synbiotics offer great potential as a preventative strategy. Synbiotic intervention in animal models of colon carcinogenesis has yielded very promising results. Current studies in humans should further elucidate the mechanisms involved in their

preventative action and their possible role in an early intervention strategy. In this area there is a need for well-designed and executed intervention studies. The synbiotic combination should be selected from well characterised pre- and probiotics and based on its ability to affect the disease process. The study design should be double-blinded and placebo controlled and should investigate a well-defined population. The use of proven biomarkers is essential in assessing the efficacy of the intervention.

Progress in the area of detection of cancer and the development of early preventive measures, such as synbiotics, will lead to a dramatic change in the morbidity and mortality pattern of colorectal cancer in the developed world.

11. Bibliography

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Chapter 2

General Methods

Study Outline

A twelve week randomised, double blind, placebo controlled trial of a synbiotic food supplement, containing a synbiotic mixture of *Lactobacillus rhamnosus* GG (LGG) and *Bifidobacterium lactis* Bb12 (Bb12) and the inulin-type prebiotic Raftilose[®] Synergy[®]1 (inulin) for reduction in cancer risk biomarkers was carried out in The Mercy University Hospital, Cork, Ireland. This study was evaluated and approved by the Cork University Hospitals Ethics Committee.

The strains selected for this study were *Lactobacillus rhamnosus* GG (Valio, Finland) and *Bifidobacterium lactis* Bb12 (Christian Hansen Labs, Sweden) the properties of which were discussed previously (*chapter 1 sections: 8.1.4 & 8.1.5*). It was thought that a combination of two probiotic strains would be more effective than a single strain alone. Studies have shown that adherence of Bb12 to ileostomy glycoproteins and to the intestinal mucus of infants is enhanced in the presence of LGG indicating that simultaneous use of these strains may be more efficacious than individual use (Juntunen *et al.* 2001; Ouwehand *et al.* 2000). From a practical point of view it was decided to use existing commercial strains with a history of anticancer properties in experimental models.

The prebiotic selected for use in this study is a 1:1 mixture of long chain and short chain fractions of inulin extracted from chicory roots (*Cichorium Intybus*) and known as oligofructose-enriched inulin or 'Synergy[®]1' (Raftilose Synergy[®]1, Orafit, Belgium). One fraction is composed of linear β (2-1) fructans with a degree of polymerisation (DP) ranging between ten and sixty-five (average DP 25) and the other fraction contains similar molecules but with chain length varying between two

Inclusion Criteria:

Subjects must, within the last 5 years, have

- had a biopsy- and histologically-confirmed adenomatous polyp
- or
- undergone resection for histologically-confirmed colon cancer

Both retrospective and prospective polypectomy and colon carcinoma resected subjects may be enrolled.

Exclusion Criteria:

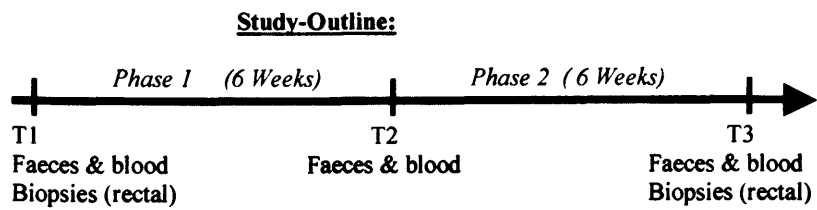
- Subjects over 75 years of age.
- Pregnancy or a desire to become pregnant during the study period
- Subjects with known lactose intolerance and subjects with clinically significant immunodeficiency
- Subjects who are considered to be poor clinic attendees or unlikely for any reason to be able to comply with the trial
- Concomitant Drug usage – subjects will be excluded if they require COX-2 inhibitors or other selected NSAIDs
- Subjects who have been on antibiotics within the previous month or are likely to require antibiotics during the trial period will be excluded
- Subjects with additional gastrointestinal disorders, (e.g. Crohn's disease or ulcerative colitis)
- Malignancy or any concomitant end-stage organ disease

Figure 1: Study inclusion and exclusion criteria

and eight (average DP 4). The latter molecules are rapidly fermented by the colonic flora leading to a modification of the composition of the intestinal flora in the proximal part of the colon. The longer chains are typically fermented at a slower rate, but more selectively than the short-chains. This longer chain fraction has the ability to maintain the metabolic activity of the improved flora for a lengthy time period, or spatially, in more distal parts of the intestine. In experimental models this mixture of inulin-type fructans reduces the incidence of aberrant crypt foci significantly more than its individual components alone, (Verghese *et al.* 2003).

A dedicated study nurse was employed to identify subjects from the patient lists of consultant gastroenterologists in The Mercy University Hospital, Cork, Ireland, who fulfilled the inclusion and exclusion criteria of the study, (*Figure 1*). The study nurse then contacted potential subjects and outlined the study protocol. Interested subjects met with the study nurse in her consulting room, and a full patient history and informed written consent were obtained at least one week before the subject began the intervention.

The outline of the study was as follows:



The subjects attended appointments with the study nurse at T1 (before intervention), T2 (following six weeks intervention) and T3 (following twelve weeks intervention).

Figure 2: Faecal parameters investigated

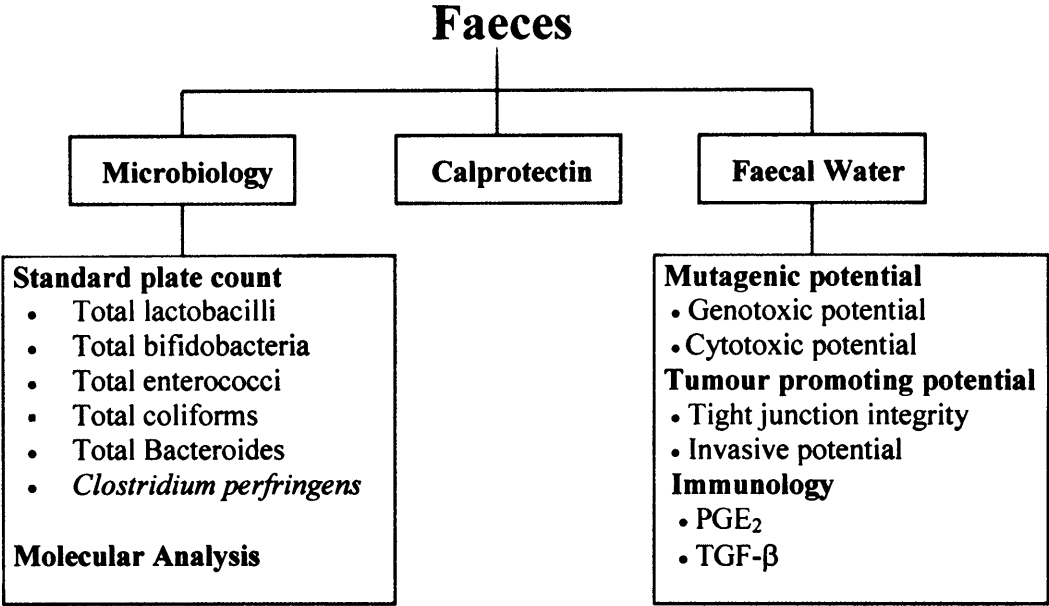


Figure 3: Blood parameters investigated

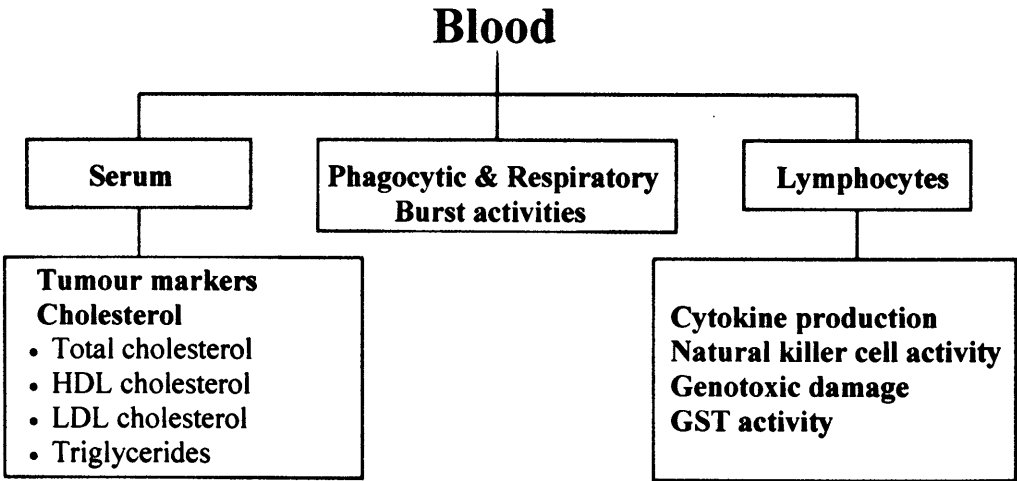
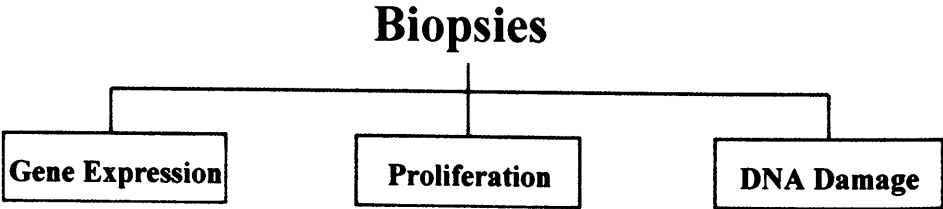


Figure 4: Biopsy parameters investigated



Subjects were randomly assigned to two groups. One group received the synbiotic product consisting of encapsulated probiotic bacteria (10^{10} cfu LGG and 10^{10} cfu Bb12) and a 12g sachet of Synergy[®]1 and the other group received the placebo product, consisting of encapsulated maltodextrin and a 12g sachet of maltodextrin. Subjects kept a six-week diary detailing general health, appetite, smoking and alcohol consumption for each phase of the intervention. At T1 and T2 the subjects received a box containing sufficient product for six-weeks. At T2 and T3 subjects were interviewed by the study nurse, and reactions to the product, medications taken and any adverse events, which had occurred in each six-week period were recorded. The amount of product returned was also recorded to confirm compliance.

Subjects provided samples at three time points. Faecal (*Figure 2*) and blood (*Figure 3*) samples were provided at the beginning of intervention (week 0), mid-way through intervention (week 6) and at the end of intervention (week 12) and numerous parameters were investigated.

Biopsy samples (*Figure 4*) were provided at the beginning of intervention (week 0) and at the end of intervention (week 12). Because bowel preparations (i.e. enemas and laxatives) used before endoscopic examinations have been reported to affect colon proliferation, (Kleibeuker *et al.* 1995) patients were instructed not to take any laxatives or enemas before endoscopy, which was performed in either the morning or afternoon (between 10 am and 3 pm). Rectal biopsies were obtained without sedation, by rigid sigmoidoscopy from normal-appearing mucosa at 10 to 15 cm from the anal verge and transferred immediately to appropriate storage media.

Figure 5: Recruitment and randomisation process

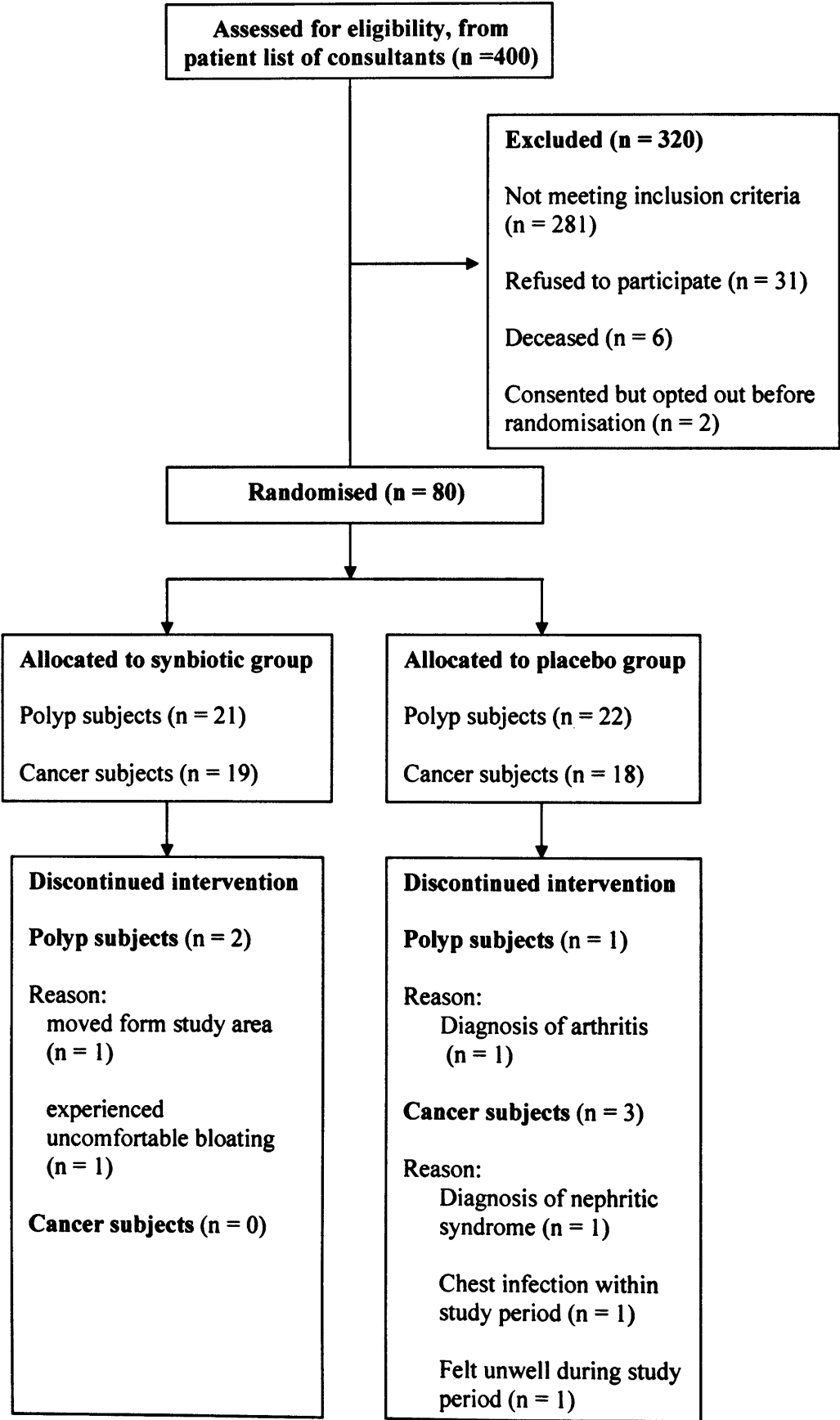


Figure 5 shows the recruitment and randomisation processes. A total of eighty subjects (forty three polyp, thirty seven cancer) were recruited to the intervention study from which six patients subsequently withdrew. Overall, thirty four cancer subjects completed the trial, of which thirteen were female and twenty one were male, with an average age of 60.1 ± 5.8 and 62.1 ± 5.3 (mean $\pm$ SD), respectively. In this group, fifteen subjects consumed placebo and nineteen consumed synbiotic. A total of forty polyp subjects successfully completed the trial, eighteen were female and twenty-two were male, with an average age of 56.0 ± 9.8 and 58.0 ± 9.7 (mean $\pm$ SD), respectively. In the group of polyp subjects, twenty-one subjects consumed placebo and nineteen subjects consumed synbiotic.

There were no adverse effects of intervention, however some subjects consuming the synbiotic reported a feeling of fullness and excessive bloating, both of which are commonly associated with prebiotic consumption, (Cummings *et al.* 2001). Feedback from most subjects was positive with many patients expressing the wish to continue on the product after intervention was completed.

Blinding of the Study

The study was conducted in a double-blinded manner. Subjects were coded from Syn-01 to Syn-80 and each time point was coded either X, Y or Z. Coded samples were processed and analysed in a blinded manner. For the duration of the study the code was stored by one individual and following completion of all analyses was disclosed.

Product Transit

A common criticism of probiotic research is that the fate of the introduced bacterium in the GI tract is not always investigated. It is important to confirm that introduced probiotic strains transit the GI tract so that any observed effect may be associated with probiotic consumption. In the human intervention study subjects consumed wild type probiotic strains, which cannot be selectively isolated from the faeces of subjects. Therefore spontaneously occurring rifampicin-resistant (rif^R) mutants ($50\mu\text{g/ml}$) were selected from Bb12 and LGG. Previous experience in humans demonstrated that such rif^R strains were not normally present in stool samples, and that rifampicin resistance is a suitable marker to allow selective isolation and enumeration of introduced probiotic strains from faeces, (Dunne *et al.* 1999, 2001). The rifampicin resistant strains were freeze-dried and the resultant freeze-dried product was checked for purity.

Briefly a 1.5L fermenter containing modified De Man, Rogosa, Sharpe (MRS) broth [0.4% yeast extract, 2% glucose, 0.2% K_2HPO_4 , 0.5% Na-acetate, 0.2% $(\text{NH}_4)_3$ citrate, 0.02% MgSO_4 , 0.0046% MnSO_4 and 1.8% peptone] for LGG rif^R or modified MRS broth containing 0.05% cysteine for Bb12 rif^R was inoculated with overnight cultures of rif^R strains. Fermentation was allowed to proceed for sixteen hours at 37°C and constant pH 5.5 with agitation. The suspension was centrifuged, the pelleted bacteria resuspended in 10% skim milk, 2% sucrose and ascorbic acid and frozen at -80°C for at least one hour. The bacteria were freeze-dried for 48 hours and viable bacteria per gram of freeze-dried product were enumerated. Freeze-dried powder was serially diluted 10-fold in sterile PBS / 0.05% cysteine and appropriate dilutions were spread-plated on rifampicin containing MRS agar (LGG) and

modified raffinose bifidobacterium agar, (Hartemink *et al.* 1996), where raffinose was replaced with lactose, in order to exclude the growth of LGG and isolate Bb12. Bb12 rif^R freeze-dried powder was found to contain 5.9×10^{10} c.f.u./g and LGG rif^R powder contained 4.7×10^{10} c.f.u./g.

The freeze-dried rif^R strains and the wild-type parent strains were found to be identical using API (a rapid biochemical test). In this project it was considered desirable that the probiotics reach the colon in a viable form in order to exert beneficial health effects. As the acid environment in the stomach is the main reason for bacteria not surviving passage through the upper intestinal tract, the probiotics were administered in a coated capsule. The coating resists the acid conditions in the stomach, and releases it's contents in the small intestine. The probiotic strains selected have previously been shown to resist the bile acids that are secreted in the small intestine.

The freeze-dried strains were encapsulated and a pilot study to confirm probiotic transit was conducted with three healthy volunteers who consumed one capsule containing 10^{10} cfu of both LGG rif^R and Bb12 rif^R with a sachet of Synergy[®]1 (12g) every day for a seven-day period. The volunteers provided faecal samples before feeding and at the end of feeding. The number of rif^R bacteria present in faeces before and after seven days of feeding were enumerated using standard plate count techniques. Total stool from one passage was collected in a plastic container and mixed using a sterile wooden spatula. An aliquot was used to make a faecal slurry with sterile 60% phosphate buffered saline solution (PBS) / 40% Glycerol / 0.05% cysteine which was serially diluted 10-fold in sterile PBS / 0.05% cysteine.

Appropriate dilutions were spread-plated on rifampicin containing MRS agar (LGG) and modified raffinose bifidobacterium agar (Bb12) as described previously.

In three healthy volunteers fed for seven days with rif^R Bb12 and LGG (10¹⁰ cfu of each strain/day) and Synergy[®]1, average counts of 4.17 x 10⁶ and 2.65 x 10⁷ cfu/g of faeces of rif^R lactobacilli and rif^R bifidobacteria respectively were recovered from the faeces (**Figure 6**). There were no rif^R bacteria present in the faeces before feeding. This result indicates that the probiotic strains consumed in this study possess the ability to transit the human gastrointestinal tract.

In the intervention study, subjects in the symbiotic group consumed probiotic capsules every day for a twelve-week period. Subjects received a six-week supply of probiotic capsules.

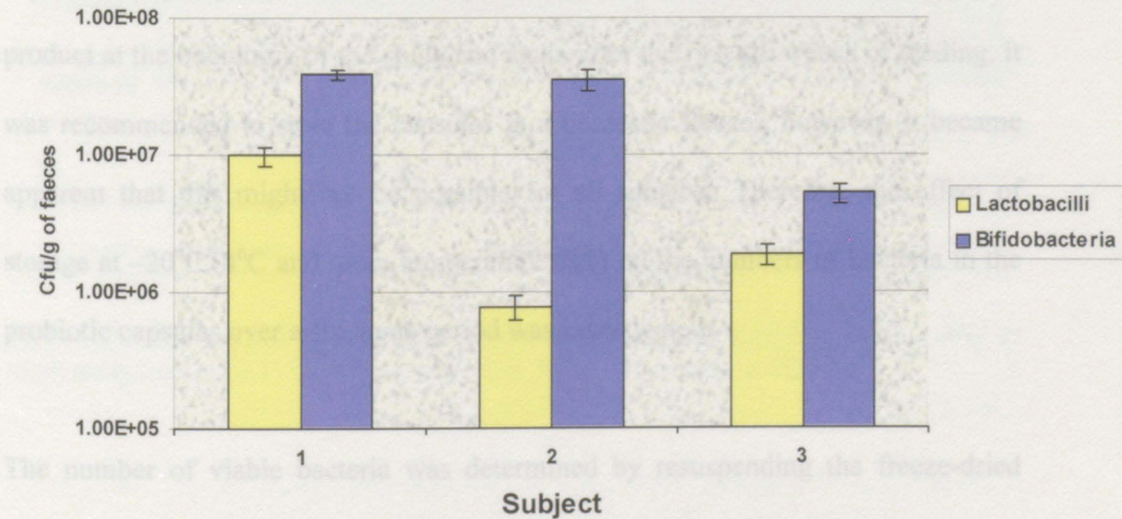


Figure 6: Numbers of rifampicin resistant lactobacilli and bifidobacteria recovered from faeces of healthy volunteers after 7 days feeding with rif resistant Bb12 and LGG.

It was found that the optimal temperature for storage of the probiotic capsules was at -20°C, however, storage at 4°C or room temperature did not result in a significant decrease in numbers as counts remained above 9.5 x 10⁹ cfu / capsule irrespective of

Product Quality & Stability

Before beginning the intervention study and during the study period product quality and stability was investigated. Batches of synbiotic and placebo product were received from Orafiti, (Tienen, Belgium), as required throughout the intervention. Microbial analysis was performed on each new batch and the numbers of probiotic bacteria in the synbiotic capsules was consistently found to be 10^{10} cfu of each bacterial strain. The sachets of Synergy[®]1 and maltodextrin as well as the maltodextrin capsules were free from detectable contaminants.

In the intervention study, subjects in the synbiotic group consumed probiotic capsules every day for a twelve-week period. Subjects received a six-week supply of product at the beginning of the study and again after the first six weeks of feeding. It was recommended to store the capsules in a domestic freezer, however, it became apparent that this might not be possible for all subjects. Therefore the effect of storage at -20°C , 4°C and room temperature (RT) on the numbers of bacteria in the probiotic capsules over a six-week period was investigated.

The number of viable bacteria was determined by resuspending the freeze-dried contents of a capsule in PBS / 0.05% cysteine and serially diluting 10-fold in sterile PBS / 0.05% cysteine. Appropriate dilutions were spread plated on MRS agar (LGG) and modified raffinose bifidobacterium agar (Bb12) as described previously.

It was found that the optimal temperature for storage of the probiotic capsules was at -20°C , however, storage at 4°C or room temperature did not result in a significant decrease in numbers as counts remained above 9.8×10^9 cfu / capsule irrespective of

the storage condition (**Figure 7**). Therefore in the human intervention study, subjects consumed consistent numbers of viable probiotic bacteria throughout irrespective of the storage temperature.

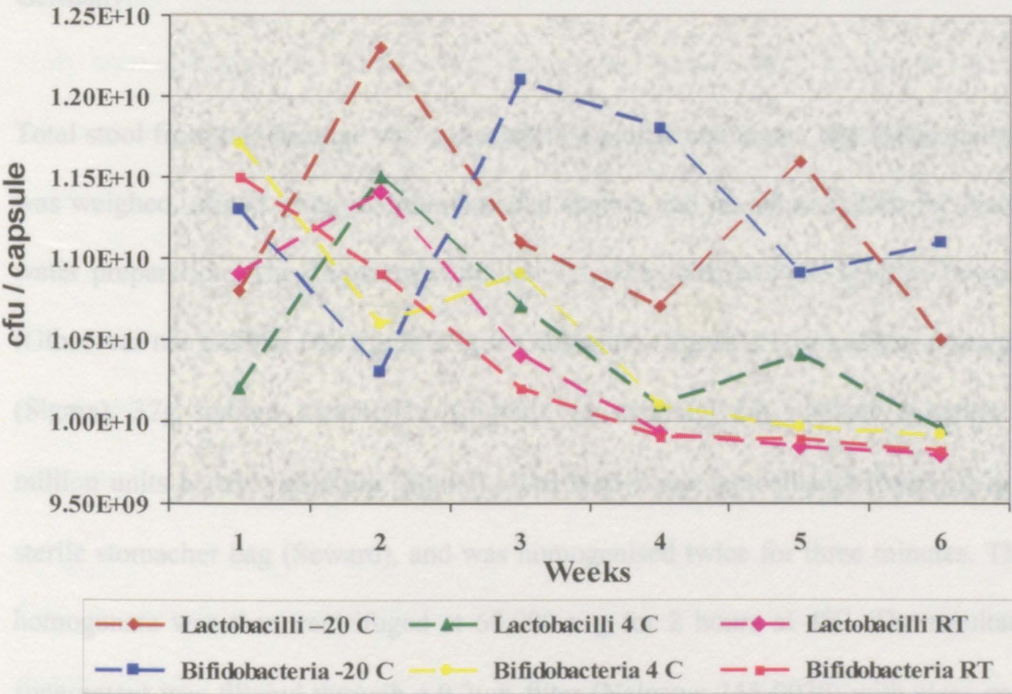


Figure 7: Survival of encapsulated freeze-dried bacteria stored at -20°C , 4°C or room temperature (RT) for a 6 week period. Values are mean $\pm$ SD.

Faecal Water (FW) Isolation

The method employed for the isolation of FW in this study was developed in collaboration with Dr. Annett Klinder and Prof. Beatrice Pool-Zobel at the Department of Nutritional Toxicology, Friedrich-Schiller University of Jena, Jena, Germany.

Total stool from one passage was collected in a plastic container. The faecal sample was weighed, mixed using a sterile wooden spatula and an aliquot taken for faecal water preparation. The aliquot was diluted 1:1 with DMEM [10x DMEM powder (Gibco cat no. 52100) 10L H₂O, 2.9g L-Glutamine (Sigma), 1.1g sodium pyruvate (Sigma), 37g sodium bicarbonate (Sigma), 1g streptomycin sulphate (Sigma), 1 million units benzyl penicillin (Sigma), 40µl Beta-2-mercaptoethanol (Merck)] in a sterile stomacher bag (Seward), and was homogenised twice for three minutes. The homogenate was then centrifuged at 60,000 x g for 2 hours at 4°C. The resultant supernatant was filtered through a 0.2µm filter (Nalgene; 155-0020) with glass pre-filter (Nalgene, DS0281-5000). 1ml aliquots were stored at -80°C. FW levels of Prostaglandin E₂ (PGE₂) and transforming growth factor – beta 1 (TGF-β), (*chapter 3*), and the tumour promoting (*chapter 4*), genotoxic (*chapter 5*) and cytotoxic (*chapter 6*) potential of FW were determined. We have previously shown that this method yields a large sample volume allowing the determination of many parameters without influencing the properties of the FW (Klinder *et al.* 2005).

Peripheral Blood Mononuclear Cell (PBMC) Isolation

Whole blood (9ml to 30ml) was collected in heparin treated collection tubes (Sarstedt) and PBMCs isolated by density gradient centrifugation. Briefly blood was diluted 5:3 with PBS, (Gibco Life Sciences). The PBS-blood mixture was carefully layered onto Histopaque-1077 (Sigma) and centrifuged at 400 x g for 30 min. The buffy layer containing the PBMCs was removed and transferred to a 15ml centrifuge tube and washed twice by centrifuging at 400 x g for 10 mins in serum free RPMI-1640 (Gibco Life Sciences) containing L-glutamine (2mmol/L), penicillin (100,000 U/L) and streptomycin (100mg/L). The resultant pellet was resuspended in 3ml complete RPMI-1640 [RPMI-1640 containing 10% heat-inactivated foetal bovine serum (FBS)] and cell number and viability was determined in a haemocytometer (improved nuebauer) using trypan blue exclusion.

Cell concentration was adjusted to 1×10^6 cells/ml of complete RPMI-1640. The isolated PBMCs were stimulated for cytokine production (*chapter 3*), the remaining PBMCs were cryopreserved for analysis of Natural Killer Cell (NK cell) activity (*chapter 3*), for determination of GST activity (*chapter 4*) and genotoxic damage (*chapter 5*).

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Chapter 3

The systemic immune system in cancer and polyp subjects and the influence of synbiotic consumption on immune function

Abstract

Probiotics and prebiotics are known to modulate the immune system in animals and humans and to inhibit colon carcinogenesis in experimental animal models, but the influence of synbiotics in these processes is not well documented. We have previously shown in Azoxymethane (AOM) treated rats that consumption of a synbiotic combination of *Lactobacillus rhamnosus* GG (LGG), *Bifidobacterium lactis* Bb12 (Bb12) and Synergy[®]1 for 31 weeks modulated the immune system with a concomitant reduction in the incidence of colonic tumours and a stimulation of natural killer cytotoxicity with increased IL-10 production in the Peyer's patches (Femia *et al.* 2002). The aim of this study was to investigate the influence of twelve-week consumption of a synbiotic combination of LGG, Bb12 and Synergy[®]1 on the systemic immune system in cancer subjects who had undergone previous curative resection, and in polypectomised subjects, and to identify immune biomarkers suitable for use in intervention studies. A secondary aim was to investigate if the functioning of the systemic immune system was different in cancer as compared to polyp subjects and to identify putative immune biomarkers which may be useful as cancer risk biomarkers. We found that synbiotic consumption did not significantly modulate the systemic immune system in cancer and polyp subjects. We did however identify significant differences between cancer and polyp subjects in phagocytic activity, natural killer cell activity, IL-12 production by PBMCs and PGE₂ levels in FW at the beginning of the study. Further work is needed to confirm the usefulness of these markers as biomarkers for cancer risk.

Introduction

The gastrointestinal tract is the largest immune organ and is also home to the largest concentration and diversity of resident microbial antigens in the human body. The antigens of the gut are derived from the diet and the resident microbiota. In order to prevent unnecessary and potentially harmful reactions, the gut immune system must be able to distinguish the normal microflora and dietary antigens from pathogens (oral tolerance).

It is known that there is a lifelong dialogue between the commensal microflora and the host immune system, which is initiated at birth. Evidence from studies with germ free animals show that in the absence of the commensal microflora, mucosal associated lymphoid tissue is underdeveloped and cell mediated immunity is defective, (Schaafsma 1997). The normal flora also acts as a physical barrier against pathogenic and opportunistic infection by a process called colonisation resistance. Germ-free animals, which lack a gut flora, are very susceptible to infections.

The importance of the commensal flora in stimulation of non-damaging immune responses has become more evident recently. Increases in observed incidence and severity of allergies as well as conditions such as inflammatory bowel disease in the Western world have been linked with increases in standards of hygiene and sanitation, concomitant with a decrease in the number and range of infectious challenges encountered by the growing and developing host. This lack of immune education may cause the host immune system to over-react to antigens of the non-pathogenic normal flora resulting in inflammation, allergy and autoimmunity, (Dunne *et al.* 1999).

The commensal microflora influences the immune response through signalling with the gut epithelium (Shanahan 2000). Certain members of the gut flora have been shown to beneficially influence this intracellular signalling in *in vitro* studies (Haller *et al.* 2000). Interactions with Toll-like receptors and dendritic cells in the gut are believed to be involved in this process (Marteau *et al.* 2002; Shanahan 2002).

The normal microflora plays a central role in the initiation and maintenance of appropriate immune function. Therefore modification of the composition and metabolic activity of the flora seems a sensible means to beneficially enhance host health. Probiotics, prebiotics and synbiotics have proven effective in altering the composition of the flora and modulating the immune system in a health-promoting manner.

One of the selection criteria for probiotic bacteria is that they exhibit the ability to modulate the immune system, (Dunne *et al.* 1999). Although there is clear evidence from experimental and human studies that probiotics influence host resistance against intestinal infections as well as a number of immune cell functions, not all studies report effective immune modulation by probiotics. In addition, the underlying mechanisms by which probiotics exert their immunological effects are not well defined. Several recent reviews summarise the available experimental evidence of the immuno-modulatory effects of probiotics (Blum & Schiffrin 2003; Cross 2002; Erickson & Hubbard 2000; Meydani & Ha 2000). Immune enhancing effects associated with probiotics include enhanced macrophage phagocytosis (Perdigon *et al.* 1988), promotion of IgA responses (Isolauri *et al.* 1993; Link-

Amster *et al.* 1994; Malin *et al.* 1996), and altered cytokine production (Miettinen *et al.* 1996).

Current research indicates that consumption of prebiotics with probiotic strains enhances the growth of the introduced strain in the gut. This is known as the synbiotic effect and the term synbiotic is applied to a product, which contains both prebiotics and probiotics. A synbiotic is defined as 'a mixture of a probiotic and a prebiotic that beneficially affects the host by improving the survival and the implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria', (Roberfroid 1998).

Consumption of lactic acid bacteria has been reported to increase the phagocytic and respiratory burst activities of human peripheral blood cells. Schiffrin *et al.* (1995) found that administration of milk fermented by *Lactobacillus johnsonii* La1 or *Bifidobacterium lactis* Bb12 enhanced phagocytosis of *E.coli* compared to pre-feeding levels. The same fermented milk product was found to increase phagocytic and respiratory burst activities in humans consuming milk fermented with 10^9 cfu but was unaltered in the group consuming milk fermented with 10^8 (Donnet-Hughes *et al.* 1999). Results from studies investigating the influence of probiotic consumption on these parameters are not always consistent. Spanhaak *et al.* (1998) reported that consumption of milk fermented with *Lactobacillus casei* Shirota did not influence the phagocytic activity of blood cells.

Cytokines are biologically active signalling proteins that function as intercellular communicators. They act in the local environment by interacting with specific receptors on the surface of cells and are involved in pro- and anti-inflammatory responses mediating the immune response to pathogenic and commensal bacteria and antigens. They also have an important role in the development of the immune system. Studies have shown that probiotics can alter cytokine expression profiles in healthy and diseased states in both animals and humans, (McCarthy *et al.* 2003; Miettinen *et al.* 1996 & 1998; Sheil *et al.* 2004).

The modulation of cytokine production may also be mediated by constituents of probiotic bacteria and may not necessarily require whole bacteria. Lammers *et al.* (2003) showed that bifidobacterium genomic DNA induced secretion of IL-10 by PBMCs. Total bacterial DNA from faeces collected after probiotic administration modulated the immune response with a decrease of IL-1 β and an increase of IL-10 production being recorded.

Many studies have identified the prostaglandin synthesis pathway as a potential target for the treatment or prevention of colorectal cancer (Gupta & Dubois 2001; Ota *et al.* 2002). The two isoforms of the cyclooxygenase (COX-1 and COX-2) enzyme catalyse the initial step in the formation of biologically important prostanoids. The predominant prostaglandin species in benign and malignant colorectal tumours is Prostaglandin E₂ (PGE₂). Several studies have shown that levels of PGE₂ are significantly higher in benign and malignant colorectal tumours compared with paired histologically normal mucosa (Pugh & Thomas 1994; Yang *et al.* 1998). A study by Narisawa *et al.* (1990) found high levels of PGE₂ in the

carcinoma tissue, the local venous blood draining the carcinoma and in the peripheral blood in cases with liver or lung metastasis in 44 subjects with colorectal cancer. Pugh & Thomas (1994) investigated the levels of colonic mucosal PGE₂ in subjects with adenomatous polyps and carcinomas as well as in normal healthy subjects. Polyp and cancer subjects were found to express significantly higher amounts of PGE₂ than normal subjects.

Measuring PGE₂ levels in faecal water (FW) may provide a useful non-invasive biomarker for monitoring colorectal cancer risk and the effectiveness of treatment/prevention strategies. Lauritsen *et al.* (1984) reported that PGE₂ levels in FW from patients with active ulcerative colitis (UC) was significantly higher than levels in FW from healthy volunteers. They also found in UC patients in complete remission that withdrawal of sulphasalazine increased PGE₂ concentrations to values above normal levels, which then returned to pre-trial values following treatment with another salicylic derivative.

Transforming growth factor β (TGF- β) is a multifunctional polypeptide. Its role in carcinogenesis can be either suppressive or promoting depending on tumour developmental stages and cellular context. TGF- β is regarded as a major tumour suppressor during early tumour development because it inhibits cell-cycle progression and tumour growth. However, in late stages of progression, the majority of transformed cells become either partly or completely resistant to TGF- β growth inhibition due to inactivation of its signalling pathway or aberrant regulation of cell cycle machinery and the role of TGF- β signalling is often switched from tumour suppression to promotion. Development of resistance to TGF- β growth inhibition

may also be due to alterations in the Smad-dependant anti-proliferative response such as early termination of Smad signaling through Smad4 degradation or negative regulation of Smad2/3 through phosphorylation caused by the presence of oncogenic Ras (Saha *et al.* 2001).

It has been reported that in the later stages of cancer development TGF- β is actively secreted by tumour cells and contributes to tumour growth, invasion, and metastasis and decreases host-tumour immune responses (Pasche 2001). TGF- β has been reported to be over-expressed in human cancers such as pancreatic, colorectal, stomach, lung, prostate and breast (Gold 1999). Measurement of TGF- β in FW may provide a non-invasive biomarker for colorectal cancer.

Natural killer (NK) cells are a sub-population of lymphocytes important for the recognition and elimination of virally infected cells and tumour cells. NK cells are thought to play a role in the non-specific mechanism in immuno-surveillance for cancer. Studies have suggested that low NK cell activity is associated with an unfavourable prognosis, (Tartter *et al.* 1987).

Matsuzaki & Chin (2000) demonstrated that in adult mice fed the probiotic *Lactobacillus casei* strain Shirota for five days the natural killer activity of spleen cells was significantly higher than the NK activity of spleen cells from mice not fed the probiotic. Takagi *et al.* (2001) found that consumption of *Lactobacillus casei* Shirota by mice treated with a chemical carcinogen enhanced the cytotoxicity of NK cells and delayed tumour onset.

The aims of this study were:

- 1) To investigate the influence of twelve-week consumption of a synbiotic combination of *Lactobacillus rhamnosus* GG, *Bifidobacterium lactis* Bb12 and Synergy 1 on the systemic immune system in cancer subjects who had undergone previous curative resection and in polypectomised subjects.
- 2) To investigate if the functioning of the systemic immune system in cancer subjects who had undergone previous curative resection is different from that of polypectomised subjects.

Methods

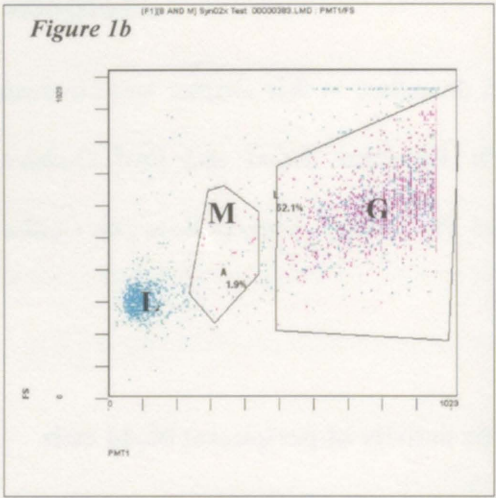
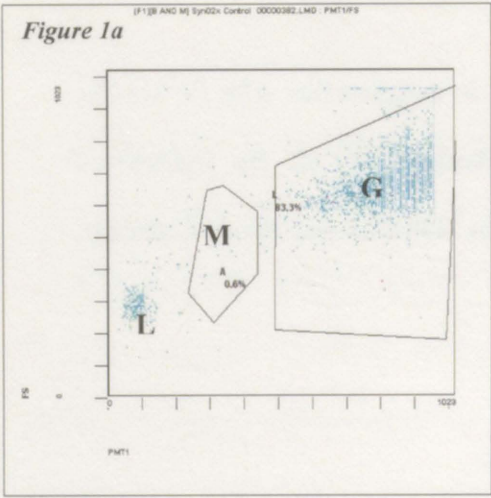
The work described in this chapter was conducted in collaboration with Dr Monika Roller, Dr Bernhard Watzl and Prof Gerhard Rechkemmer of the Institute of Nutritional Physiology, Federal Research Centre for Nutrition and Food, Karlsruhe, Germany.

Phagocytic activity of peripheral blood cells

Leukocyte phagocytosis in human whole blood was assessed using opsinised fluorescein isothiocyanate (FITC)-labelled *E. coli* [$(1 \times 10^9$ *E. coli* / ml), Molecular Probes] and flow cytometric analysis. Blood was collected in heparin treated collection tubes (Sarstedt). 100 μ l of blood was aliquoted to two flow cytometer tubes and placed on ice for 10 min. 5 μ l of pre-cooled opsinised FITC-labelled *E. coli*, was added to each tube and one tube was incubated for 10 mins at 37°C in a waterbath while the other tube was placed on ice for 10 min.

Following incubation, tubes were placed on ice and 100 μ l of ice-cold quenching solution (10% Trypan blue in PBS) was added to quench extracellular bacteria. 3ml of washing solution (PBS) was then added and the tubes were centrifuged at 250 x g for 5 min. The supernatant was removed and the pellet washed again. 2ml of 1 x FACS-Lysing Solution, (Becton Dickinson) was added to lyse red blood cells, tubes were then incubated at room temperature for 20 min protected from light.

Following incubation tubes were centrifuged at 250 x g for 5 min and the supernatant removed. Cells were then washed in 3ml of PBS and centrifuged at 250 x g for 5 min. The supernatant was removed and leukocyte DNA was stained by

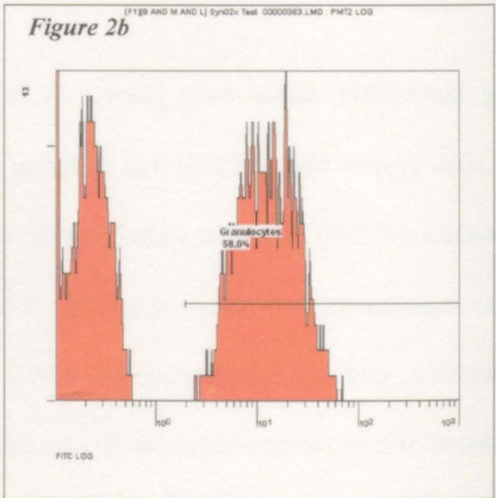
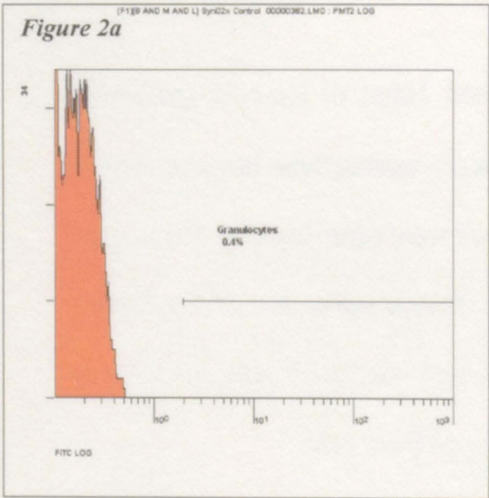


L= lymphocytes

M= monocytes

G= granulocytes

Cell populations in a control (*Figure 1a*) and test (*Figure 1b*) sample being assayed for phagocytic activity represented as a dot plot of forward scatter versus side scatter. Regions were set around monocytes and granulocytes based on morphology.



Phagocytic activity in a control (*Figure 2a*) and test (*Figure 2b*) sample being assayed for phagocytic activity measured by the number of fluorescent granulocytes presented as a percentage of total granulocytes.

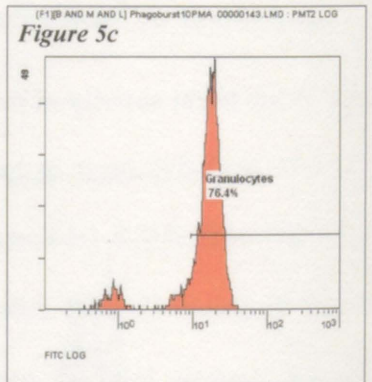
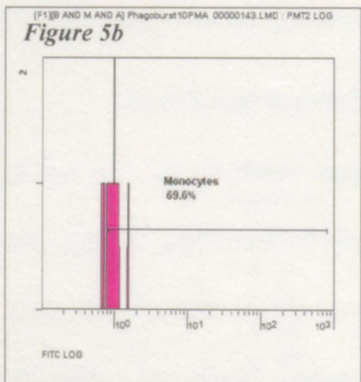
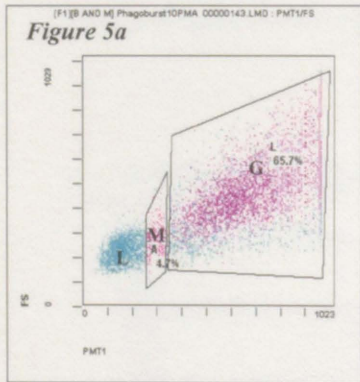
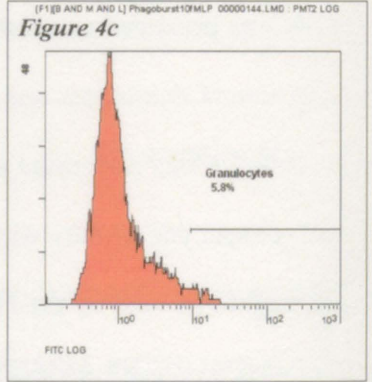
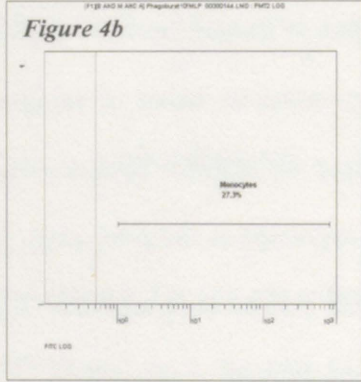
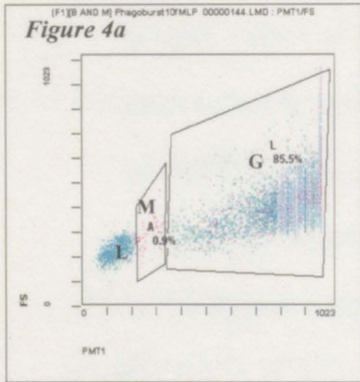
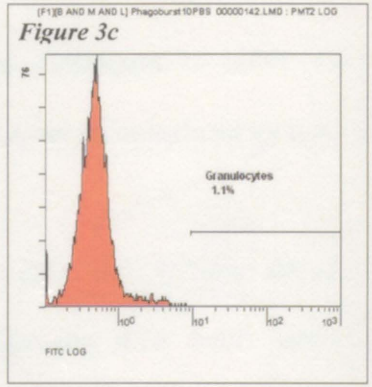
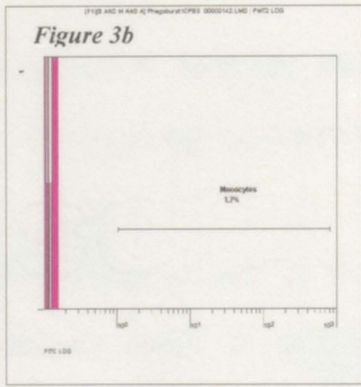
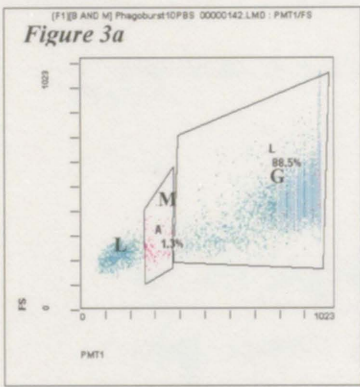
adding 100µl of propidium iodide (0.5µg/ml Molecular probes) to each tube followed by incubation on ice protected from light for 10 mins.

Phagocytic activity was measured by flow cytometry in an Epics Elite flow cytometer fitted with an argon laser at 488nm (Beckman Coulter). During acquisition a 'Live-Gate' was set in the histogram displaying diploid cell content. This data was presented as a dot plot of forward scatter versus side scatter. Regions were set around monocytes and granulocytes based on morphology (*Figures 1a-b, 2a-b*). Analysis was performed using the software package Expo Elite32 (Beckman Coulter). Phagocytic activity was expressed as the percentage of active granulocytes and monocytes as well as the mean fluorescence intensity, which correlates to the number of bacteria per individual granulocyte or monocyte.

Respiratory burst activity of peripheral blood cells

Leukocyte oxidative burst in human whole blood was determined by stimulating blood cells with fMLP (N-Formyl-Met-Leu-Phe) (Sigma) and PMA (Phorbol-12-myristat-13-acetat) (Sigma) followed by incubation with DHR (Dihydrorhodamine 123) and analysis by flow cytometry. Blood was collected in heparin treated collection tubes (Sarstedt). 100µl of blood was aliquoted into each of three flow cytometer tubes and placed on ice for 10 mins.

20µl of PBS was added to the first tube (control), 20µl of fMLP (mild stimulant) to the second tube and 20µl of PMA (high stimulant) to the third tube. The test tubes were incubated at 37°C in a waterbath for 10 mins. Following incubation 1.7µl DHR (final concentration 0.4mM), was added to each tube followed by mixing on a vortex



L= lymphocytes

M= monocytes

G= granulocytes

Cell populations in a control (**Figure 3a**), mildly stimulated (**Figure 4a**) and highly stimulated (**Figure 5a**) sample being assayed for respiratory burst activity represented as a dot plot of forward scatter versus side scatter. Regions were set around monocytes and granulocytes based on morphology.

Respiratory activity was measured by the number of fluorescent monocytes (**Figures 3b, 4b, 5b**) and granulocytes (**Figures 3c, 4c, 5c**) presented as a percentage of total monocytes and granulocytes.

and a further 10 minute incubation at 37°C was performed. DHR is a non-fluorescent substrate, which becomes oxidised by cellular enzymes and superoxide ions to a fluorescent product which binds to the mitochondria of the phagocyte. The fluorescence emitted gives a measure of respiratory burst activity.

Following incubation 2ml of 1x FACS-Lysing Solution was added, tubes were mixed on a vortex and incubated at room temperature for 20 minutes protected from light. Tubes were centrifuged at 250 x g for 5 min and the supernatant removed. Finally cells were washed in 3ml of PBS, centrifuged at 250 x g for 5 min and stained by adding 300µl of propidium iodide (33.3µg/ml). Tubes were incubated on ice protected from light for 10 min.

Respiratory burst activity was measured by flow cytometry in an Epics Elite flow cytometer fitted with an argon laser at 488nm (Beckman Coulter). During acquisition a 'Live-Gate' was set in the histogram displaying diploid cell content. This data was presented as a dot plot of forward scatter versus side scatter. Regions were set around monocytes and granulocytes based on morphology (*Figures 3a-c, 4a-c, 5a-c*). Analysis was performed using the software package Expo Elite32 (Beckman Coulter). Respiratory burst activity was expressed as the percentage of active granulocytes and monocytes as well as the mean fluorescence intensity of granulocytes and monocytes following stimulation with PMA.

PBMC stimulation

PBMCs were isolated from whole blood by density gradient centrifugation (general methods). 100µl of PBMCs (1×10^6 cells/ml) were incubated in complete RPMI-1640

in a 96 well microtitre plate at 37°C in a humidified atmosphere containing 5% CO₂ in air. PBMCs were stimulated in quadruplicate with 100µl of 10mg/L Con A (Concanavalin A) (Sigma) for the production of IL-2 and IFN-γ, 100µl of 2µg/L LPS (*E. coli* 0111:B4) (Sigma) for IL-12 and TNF-α production and in duplicate with 100µl of 2mg/L PHA (Lectin of *Phytolacca Americana*) (Sigma) for IL-10 production. Cells thus treated were incubated for 24 hours. Following incubation the microtitre plate was centrifuged at 200 x g for 5 min. The resultant supernatants were harvested and aliquoted and stored at -80°C until analysis for cytokine production by ELISA (BD Pharmingen) was performed.

Cryopreservation of PBMCs for Natural Killer (NK) cell activity

Six ml of PBMCs at a concentration of 1×10^6 cells/ml in complete RPMI-1640 (RPMI-1640 medium supplemented with 5% foetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine) were centrifuged at 400 x g for 8 minutes. The cell pellet was resuspended in 300µl of cryoprotective medium [70% RPMI-1640, 20% FBS, 10% DMSO (Dimethyl sulfoxide)], aliquoted into cooled cryogenic vials (Nunc) and placed overnight at -80°C in a cryopreservation vessel (Bicell). The cryogenic vials were transferred to liquid nitrogen after 24 hours and stored for further analysis.

Cytotoxicity of Natural Killer cells

Lytic activity of NK cells was measured using a flow cytometric method (Chang *et al.* 1993). This assay uses a cyanine membrane dye to stain live K562 target cells and an iodide nuclear dye to quantitate dead cells.

K562 human myelogenous leukaemia cells (German Cancer Research Centre, Heidelberg, Germany) at a concentration of 1×10^6 cells/ml were labelled with $10 \mu\text{l}$ of 3,3'-dioctadecyloxacarbocyanine perchlorate (3mmol/L; DiO; Molecular Probes) and incubated at 37°C for 20 min. The cell concentration was then adjusted to a working concentration of 1×10^5 cells/ml

Cryopreserved PBMCs were thawed rapidly in a water bath at 37°C , then added to 10ml complete RPMI-1640, centrifuged at $400 \times g$ for 8 mins and resuspended in 3ml of fresh medium and incubated for 18 h at 37°C , then washed with fresh medium and following counting (Coulter Z2) the cell concentration was adjusted to 2.5×10^6 cells/ml.

PBMCs were pipetted in triplicate ($100 \mu\text{l}$) in tubes to which $200 \mu\text{l}$ of appropriately diluted DiO-labelled target cells were added for final effector: target ratio of 12.5:1. Finally, to each tube $20 \mu\text{l}$ propidium iodide (525 mg/L) was added and cells were incubated for 135 min at 37°C in a humidified atmosphere.

Damaged target cells appear double-stained as their membranes become permeable to the nuclear stain, propidium iodide, during incubation. During data acquisition a live gate is set on DiO-labelled target cells, which allows differentiation between effector and target cells. The percentage of double-stained target cells was quantified and the lytic activity was calculated as the percentage of dead target cells in the test samples less the percentage of dead target cells in the control samples without effector cells.

Quantification of Prostaglandin E₂ (PGE₂) in FW

FW was prepared by diluting faeces 1:1 with DMEM followed by ultra-centrifugation as described in general methods. For this assay faecal water samples were centrifuged at 2,500 x g, for 5 mins at 20°C. 100µl of the supernatant was diluted 1:10 with water and passed through a 0.2 µm filter. 500µl of the filtrate was added to 500µl of an ice cold ethanol solution (1:4, water:ethanol). 10µl glacial acetic acid was added, gently mixed and incubated for 5 min at room temperature, followed by centrifugation at 2500 x g for 3 min at 20°C.

The supernatant was applied to a C18 reverse phase minicolumn (Amprep C18 500mg/2.8 ml RPN1910) (Amersham), which had been primed with 5.6 ml of 10 % ethanol. The column was washed with 2.8ml of distilled water followed by 2.8ml hexane and PGE₂ was eluted with 3ml ethyl acetate. The ethyl acetate was evaporated in a vacuum centrifuge and the resultant pellet was resuspended in 250µl assay buffer. PGE₂ was measured by a commercial enzyme immunoassay (Amersham), according to the manufacturers instructions.

Quantification of transforming growth factor – beta 1 (TGF-β) in FW

In order to quantify TGF-β in faecal water, latent TGF-β was first activated in the FW sample. FW samples were centrifuged at 2,500 x g for 5 mins at 20°C. 80µl of the supernatant was mixed with 80µl of 2.5M acetic acid / 10M urea and incubated for 10 min at room temperature. The samples were neutralised with 80µl of 2.7M NaOH / 1M HEPES. TGF-β was measured immediately by ELISA (R&D systems), according to the manufacturers instructions.

Results

Phagocytic Activity of Peripheral Blood Cells

Whole blood was incubated with fluorescently labelled *E. coli* and phagocytic activity was determined by measuring the percentage of active granulocytes and monocytes and the mean fluorescence intensity of granulocytes and monocytes using flow cytometry.

Cancer subjects

Phagocytic activity was measured as the percentage of active granulocytes as well as the mean fluorescence of granulocytes. There was no significant difference of these parameters at the end of (week 12) or midway through intervention (week 6) as compared to the beginning of intervention (week 0) in both the synbiotic (n=13) and placebo (n=12) group of cancer subjects (*Table 1(a)*). Similarly the percentage of active monocytes and the mean fluorescence of monocytes were unaltered at all three time-points in both groups of cancer subjects (*Table 1(b)*).

Polyp subjects

In polyp subjects consuming the synbiotic (n=13) the percentage of active granulocytes as well as the mean fluorescence of granulocytes was unaltered during intervention. Unexpectedly the percentage of active granulocytes was significantly increased at week 12 compared to week 0 ($p=0.0175$) in the group of polyp subjects consuming placebo (n=16) (*Table 2(a)*). In both the synbiotic (n=13) and placebo (n=16) group of polyp subjects the percentage active monocytes as well as the mean fluorescence of monocytes were unaltered during the twelve-week intervention period (*Table 2(b)*).

The phagocytic activity of cancer (synbiotic & placebo) subjects and polyp (synbiotic & placebo) subjects at week 0, was analysed. The phagocytic activity of granulocytes (% active granulocytes) was significantly higher in cancer subjects compared to polyp subjects ($p=0.0173$). However there was no significant difference in the percentage of phagocytic activity in monocytes or the mean fluorescence of granulocytes or monocytes (*Table 3*).

Respiratory Burst Activity of Peripheral Blood Cells

The respiratory burst activity of granulocytes and monocytes in whole blood following stimulation with phorbol-12-myristat-13-acetat (PMA) was analysed by flow cytometry.

In cancer subjects consuming synbiotic ($n=13$) there was an increase although not significant ($p=0.0669$) in the percentage of active granulocytes at week 12 compared to week 0. The percentage of active granulocytes was unaltered in the placebo group of cancer subjects ($n=12$), and both the synbiotic ($n=15$) and placebo ($n=18$) group of polyp subjects (*Figure 6(a)*).

The mean fluorescence of granulocytes was significantly increased at week 12 compared to week 0 in synbiotic consuming cancer ($p=0.0090$) and polyp ($p=0.0344$) subjects as well as in the placebo ($p=0.0390$) group of cancer subjects. The mean fluorescence of granulocytes in polyp placebo subjects was unaltered (*Figure 6(b)*).

The respiratory burst activity of cancer (synbiotic & placebo) subjects and polyp (synbiotic & placebo) subjects at week 0, was analysed. The respiratory burst activity of granulocytes was not significantly different in cancer and polyp subjects (*Table 3*).

Cytokine Expression in Supernatants from Mitogen Stimulated PBMCs

Peripheral blood mononuclear cells were isolated from whole blood by density gradient centrifugation and were then stimulated for 24 hours with a range of mitogens following which cell free supernatants were analysed by ELISA for the levels of the cytokines IL-2, IL-10, IL-12, IFN- γ and TNF- α .

Cancer subjects

Mitogen induced cytokine levels in PBMCs isolated from the cancer synbiotic (n=19) group at week 0, week 6 and week 12 were analysed by ELISA. Synbiotic consumption significantly increased the levels of IFN- γ at week 12 compared to week 6 (p=0.0107) and week 0 (p=0.0376). IL-2, IL-10, IL-12, and TNF- α levels were unaltered (*Figure 7(a)*).

In the group of cancer subjects consuming placebo (n=15) cytokine levels were unaltered during the twelve-week intervention period (*Figure 7(b)*).

Polyp subjects

Synbiotic intervention did not alter cytokine levels in polyp (n=19) subjects over the twelve-week intervention period (*Figure 8(a)*). However in the group of polyp subjects consuming placebo (n=20), IL-2 production was significantly increased at

week 12 compared to week 6 ($p=0.0209$) and week 0 ($p=0.0022$). IL-12 production was also significantly increased at week 12 compared to week 0 ($p=0.0050$). IL-10, IFN- γ and TNF- α expression was unaltered (*Figure 8(b)*).

Cytokine expression in PBMCs derived from cancer (synbiotic & placebo) subjects and polyp (synbiotic & placebo) subjects at week 0, was analysed. IL-12 protein expression was significantly higher ($p=0.0034$) in cancer than in polyp subjects. IL-2, IL-10, IFN- γ and TNF- α levels were not significantly different (*Table 3*).

Natural Killer Cell Activity

The natural killer cell activity of PBMCs was assessed by flow cytometry. There was no significant change in the lytic activity of natural killer cells in cancer subjects consuming synbiotic ($n=7$) or placebo ($n=6$) as well as polyp subjects consuming synbiotic ($n=5$) or placebo ($n=6$) over the twelve-week intervention period (*Table 4*).

The natural killer cell activity of PBMCs derived from cancer (synbiotic & placebo) subjects and polyp (synbiotic & placebo) subjects at week 0, was analysed. The lytic activity of natural killer cells was significantly higher in polyp subjects than in cancer subjects ($p=0.0178$) (*Table 3*).

Quantification of Prostaglandin E₂ (PGE₂) in FW

PGE₂ was isolated from FW using a C18 reverse phase minicolumn and PGE₂ levels were measured by ELISA. There was no significant change in PGE₂ levels in synbiotic ($n=17$) and placebo ($n=14$) cancer subjects or in polyp synbiotic ($n=17$) and placebo ($n=19$) subjects (*Table 5*). There was a large inter and intra-individual

variation in levels of faecal PGE₂ in this study suggesting that this marker is not suitable as an biomarker in an intervention study of this type.

The level of PGE₂ in FW derived from cancer (synbiotic & placebo) subjects and polyp (synbiotic & placebo) subjects at week 0, was analysed. PGE₂ levels in FW was significantly higher in cancer subjects compared to polyp subjects (p=0.0032) (*Table 3*).

Quantification of Transforming Growth Factor – Beta 1 (TGF-β) in FW

The level of TGF-β in faecal water was measured by ELISA. During the twelve-week intervention period there was no change in the levels of TGF-β in FW in either cancer (synbiotic n=17, placebo n=14) or polyp (synbiotic n=17, placebo n=20) subjects (*Table 6*). Similar to PGE₂ there was a large inter and intra-individual variation in levels of faecal TGF-β in this study suggesting that this marker is not suitable as an biomarker in an intervention study of this type.

The level of TGF-β in FW derived from cancer (synbiotic & placebo) subjects and polyp (synbiotic & placebo) subjects at week 0, was analysed. The level of TGF-β in FW was not significantly different in cancer and polyp subjects (*Table 3*).

Table 1(a)

Percentage active granulocytes and the mean fluorescence of granulocytes in both the synbiotic and placebo group of cancer subjects at the beginning of (week 0), midway through (week 6) and at the end of intervention (week12). There was no influence of synbiotic consumption on the phagocytic activity of granulocytes. Results are expressed as mean $\pm$ standard error.

Table 1(b)

Percentage active monocytes and the mean fluorescence of monocytes in both the synbiotic and placebo group of cancer subjects at week 0, week 6 and at week 12. There was no influence of synbiotic consumption on the phagocytic activity of monocytes. Results are expressed as mean $\pm$ standard error.

Table 1(a): Granulocyte phagocytic activity in cancer subjects

Group	% Active Granulocytes			P value
	Week 0	Week 6	Week 12	
Synbiotic cancer (n=13)	47.6 ± 4.5	34.1 ± 6.0	43.7 ± 4.5	n.s.
Placebo cancer (n=12)	49.9 ± 6.0	38.3 ± 5.4	40.4 ± 5.4	n.s.

Group	Mean Fluorescence Granulocytes			P value
	Week 0	Week 6	Week 12	
Synbiotic cancer (n=13)	21.7 ± 1.4	16.7 ± 2.0	22.1 ± 2.2	n.s.
Placebo cancer (n=12)	22.4 ± 3.4	16.7 ± 1.4	22.8 ± 2.6	n.s.

Table 1(b): Monocyte phagocytic activity in cancer subjects

Group	% Active Monocytes			P value
	Week 0	Week 6	Week 12	
Synbiotic cancer (n=13)	19.0 ± 2.9	15.6 ± 2.5	18.4 ± 1.9	n.s.
Placebo cancer (n=12)	21.9 ± 3.2	16.5 ± 2.5	19.7 ± 3.4	n.s.

Group	Mean Fluorescence Monocytes			P value
	Week 0	Week 6	Week 12	
Synbiotic cancer (n=13)	21.3 ± 3.0	14.8 ± 1.8	16.0 ± 1.1	n.s.
Placebo cancer (n=12)	27.5 ± 8.4	16.9 ± 3.1	20.1 ± 1.8	n.s.

Table 2(a)

Percentage active granulocytes and mean fluorescence of granulocytes in both the synbiotic and placebo group of polyp subjects at the beginning of (week 0), midway through (week 6) and at the end of intervention (week12). In the group of polyp subjects consuming placebo the percentage of active granulocytes was significantly increased at week 12 compared to week 0. The percentage of active granulocytes was unaltered in synbiotic consuming subjects. Results are expressed as mean $\pm$ standard error.

Table 2(b)

Percentage active monocytes and the mean fluorescence of monocytes in both the synbiotic and placebo group of polyp subjects at week 0, week 6 and at week 12. There was no influence of synbiotic consumption on the phagocytic activity of monocytes. Results are expressed as mean $\pm$ standard error.

Table 2(a): Granulocyte phagocytic activity in polyp subjects

Group	% Active Granulocytes			P value
	Week 0	Week 6	Week 12	
Synbiotic polyp (n=13)	37.6 ± 5.2	34.3 ± 3.5	45.2 ± 6.1	n.s.
Placebo polyp (n=16)	34.4 ± 4.0	34.3 ± 4.8	45.3 ± 4.7	0.0175

Group	Mean Fluorescence Granulocytes			P value
	Week 0	Week 6	Week 12	
Synbiotic polyp (n=13)	19.3 ± 1.1	17.9 ± 2.4	23.9 ± 4.1	n.s.
Placebo polyp (n=16)	19.0 ± 1.3	17.4 ± 1.6	23.1 ± 3.1	n.s.

Table 2(b): Monocyte phagocytic activity in polyp subjects

Group	% Active Monocytes			P value
	Week 0	Week 6	Week 12	
Synbiotic polyp (n=13)	17.5 ± 2.7	16.1 ± 3.0	15.9 ± 2.3	n.s.
Placebo polyp (n=16)	15.8 ± 2.1	17.4 ± 2.0	17.5 ± 2.2	n.s.

Group	Mean Fluorescence Monocytes			P value
	Week 0	Week 6	Week 12	
Synbiotic polyp (n=13)	23.5 ± 5.9	17.2 ± 2.1	20.8 ± 3.3	n.s.
Placebo polyp (n=16)	18.4 ± 1.2	17.1 ± 1.2	21.8 ± 2.7	n.s.

Table 3

Comparison of immune parameters in all cancer subjects compared to all polyp subjects at the beginning of intervention. The percentage of active granulocytes, natural killer cell activity, the expression of IL-12 by PBMCs and the level of PGE₂ in FW was significantly different in cancer subjects compared to polyp subjects. Results are expressed as mean $\pm$ standard error. Statistical significance was assessed by un-paired t-test.

Table 3

Immune function in cancer vs. polyp subjects at the start of intervention

Immune marker (units)	Cancer Subjects Week 0	Polyp Subjects Week 0	P value
Phagocytic Activity			
Phagocytic activity (% active granulocytes)	48.33 ± 3.27 (n=30)	37.63 ± 2.91 (n=39)	0.0173
Phagocytic activity (MF granulocytes)	22.58 ± 1.53 (n=30)	20.68 ± 0.93 (n=39)	n.s.
Phagocytic activity (% active monocytes)	21.51 ± 1.96 (n=30)	17.64 ± 1.46 (n=39)	n.s.
Phagocytic activity (MF monocytes)	24.34 ± 3.60 (n=30)	21.05 ± 2.08 (n=39)	n.s.
Respiratory Burst Activity			
Respiratory burst activity (% active granulocytes)	87.19 ± 1.42 (n=30)	88.44 ± 0.74 (n= 39)	n.s.
Respiratory burst activity (MF granulocytes)	16.12 ± 0.65 (n=30)	17.72 ± 1.43 (n=39)	n.s.
Peripheral Blood Cytokine Expression			
IL-2 (pg/ml)	245.9 ± 45.7 (n=34)	160.5 ± 23.53 (n=39)	n.s.
IL-10 (pg/ml)	146.6 ± 19.1 (n=34)	108.5 ± 18.87 (n=39)	n.s.
IL-12 (pg/ml)	795.4 ± 125.3 (n=34)	378.5 ± 67.06 (n=39)	0.0034
IFN-γ (pg/ml)	723.2 ± 138.4 (n=34)	888.2 ± 146.9 (n=39)	n.s.
TNF-α (pg/ml)	9186 ± 1700 (n=34)	6861 ± 1884 (n =39)	n.s.
Natural Killer Cell Activity			
Natural Killer cell activity (% killed target cells)	13.27±1.24 (n=24)	19.93 ± 2.78 (n=15)	0.0178
FW Cytokine Expression			
TGF-β FW (pg/ml)	1644 ± 313.9 (n=34)	1622 ± 248.9 (n=40)	n.s.
PGE ₂ FW (pg/ml)	27473 ± 4994 (n=33)	11822 ± 2119 (n=39)	0.0031

Figure 6(a)

Respiratory burst activity expressed as the percentage of active granulocytes at week 0, week 6 and at week 12. The percentage of active granulocytes was unaltered in the synbiotic (n=15) and placebo (n=18) groups of polyp subjects and the placebo (n=12) group of cancer subjects. However the percentage of active granulocytes was increased, although not significantly ($p=0.0669$) at week 12 compared to week 0 in the synbiotic group of cancer subjects (n=13). Results are expressed as mean $\pm$ standard error.

Figure 6(b)

Respiratory burst activity expressed as the mean fluorescence of granulocytes at week 0, week 6 and at week 12. The mean fluorescence of granulocytes was significantly increased at week 12 compared to week 0 in synbiotic consuming cancer (n=13) and polyp (n=15) subjects as well as in the placebo group of cancer (n=12) subjects. The mean fluorescence of granulocytes in polyp placebo subjects (n=18) was unaltered. Results are expressed as mean $\pm$ standard error.

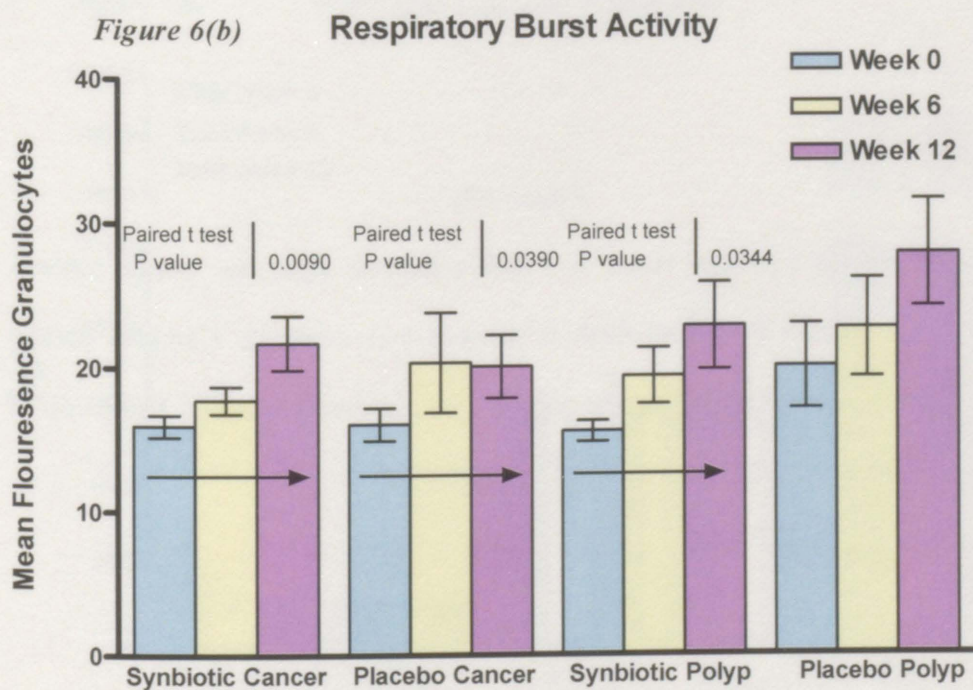
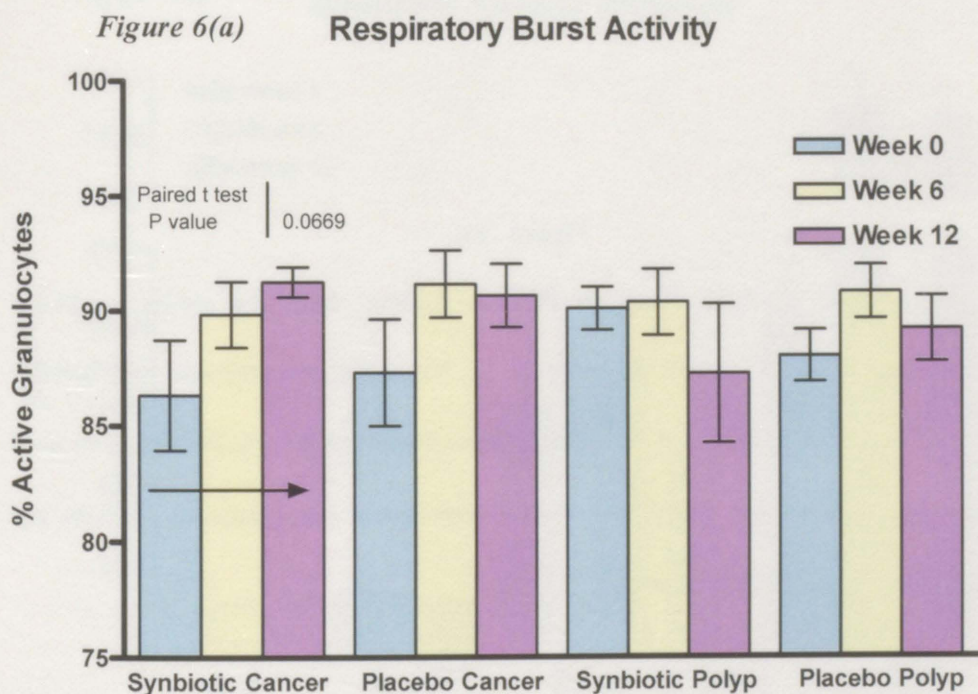


Figure 7(a)

Mitogen induced cytokine levels in PBMCs isolated from the cancer synbiotic (n=19) group at week 0, week 6 and week 12. Synbiotic consumption significantly increased the levels of IFN- γ at week 12 compared to week 6 ($p=0.0107$) and week 0 ($p=0.0376$). IL-2, IL-10, IL-12 and TNF- α expression was unaltered. Results are expressed as mean $\pm$ standard error.

Figure 7(b)

Mitogen induced cytokine levels in PBMCs isolated from the cancer subjects consuming placebo (n=15) at week 0, week 6 and week 12. Cytokine protein expression was unaltered during the twelve-week intervention period this group of subjects. Results are expressed as mean $\pm$ standard error.

Figure 7(a)

Synbiotic Cancer Subjects

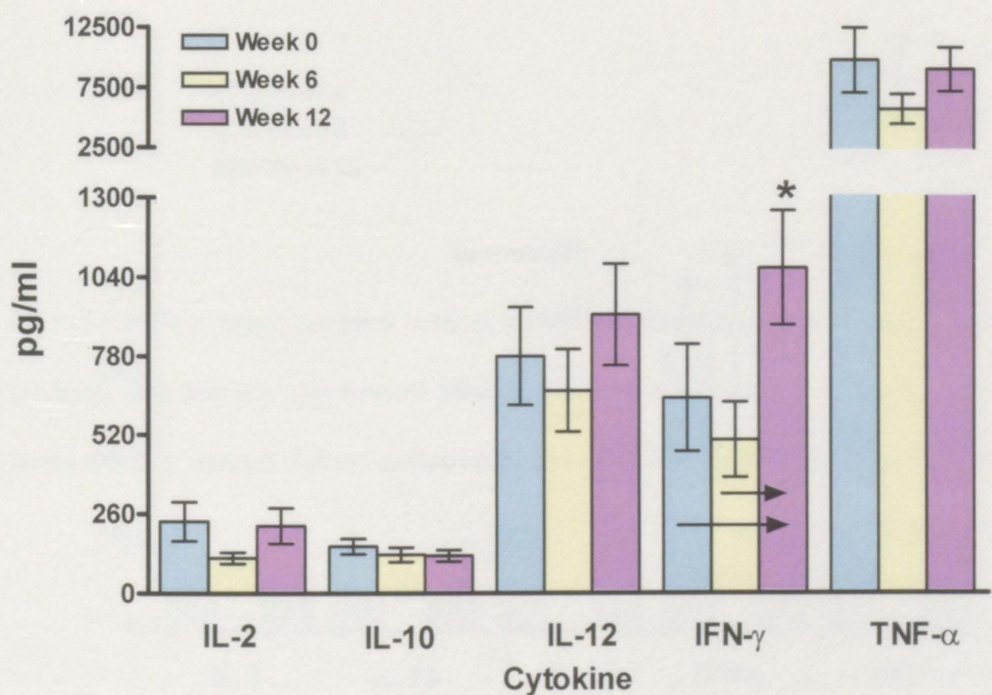


Figure 7(b)

Placebo Cancer Subjects

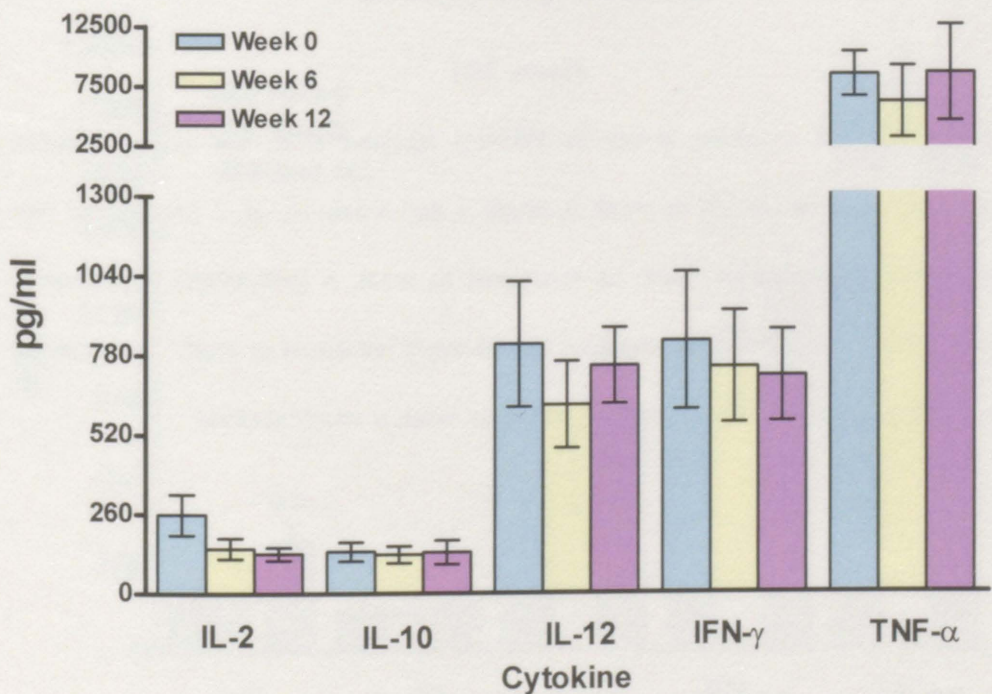


Figure 8(a)

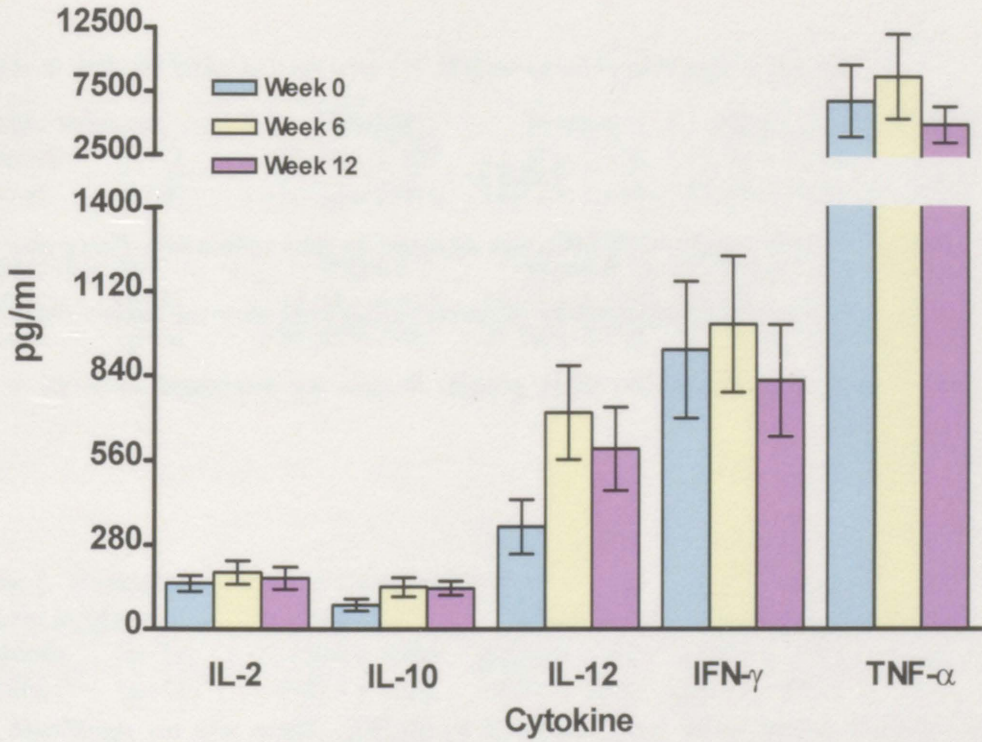
Mitogen induced cytokine levels in PBMCs isolated from the polyp synbiotic (n=19) group at week 0, week 6 and week 12. Synbiotic intervention did not alter cytokine protein expression over the twelve-week intervention period. Results are expressed as mean $\pm$ standard error.

Figure 8(b)

Mitogen induced cytokine levels in PBMCs isolated from the polyp subjects consuming placebo (n=20) at week 0, week 6 and week 12. IL-2 production was significantly increased at week 12 compared to week 6 ($p=0.0209$) and week 0 ($p=0.0022$). IL-12 production was also significantly increased at week 12 compared to week 0 ($p=0.0050$). Results are expressed as mean $\pm$ standard error.

Synbiotic Polyp Subjects

Figure 8(a)



Placebo Polyp Subjects

Figure 8(b)

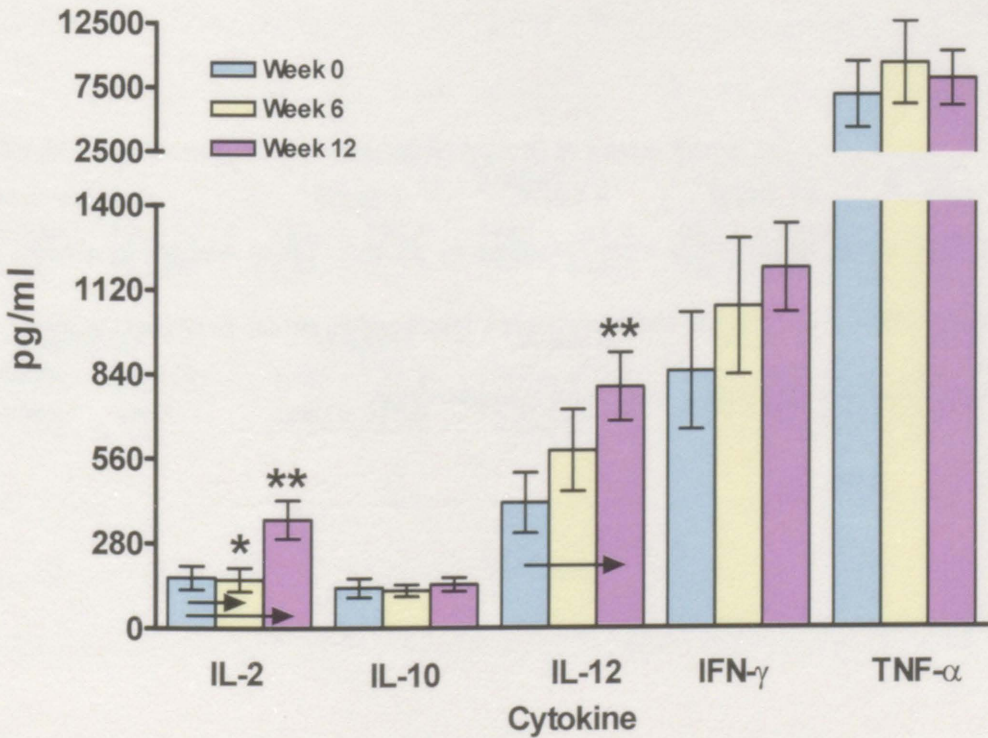


Table 4

The natural killer cell activity of PBMCs was assessed by flow cytometry. There was no significant change in the lytic activity of natural killer cells over the twelve-week intervention period in any of the study groups. Results are expressed as mean $\pm$ standard error.

Table 5

PGE₂ levels in faecal water were measured by ELISA. There was no significant change in PGE₂ levels over the twelve-week intervention period in any of the study groups. Results are expressed as mean $\pm$ standard error.

Table 6

TGF- β levels in faecal water were measured by ELISA. There was no significant change in TGF- β levels over the twelve-week intervention period in any of the study groups. Results are expressed as mean $\pm$ standard error.

Table 4: Natural killer cell activity (% of dead cells) in all subjects groups

Cancer subjects		Week 0	Week 6	Week 12	P value
Synbiotic	(n=7)	12.44 ± 2.307	13.30 ± 2.316	15.32 ± 4.213	n.s.
Placebo	(n=6)	13.32 ± 2.844	18.12 ± 3.640	12.95 ± 3.809	n.s.
Polyp subjects		Week 0	Week 6	Week 12	P value
Synbiotic	(n=5)	23.34 ± 4.954	16.65 ± 1.110	18.65 ± 1.903	n.s.
Placebo	(n=6)	24.53 ± 3.609	20.08 ± 1.830	27.77 ± 6.451	n.s.

Table 5: Prostaglandin E₂ (pg/ml) in Faecal Water

Cancer subjects		Week 0	Week 6	Week 12	P value
Synbiotic	(n=17)	25106 ± 5926	25171 ± 5782	27095 ± 5590	n.s.
Placebo	(n=14)	33817 ± 9062	34077 ± 9046	28192 ± 8515	n.s.
Polyp subjects		Week 0	Week 6	Week 12	P value
Synbiotic	(n=17)	10996 ± 2695	8140 ± 1882	10124 ± 2936	n.s.
Placebo	(n=19)	13749 ± 3597	11098 ± 3827	9922 ± 2018	n.s.

Table 6: Transforming Growth Factor-β (pg/ml) in Faecal Water

Cancer subjects		Week 0	Week 6	Week 12	P value
Synbiotic	(n=17)	1033 ± 224.5	1486 ± 485.9	671.1 ± 129.4	n.s.
Placebo	(n=14)	2263 ± 622.5	2397 ± 646.3	2357 ± 570.2	n.s.
Polyp subjects		Week 0	Week 6	Week 12	P value
Synbiotic	(n=17)	1405 ± 380.8	1994 ± 477.2	1924 ± 493.4	n.s.
Placebo	(n=20)	1682 ± 353.8	1520 ± 324.9	1654 ± 339.2	n.s.

Discussion

In the current study we evaluated the ability of a synbiotic combination of *Lactobacillus rhamnosus* GG, *Bifidobacterium lactis* Bb12 and Synergy[®]1 to modulate the systemic immune system in polypectomised subjects and in cancer subjects who had previously undergone 'curative resection'. We have previously shown in Azoxymethane (AOM) treated rats that consumption of this synbiotic combination for 31 weeks modulated the immune system with a concomitant reduction in the incidence of colonic tumours (Femia *et al.* 2002; Roller *et al.* 2004a). By investigating the influence of synbiotic consumption on the systemic immune system of subjects considered at risk for colon cancer we hoped to identify non-invasive biomarkers for use in intervention studies and also biomarkers of cancer risk.

Consumption of probiotics has been reported to enhance phagocytosis and respiratory burst activities in human and animal studies (Collins *et al.* 2001; Mattila-Sandholm *et al.* 1999; Perdigon *et al.* 1995, 1987, 1988). In humans consumption of milk fermented by *Lactobacillus johnsonii* La1 or *Bifidobacterium lactis* Bb12 enhanced phagocytosis of *E. coli* compared to pre-feeding levels (Donnet-Hughes *et al.* 1999; Schiffrin *et al.* 1995).

In contrast to the studies outlined above synbiotic consumption did not enhance the phagocytic or respiratory burst activities of granulocytes or monocytes in the current study. There was a significant increase in the percentage of active granulocytes in polyp placebo subjects at the end of intervention compared to the beginning indicating an increase in the number of phagocytosing granulocytes. However there

was no change in the mean fluorescence or the percentage of active monocytes in this group. The increase in the percentage of active granulocytes observed in placebo polyp subjects might indicate changes occurring in the GI tract of these subjects, which did not occur in polyp subjects consuming synbiotic. It may be that synbiotic consumption prevented these changes, however this study was not designed to ask this question and further work would be needed to determine the reason for this increase. The outcome of this study is not unusual as not all studies have shown enhancement of phagocytic activity in response to probiotics. Spanhaak *et al.* (1998) reported that consumption of milk fermented with *Lactobacillus casei* Shirota did not influence the phagocytic activity of blood cells.

In the current study there was a significant increase in respiratory burst activity as measured by mean fluorescence in granulocytes in both synbiotic and placebo cancer subjects and in synbiotic polyp but not placebo polyp subjects at the end of intervention compared to the beginning. Interestingly this increase in respiratory burst activity in three of the four study groups is in contrast to the phagocytosis assay where placebo polyp subjects were the only group in which the percentage of active granulocytes was altered. Similar to the phagocytosis assay the enhancement in respiratory burst activity may be attributable to some other unknown factor. From these results we conclude that phagocytic and respiratory burst activities are not suitable biomarkers in an intervention study of this type.

Modulation of cytokine expression following probiotic consumption has been reported in many studies in healthy and diseased states in both animals and humans, (McCarthy *et al.* 2003; Miettinen *et al.* 1996, 1998; Sheil *et al.* 2004). Miettinen *et*

al. (1996,1998) reported that PBMCs stimulated with several strains of non-pathogenic lactobacilli induce higher amounts of TNF- α than PBMCs stimulated with LPS. IL-6 was also produced. Five-week oral consumption of *Lactobacillus rhamnosus* GG in ten healthy human volunteers decreased the secretion of TNF- α , IL-6 and IFN- γ by PBMCs and increased IL-10 and IL-4 secretion following *in vitro* stimulation with self and non-self intestinal organisms and whole stool preparations, (Schultz *et al.* 2003). Previous work in our lab has shown that different probiotic strains induce distinct cytokine responses (Sheil 2003).

In this study synbiotic consumption by cancer subjects increased IFN- γ production by PBMCs but did not alter production of IL-2, IL-10, IL-12, and TNF- α . In the polyp placebo group there was a significant increase in IL-2 and IL-12 production, however there was no alteration in cytokine production by polyp subjects consuming the synbiotic, suggesting a dampening of the production of the pro-inflammatory cytokines IL-2 and IL-12 in polyp subjects due to synbiotic consumption. Roller *et al.* (2004a) found that consumption of the synbiotic combination used in the current study increased IL-10 production in Peyer's patches from AOM treated mice with a lower incidence of colon tumours compared to mice not fed the synbiotic.

Natural killer (NK) cells are thought to play a role in the non-specific mechanism of immuno-surveillance for cancer. Medium and high NK cell activity of PBMCs is associated with reduced cancer risk, whereas low activity is associated with increased cancer risk (Imai *et al.* 2000). Natural killer cells from probiotic fed mice have been reported to demonstrate increased activity against tumour cells (Gill 1998; Kato *et al.* 1984; Matsuzaki & Chin 2000). In the current study there was no effect

of synbiotic intervention on the natural killer activity of PBMCs from cancer and polyp subjects. In contrast Roller *et al.* (2004b) reported an increase in natural killer cell activity in Peyer's patches from AOM treated rats fed the same synbiotic as that used in this study compared to rats not fed the synbiotic.

Prostaglandin E₂ (PGE₂) and Transforming Growth Factor- β (TGF- β) have been reported to be over-expressed in human cancers. PGE₂ levels are significantly higher in colorectal tumours compared with paired histologically normal mucosa (Pugh & Thomas 1994; Yang *et al.* 1998). TGF- β is actively secreted by tumour cells and contributes to tumour growth, invasion, and metastasis and decreases host-tumour immune responses (Pasche 2001). In the current study there was no influence of synbiotic consumption on the levels of PGE₂ and TGF- β in FW from cancer and polyp subjects.

We compared the levels of immune parameters in cancer subjects to polyp subjects at the beginning of intervention. The phagocytic activity of granulocytes (% active granulocytes) was significantly higher in cancer subjects compared to polyp subjects.

Natural killer cell activity was significantly higher in polyp subjects compared to cancer subjects. This result agrees with the findings of Imai *et al.* (2000) who investigated NK cell activity in 3625 Japanese people mostly older than forty and carried out a follow-up survey of cancer incidence for eleven years. Their results indicate that medium and high NK cell activity of PBMCs is associated with reduced cancer risk, whereas low activity is associated with increased cancer risk. Natural

killer cell activity may be useful as a biomarker of cancer risk but further work is needed to confirm its' potential in this area.

IL-12 production by PBMCs from cancer subjects was significantly higher than production by PBMCs from polyp subjects following in vitro stimulation with LPS. In contrast, studies have shown that CRC patients have lower preoperative levels of IL-12 compared to healthy controls and lower levels of IL-12 production was associated with advanced disease (O'Hara *et al.* 1998a,b).

The level of PGE₂ in FW of cancer subjects was significantly higher than in FW from polyp subjects. This increase may be due to inflammation of the colonic mucosa. Lauritsen *et al.* (1984) reported that the level of PGE₂ in FW from patients with active ulcerative colitis is significantly higher than in FW from healthy volunteers. Further work is needed but measurement of PGE₂ in FW is a potential non-invasive biomarker of cancer risk.

In conclusion consumption of a synbiotic combination of *Lactobacillus rhamnosus* GG, *Bifidobacterium lactis* Bb12 and Synergy[®]1 for twelve weeks did not significantly modulate the systemic immune system in cancer and polyp subjects. Previous work by our group has shown that consumption of this synbiotic combination by AOM treated rats modulated the immune system and reduced tumour incidence. This apparent paradox may be explained by the fact that in the rat study immune responses were assessed in tissues of the immune system (i.e. Peyer's patches, mesenteric lymph nodes) while in the human study we did not have access to these tissues and so the immune modulating effects of the synbiotic were

investigated in the systemic immune system. However it may also call into question the degree to which observations from animal studies can be directly interpreted in the human context.

Comparison of immune function in cancer and polyp subjects identified significant differences in phagocytic activity, natural killer cell activity, IL-12 production by PBMCs and PGE₂ levels in FW as possible biomarkers of cancer risk. Further work however is needed to confirm the efficacy of these biomarkers.

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Chapter 4

Synbiotic consumption reduces the proliferative activity of the colonic mucosa but does not influence the tumour promoting activity of faecal water (FW)

Abstract

Animal and human studies have shown that prebiotics and probiotics influence cancer risk in a protective manner, however the influence of synbiotic consumption has not been well studied. We have previously shown in azoxymethane (AOM) treated rats that consumption of a synbiotic (*Lactobacillus rhamnosus* GG, *Bifidobacterium lactis* Bb12, Synergy[®]1) significantly reduced the number of colonic tumours compared to control rats. In humans cancer develops over a long time period and currently available tumour markers do not have the ability to detect early stage colorectal cancers. The aim of this study was to determine if synbiotic consumption could influence the proliferative activity of the colonic mucosa, the tumour promoting potential of faecal water (FW), the expression of glutathione-S-transferases (GSTs) and the levels of CEA and CA19-9. A secondary aim was to determine which, if any, of the above markers might be suitable as cancer risk biomarkers and / or biomarkers suitable for use in intervention studies. We found that synbiotic consumption significantly reduced the proliferative activity in biopsies from polyp subjects and increased GST activity in cancer subjects. There was no change in the tumour promoting activity of FW as measured by comet assay. Neither was there an effect of synbiotic consumption on the levels of CEA and CA19-9. The influence of FW on tight junction integrity in Caco-2 cells was significantly different for FW derived from polyp subjects compared to FW derived from cancer subjects indicating that the composition of FW is different in cancer and polyp subjects. In conclusion synbiotic consumption influences the proliferative activity of the colonic mucosa and the expression of detoxifying enzymes in a health promoting manner. The composition of FW derived from cancer subjects is different to FW derived from polyp subjects at the beginning of intervention and at all time points.

Proliferative activity, GST expression and the influence of FW on tight junction integrity may be useful as biomarkers of cancer risk.

Introduction

The long time period between exposure to a carcinogen and tumour formation in humans poses a difficult problem when studying the relationship between suspected aetiological agents of colon cancer and the end point of tumour formation. The currently available biochemical markers for colorectal cancer (CRC) are not markers of cancer risk and therefore are not particularly useful in cancer risk lowering studies or in evaluating the effectiveness of prevention strategies. There is a real need for the development of new non-invasive reliable markers predictive of tumour formation, which are altered by changing risk.

The colonic epithelium is a homeostatic tissue with strict control of proliferation, growth, arrest and apoptosis. Disruption of the balance between cell growth and cell death can result in clonal expansion and development of carcinomas. A gradual inhibition of apoptosis, leading to increased proliferation has been reported in the conversion of normal colonic epithelium into carcinomas (Bedi *et al.* 1995)

Normal colonic epithelium is organised into crypts where cell proliferation, migration, differentiation and apoptosis are topographically organised in a linear fashion along the crypt axis. Normal colonic crypts are composed of stem cells at the base, a proliferation and a differentiation zone in the lower third of the crypt, a migration zone in the upper two thirds, and the surface epithelium where senescent cells are eliminated by apoptosis. However in the abnormal situation, e.g. polyp or tumour formation, proliferation occurs throughout the entire crypt often with the zone of maximum activity occurring at the top of the crypt.

Proliferative activity is one of the most commonly used biomarkers to assess cancer risk and to monitor the effectiveness of risk lowering strategies. Studies have shown that subjects at high risk of colon cancer, such as subjects with familial adenomatous polyposis (Mills *et al.* 2001), ulcerative colitis (Shinozaki *et al.* 2000) or sporadic colon cancer (Roy *et al.* 1999) have a high mucosal proliferative activity as compared to controls. de Leon *et al.* (1988) reported that proliferation in the upper crypt was significantly increased in patients with polyps and colorectal cancer compared to healthy controls. In animal models of carcinogenesis, the carcinogen often increases cell proliferation and causes a shift in the zone of proliferation.

Studies have shown that consumption of prebiotics, probiotics and synbiotics decrease proliferative activity in humans and animals. Biasco *et al.* (1991) reported that elevated cell proliferation in the colonic mucosa was decreased by probiotic consumption in some patients with benign tumours in the colon. We have previously shown that feeding azoxymethane (AOM) treated rats the prebiotic Synergy 1 significantly decreases the proliferative activity of the colonic mucosa. AOM treated rats fed the probiotics *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12 and rats fed a synbiotic combination of both probiotic strains and Synergy[®]1 tended to have a lower proliferative activity than control AOM treated rats. In synbiotic fed rats there were significantly less colonic tumours than in control rats (Femia *et al.* 2002).

A major function of intestinal epithelial cells is to provide a physical barrier between the potentially hostile intestinal lumen and the highly immunoreactive subepithelial tissue. Epithelial barrier function is provided by the epithelial cells and the tight

junctions (TJs) which connect them. These junctions express a high degree of plasticity and may be greatly attenuated in disease states.

TJs are critical for the control of paracellular transport between the luminal and the basolateral fluid compartments (Madara, 1998). Disruption of TJs leads to a 'leaky gut' with an increase in intestinal paracellular permeability, (Sugi *et al.* 2001). Soler *et al.* (1999) demonstrated the association between increased tight junction permeability and colonic tumour development. They found that TJs were highly permeable in both rat and human colon tumours and also in the colonic epithelium of rats with DMH induced carcinogenesis. This study also found in a limited number of samples that human colonic polyps of the type more frequently associated with colon cancer, (e.g. villous adenoma and adenomatous polyps) had more permeable TJs than hyperplastic polyps which do not increase the risk of colon cancer and whose TJ permeability was more similar to that of normal colonic epithelium.

Intestinal epithelial intercellular junctional integrity can be investigated *in vitro* by measuring the transepithelial electrical resistance (TER) across a confluent monolayer of cells grown on a permeable membrane. Transepithelial resistance to the flow of an electrical current represents a direct measure of monolayer resistance to passive ion permeation and is used as a measure of junctional permeability. Using this method resistance is detected using a handheld volt ohm meter. It is also possible to measure TJ integrity in the same system by measuring the paracellular transport of a labeled probe e.g. radiolabelled mannitol ^{14}C .

Metastasis is the escape of tumour cells from a primary site and their re-establishment with secondary tumour formation at a distant secondary site. In recent years there has been much development in the areas of diagnosis, surgery and adjuvant therapies, however most deaths from cancer are due to metastases which are resistant to conventional therapies. Metastases may occur in areas far distant from the primary tumour and often go undetected at surgery.

The metastatic process involves a complex series of interactions between the cancer and its environment. Fidler & Radinsky (1996) outlined the step leading to cancer metastasis:

- Escape of the cells from the primary tumour
- Intravasation (entry of cells into lymphatic or blood circulation)
- Survival and transport in the circulation
- Arrest in distant organs
- Extravasation (escape of cells from the circulation), growth of cells to form secondary tumours in the new organ or environment

It is well known that the majority of cells within a tumour cannot complete the multistep process of metastasis and are eliminated. Less than 0.1% of cells entering the bloodstream successfully form clinically detectable lesions, (Welch & Rink-Schaeffer 1999).

The final step of the metastasis process is the development of a new tumour. In order to achieve this, the metastatic cells must first invade the tissue, a process which involves adherence to the basement membrane (BM), production and secretion of proteolytic enzymes capable of degrading the extracellular matrix (ECM) and basal

membrane (BM), adherence to and degradation of the ECM components of host cells and regulation of genes encoding for proteolytic enzymes for ECM degradation and adhesion. The genes involved in the degradation of the ECM are those coding for the Matrix Metalloproteinases (MMPs).

The metastatic potential of epithelial cells can be demonstrated in vitro using the Matrigel Invasion assay (Albib *et al.* 1987; Kermorgant *et al.* 2001). The aqueous phase of faeces called faecal water (FW), contains most of the free reactive and soluble factors, which interact with the mucosa in vivo (Rafter *et al.* 1987). Investigating the effect of FW on cells in the Matrigel Invasion assay determines the invasion promoting potential of FW.

One of the most important groups of phase II detoxifying enzymes are the glutathione-S-transferases (GSTs) which are known to inactivate a number of genotoxic and carcinogenic substances by catalysing their conjugation with glutathione. The GST family is divided into two microsomal and several cytosolic classes (alpha, mu, kappa, pi, sigma, theta and zeta). Several GSTs are polymorphic and some allelic variants causing impaired enzyme activity are suspected to increase susceptibility to cancer. Extensive deletions in GSTM1 and GSTT1 result in complete loss of enzyme function, which may influence colorectal cancer susceptibility (Seow *et al.* 2002).

Szarka *et al.* (1995) measured the GST activity of blood lymphocytes of healthy control individuals and individuals at high risk of colon cancer. They found that the GST activity of blood lymphocytes from high-risk individuals was significantly

lower than that of blood lymphocytes from control individuals. They also measured GST activity in colon tissue from the high-risk individuals and paired analysis indicated a strong correlation between the GST activity of blood lymphocytes and colon tissue. Dietary intervention can modulate the activity of GSTs. Treptow- van Lishaut *et al.* (1999) reported that consumption of a resistant starch, induced GST π in the rat colon and that germ free rats had lower levels of colonic GSTs than conventional rats. Thus the inducibility of GSTs in normal tissues is a marker of chemoprevention.

However human colonic tumours have been reported to express high levels of GST. Hengstler *et al.* (1998) reported that the expression of GST α and π was significantly increased in tumour tissue compared to adjacent normal tissue in the colon. Sutoh *et al.* (2000) reported increased GST π and P-glycoprotein expression was associated with failure of cancer chemotherapy and poor patient survival. Further work is needed but the use of GST activity as a biomarker for cancer risk and as a means of monitoring responsiveness to treatment is a promising prospect.

Carcinoembryonic antigen (CEA) has been in use for approximately 30 years and is one of the most widely used tumour markers worldwide. It is a high molecular weight glycoprotein belonging to the immunoglobulin superfamily of molecules. It has been postulated to play a role in a number of biological processes including cell adhesion, immunity and apoptosis. After CEA, CA 19-9 is the most widely used tumour marker. It is the best available marker for pancreatic cancer but it is less sensitive than CEA for detecting colorectal carcinoma (Duffy *et al.* 2002). As these markers are the most commonly used tumour markers for colorectal cancer we

decided to investigate if synbiotic intervention in subjects at risk of colorectal cancer could affect their levels.

The aims of this study were:

- 1) To investigate the influence of twelve-week consumption of this synbiotic combination of *Lactobacillus rhamnosus* GG, *Bifidobacterium lactis* Bb12 and Synergy[®]1 on proliferative activity in the colonic mucosa of polypectomised subjects.
- 2) To investigate the influence of twelve-week consumption of a synbiotic combination on the expression of tumour markers (CEA and CA19-9) as well as the tumour promoting activities of faecal water in polypectomised subjects and in cancer subjects who had previously undergone 'curative resection'.
- 3) To investigate if synbiotic intervention can modulate the activity of glutathione-S-transferases
- 4) To investigate if the expression of tumour markers and the tumour promoting activities of faecal water in cancer subjects who had undergone previous curative resection is different from that of polypectomised subjects.

Methods

Work described in this chapter was carried out in collaboration with Dr Roisin Hughes and Prof. Ian Rowland of the Northern Ireland Centre for Food & Health, University of Ulster, Coleraine, Northern Ireland, Dr Giovanna Caderni, of the Department of Pharmacology, University of Florence, Italy, Dr Annett Klinder and Prof. Beatrice L. Pool-Zobel of the Department of Nutritional Toxicology, Friedrich-Schiller University of Jena, Germany and Dr Tony Fitzgerald of the Biochemistry Department, The Mercy University Hospital, Cork.

Proliferation Assay

Proliferative activity was assessed *in vitro* by [^3H] thymidine incorporation in colorectal biopsy specimens from polyp subjects. Proliferative activity was not assessed in cancer subjects due to sample limitation. Because bowel preparations used before endoscopic examinations have been reported to affect colon proliferation (Kleibeuker *et al.* 1995), patients were instructed not to take any laxatives or enemas before endoscopy.

Two rectal biopsies were obtained by sigmoidoscopy from normal appearing mucosa at about 15 cm from the anal verge of polyp subjects on placebo or synbiotic diet and placed in a collection tube containing DMEM (Gibco). Biopsies were washed well with sterile saline to remove any faeces and mucus and immediately transferred to a 20 ml sterile vial fitted with a sleeve-style rubber stopper, containing 1 ml DMEM (Gibco,) and 5 μl of ^3H -thymidine (specific activity, 41 Ci/mmol; final concentration, 5 $\mu\text{Ci/ml}$; Amersham Bioscience). Oxygen (25 ml) was injected into the vials, which were then incubated in a shaking water-bath at 37°C for 90 mins.



Figure 1a: Biopsy section showing longitudinal crypts and labelled proliferating cells (Magnification: 40X)



Figure 1b: Colonic crypt showing labelled proliferating cells (Magnification: 1000X)

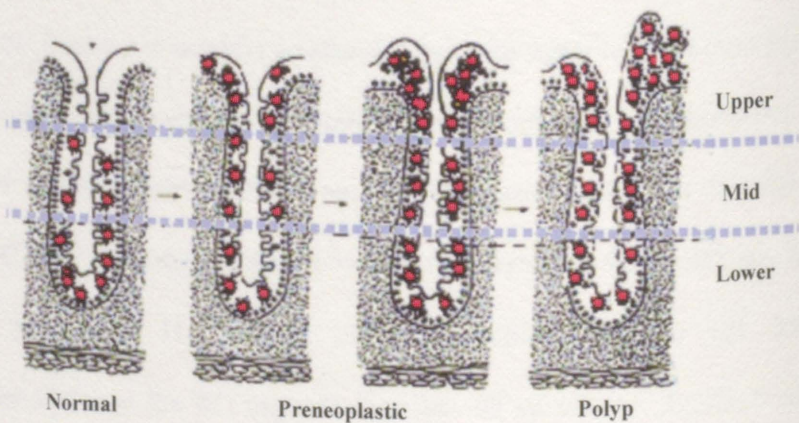


Figure 1c: Distribution of the proliferative activity along the colonic crypt

At the end of the incubation the pressure in the vial was released with a syringe and biopsies were washed 10 times with sterile saline to remove any unincorporated [³H] thymidine. Biopsies were orientated luminal side up under the microscope, set in a plastic frame and fixed in this orientation with 2% agar at 40°C. After solidification of the agar, the biopsies were fixed in 10% buffered formalin, embedded in paraffin and stored at room temperature until processing for autoradiography.

Samples were processed for autoradiography as follows. Samples were sectioned on a microtome and a series of sections, 5µm in thickness and perpendicular to the surface of the mucosa were cut, these were dipped, in the dark, in Nuclear Track Emulsion (NTB-2; Eastman Kodak), diluted 1:1 in distilled water at 40°C, and kept in the dark at 4°C for 2 weeks. Slides were then developed by standard photographic procedures and stained with haematoxylin-eosin.

For each subject, only full longitudinal crypt sections (i.e. from the base to the top of the crypt) were scored (*Figures 1(a-b)*). For each subject (n=32) a mean of 14 ± 0.7 (SE) crypts were scored (range: 10-19). The number of cells per crypt column (i.e. number of cells from the bottom to the top of the half crypt appearing in the section) and the number and position of the labelled cells along the crypt, dividing each crypt into three equal parts: lower, middle and upper compartment (*Figure 1(c)*), was recorded.

The proliferative activity was expressed as percentage labelling index (LI):

$$\frac{\text{number of labelled cells counted in all the crypts of the same subject}}{\text{total number of cells in all the crypt sections of the same subject}} \times 100$$

To evaluate the distribution of proliferative activity along the crypt, each crypt was divided into three equal compartments (lower, middle and upper) and the distribution of proliferating cells along the crypt (%LI in each compartment of the crypt) was expressed using the formula:

$$\frac{\text{total number of labelled cells in each compartment of all the crypts scored}}{\text{total number of labelled cells scored in the complete crypt}} \times 100$$

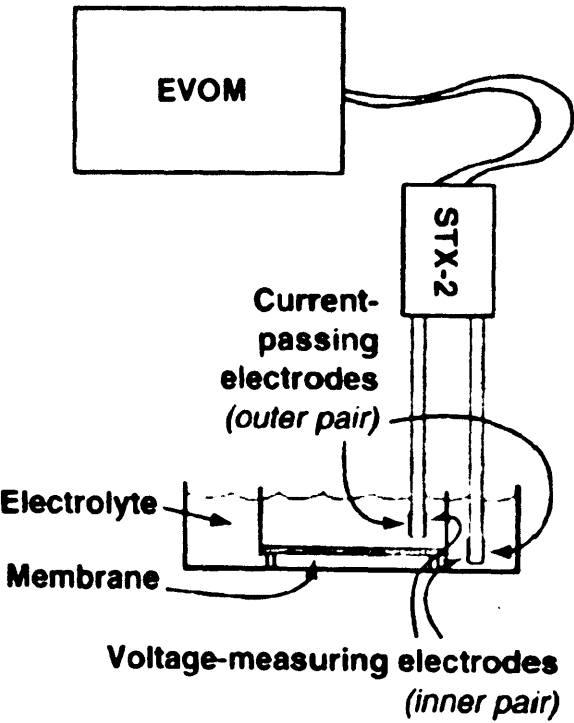
Slides were coded and read independently by two observers. The Correlation Coefficient between these two observers on a set of 46 LI scores was 0.83 ($P < 0.001$).

Tumour promotion assays: tight junction integrity

The effect of faecal water on two markers of tight junction integrity trans-epithelial resistance (TER) and ^{14}C mannitol flux in the Caco-2 cell line was measured as an indicator of tumour promotion. Caco-2 cells are capable of enterocytic differentiation forming atypical brush border membranes and tight junctions and provide a relevant model for permeability and transport studies (Pinto *et al.* 1983).

All tissue culture media and plastic ware was purchased from Life Technologies (Paisley, UK). Human Caco-2 cells were obtained from the European Collection of Animal Cell Cultures (ECACC) and cultured in tissue culture flasks with minimum essential medium supplemented with foetal bovine serum (10%), non-essential amino acids (1%) and penicillin/streptomycin (1%) in a humidified atmosphere containing 5% CO_2 in air until 75% – 85% confluent. Cells between passage 6 and 10 were used.

Figure 2: Measuring the transepithelial resistance of a cell monolayer grown on an insert using an epithelial voltohmmeter and STX2 electrode.



Caco-2 cells were seeded onto the apical surface of collagen (rat tail type I, Sigma) coated polyethylene terephthalate (PET) cell culture inserts (pore size $0.45\mu\text{m}$, surface area 4.2cm^2 , Becton Dickinson, Falcon), which were kept in six well companion plates (BD Falcon, Oxford, UK). 2.5×10^5 cells were added to each insert (i.e. 5.95×10^4 cells per cm^2) and standard growth medium (1.5ml) was added to each well. Two inserts contained cell free medium only in order to control for resistance due to the insert membrane. Spent medium was removed and fresh medium added to all inserts every forty-eight hours.

Transepithelial Electrical Resistance (TER)

TER readings were taken every 48 hours following eight days of cell culture on the inserts. TER was measured using an EVOM epithelial voltohmeter and STX2 electrode (World Precision Instruments, Stevenage, Herts, UK) and all measurements were taken after cells had adapted to room temperature (*Figure 2*). TER readings stabilised at $700\text{-}800\text{ ohms/cm}^2$ after 14-16 days and cells were then considered fully differentiated. Cells were then treated with faecal water (10% in standard growth medium) for a 12-hour period. Changes in tight junction integrity were assessed over this time by TER and results were expressed as percentage change over the 12-hour period. Samples were analysed in triplicate. Negative (standard culture media only) and positive ($250\text{ }\mu\text{M}$ deoxycholic acid) controls were included.

^{14}C Mannitol Flux

Using the same system the effect of faecal water on the paracellular transport of radiolabelled mannitol ^{14}C , was measured following the final TER reading. The

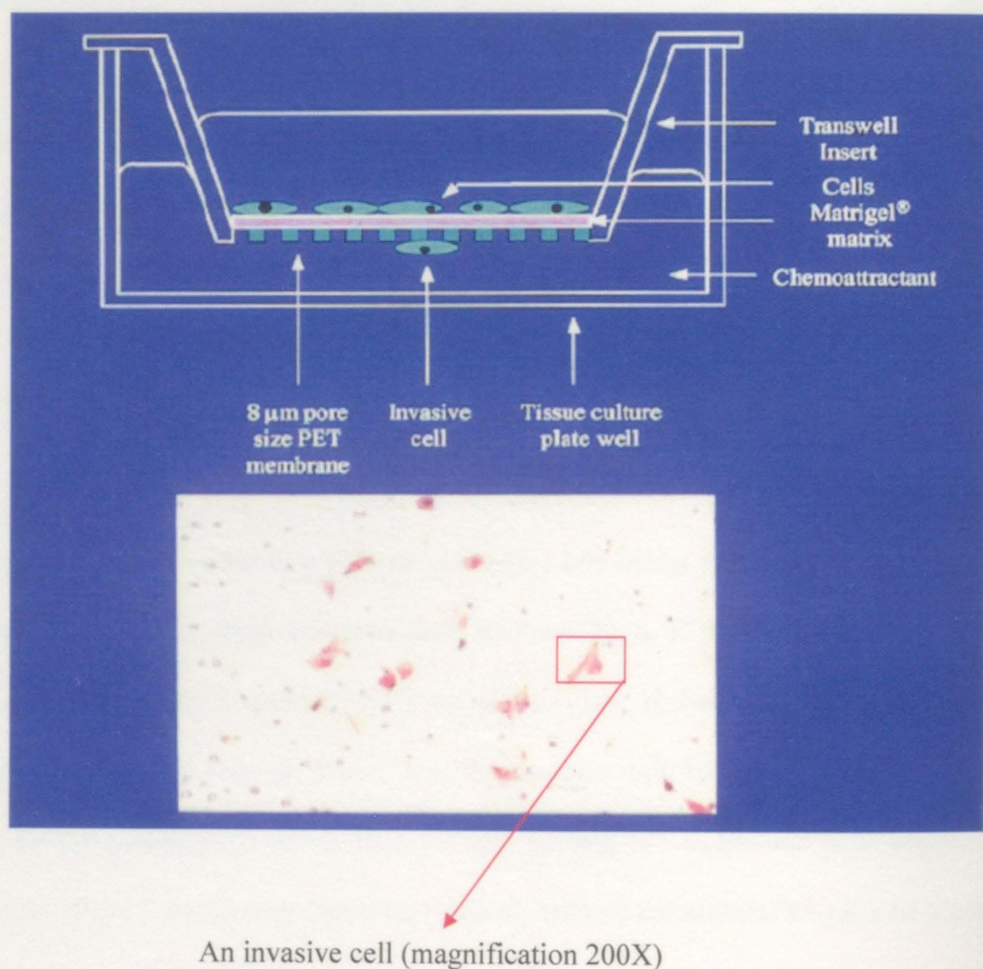
epithelial layer is normally impermeable to the sugar alcohol mannitol however permeability increases as the selective function of the tight junctions decreases. This can be measured by monitoring the apical to basolateral passage of radiolabelled mannitol ^{14}C across the monolayer and was used as another marker of tight junction integrity to confirm TER results. Results were presented as activity of ^{14}C mannitol transported across monolayer expressed as a percentage of initial activity applied to cells.

Faecal water was removed from the Caco-2 monolayers and cells were washed in Hanks Balanced Salt Solution (HBSS). ^{14}C mannitol ($1\mu\text{Ci}$ per ml in standard growth medium) was then added and cells were incubated at room temperature for one hour at which time $50\mu\text{l}$ of medium was removed from the well of the companion plate and added to 5ml scintillation fluid (Scintran Cocktail T, BDH). The scintillation count of the original solution added to the insert was also determined. The amount of ^{14}C passing into the well of the companion plate was counted on a liquid scintillation counter (Wallac) as counts per minute. Results were expressed as a percentage of the known ^{14}C counts initially added to each insert.

Tumour promotion assays: HT115 cell invasion

The number of invading HT115 cells was used as a measure of cell invasion following a 24-hour treatment with faecal water. The HT115 cell line is a highly invasive human colon cancer cell line which was obtained from the European Collection of Animal Cell culture (ECACC, UK). HT115 cells were routinely maintained in Dulbeccos minimum essential media (DMEM) supplemented with L-glutamine (1%), foetal bovine serum (10%) and penicillin/streptomycin (1%). Cells

Figure 3: Matrigel Invasion Assay



were grown in a humidified atmosphere at 37°C containing 5% CO₂ in air. Cells were grown to 80% confluence. To test the invasive ability of cells, matrigel protein (BD Biosciences, Oxford, UK) was diluted with serum free growth medium (1:2). Cell culture inserts (8µm pore size, BD Biosciences, Falcon, UK) were coated with diluted matrigel and allowed to dry at room temperature to give a final concentration of 25µg/cm² of matrigel. Chambers were then rehydrated by adding serum free medium and incubated at 37°C for two hours.

HT115 cells were then seeded onto the inserts at a density of 1x10⁵/ml in serum free growth medium. Inserts were then placed in tissue culture wells containing MRC5 monolayers. MRC5 cells which were obtained from the American Type Culture Collection (ATTC, USA) are fibroblasts, which produce a hepatocyte growth factor/scatter factor (HGF/SF), which acts as a chemo-attractant. HT115 cells were immediately treated with faecal water (10% in serum free growth medium) and incubated in a humidified incubator at 37°C for 24 hours. HT115 cells on both sides of the inserts (i.e. non invasive and invasive cells) were then fixed in 80% ethanol and stained with haematoxylin (Sigma.). Cells were counted in five random fields at a magnification of 200x (Imagedok, Kinetic Imaging) (*Figure 3*). The number of invading cells was expressed as a percentage of the total cells (non invasive + invasive) counted.

Glutathione-S-Tranferase (GST) activity in PBMCs

PBMCs were isolated by density gradient centrifugation and cell number was adjusted to 1x10⁶ cells/ml in RPMI. The cell culture medium was removed by centrifuging at 400 x g for 8mins and cells were resuspended in cryoprotective

medium (90% FBS, 10% DMSO), frozen slowly to -80°C and stored under liquid nitrogen until analysed.

Cryopreserved PBMCs were thawed on ice at 4°C, centrifuged at 400 x g for 8 mins to remove the cryoprotective medium and resuspended in 1ml PBS. Cell numbers and viabilities were determined by counting in the presence of trypan blue. Then cell suspension was again centrifuged (400 x g, 8 mins) and the pellet was resuspended in 0.5 ml sonication buffer (25 mM Tris-HCl, pH 7.8, 0.25 M sucrose, 1 mM EDTA, and 1 mM Pefabloc), and homogenised for 1 min (ultrasound Bandelin Electronics). Following ultra-centrifugation (32,000 x g, 75 min, 4°C), the supernatant was frozen at -140°C until analysed.

Total GST activity was determined by measuring spectrophotometrically the conjugation of 1 mM 1-chloro-2,4-dinitrobenzene with reduced 1 mM glutathione in the presence of cell sonicate at 30°C. Conjugation is accompanied by an increase in absorbance at 340nm (Habig *et al.* 1974). The rate of increase is directly proportional to the GST activity of the sample. Total protein content was measured according to Bradford (1976), using BSA as standard protein. All values were based on the number of cells (Treptow-van Lishaut *et al.* 1999) as well as on total protein content. Only one determination in each of these assays could be performed, due to the limited amount of material available.

CEA and CA19-9

Blood was collected in serum-gel blood collection tubes (Sarstedt). The tubes were centrifuged at 300 x g for 5mins and the upper serum layer was removed, aliquoted and stored at -80°C until analysed. Serum CEA and CA19-9 levels were determined by a microparticle enzyme immunoassay and analysed on an IMx Analyzer, (Abbott).

Results

Proliferative Activity in the Colorectal Mucosa

Colorectal biopsies were obtained from polyp subjects at the beginning (week 0) and the end of intervention (week 12). Proliferative activity was assessed *in vitro* by [3H] thymidine incorporation in colorectal biopsy specimens. The total number of cells (labelled and unlabelled) per crypt, the number of labelled cells per crypt, the labelling index (number of labelled cells per crypt x cells counted in that crypt x 100) and the distribution of the labelled cells along the crypt were scored independently by 2 observers.

Synbiotic consumption significantly reduced proliferative activity in the colonic mucosa of polyp subjects. In polyp subjects consuming the synbiotic (n=14) the number of labelled cells per crypt at the end of intervention was significantly less (p=0.0277) than at the beginning of intervention (**Figure 4(b)**). This change was not observed in the placebo polyp subjects (n=14) (**Figure 4(a)**).

The overall labelling index (LI) was also significantly reduced (p=0.0274) at week 12 compared to week 0 in the synbiotic group (**Figure 5(b)**), but was unaltered in the placebo group (**Figure 5(a)**).

The distribution of labelled cells along the crypt in both the synbiotic and placebo groups was unaltered. The significant reduction in proliferative activity in the synbiotic group occurred in the lower (1st) compartment of the crypt (p=0.0052) (**Figures 6 a-b**) while proliferative activity in the mid (2nd) and upper (3rd) crypt compartments was unaltered (**Figures 7 a-b, 8 a-b**).

The total number of cells/crypt (labelled and unlabelled) was unchanged by intervention. For synbiotic subjects the total number of cells/crypt (mean $\pm$ SE) was 62.25 ± 1.78 before and 61.83 ± 1.89 following intervention. For placebo subjects the numbers were 62.26 ± 1.73 before and 64.18 ± 1.84 after intervention.

Figure 4(a)

The number of labelled cells per crypt in colorectal biopsies from polyp subjects (n=14) consuming placebo at the beginning (week 0) and the end of intervention (week 12). The number of labelled cells per crypt was not significantly different at week 0 compared to week 12.

Figure 4(b)

The number of labelled cells per crypt in colorectal biopsies from polyp subjects consuming synbiotic (n=14) at week 0 and week 12. The number of labelled cells per crypt was significantly reduced at week 12 compared to week 0.

Figure 5(a)

The overall labelling index (LI) in colorectal biopsies from placebo polyp subjects (n=14) was not significantly different at week 0 and week 12.

Figure 5(b)

The overall labelling index (LI) in colorectal biopsies from synbiotic polyp subjects (n=14) was significantly reduced at week 12 compared to week 0

Figure 4 (a)

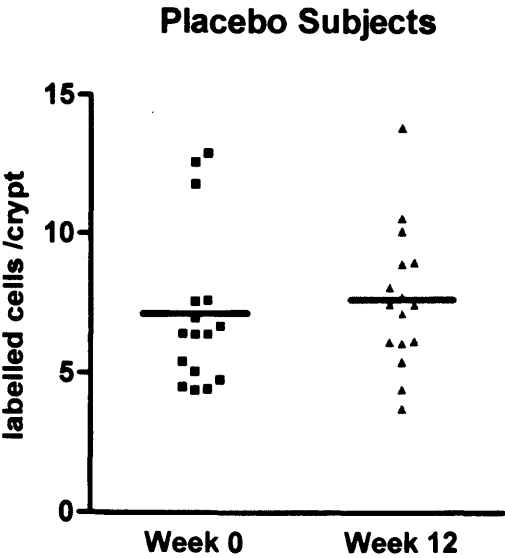


Figure 4 (b)

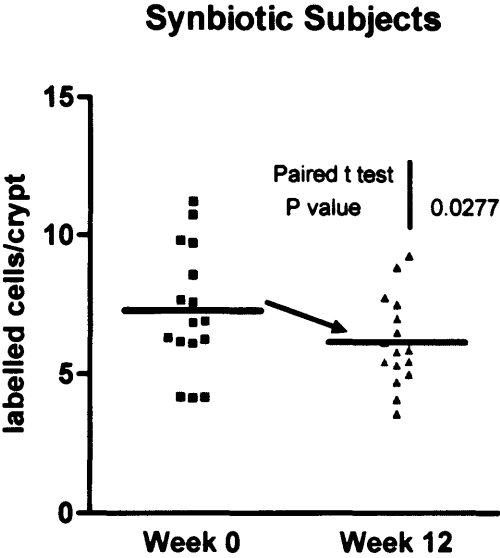


Figure 5 (a)

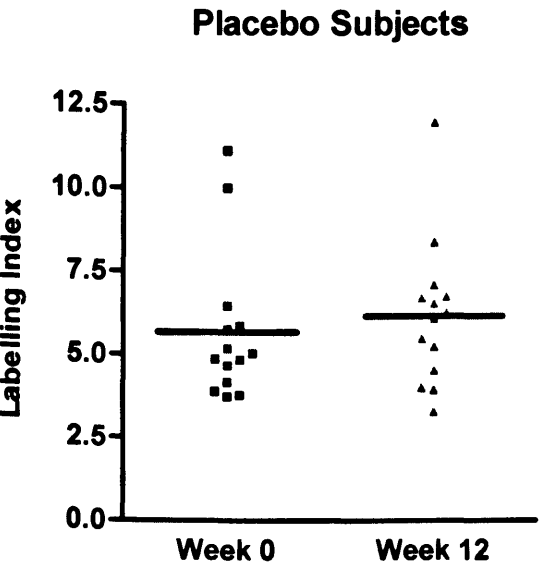


Figure 5 (b)

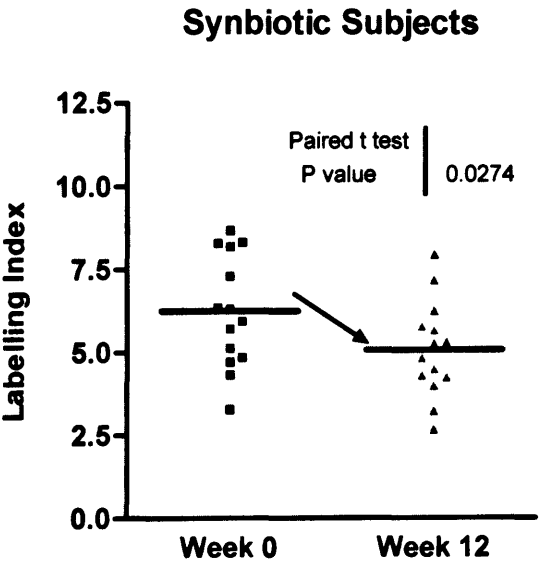


Figure 6(a)

The percentage labelling index (%LI) in the lower (1st) crypt compartment in colorectal biopsies from placebo polyp subjects (n=14). There was no significant change in the twelve-week intervention period.

Figure 6(b)

The percentage labelling index (%LI) in the lower (1st) crypt compartment in colorectal biopsies from synbiotic polyp subjects (n=14) was significantly reduced at week 12 compared to week 0.

Figure 7(a)

The percentage labelling index (%LI) in the mid (2nd) crypt compartment in colorectal biopsies from placebo polyp subjects (n=14). There was no significant change in the twelve-week intervention period.

Figure 7(b)

The percentage labelling index (%LI) in the mid (2nd) crypt compartment in colorectal biopsies from synbiotic polyp subjects (n=14) was significantly reduced at week 12 compared to week 0.

Figure 8(a)

The percentage labelling index (%LI) in the upper (3rd) crypt compartment in colorectal biopsies from placebo polyp subjects (n=14). There was no significant change in the twelve-week intervention period.

Figure 8(b)

The percentage labelling index (%LI) in the upper (3rd) crypt compartment in colorectal biopsies from synbiotic polyp subjects (n=14). There was no significant change in the twelve-week intervention period.

Figure 6 (a)

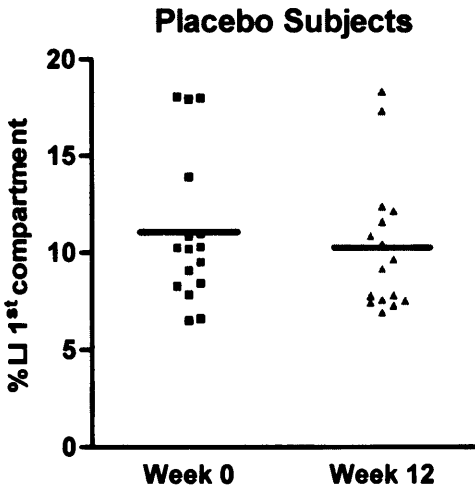


Figure 6 (b)

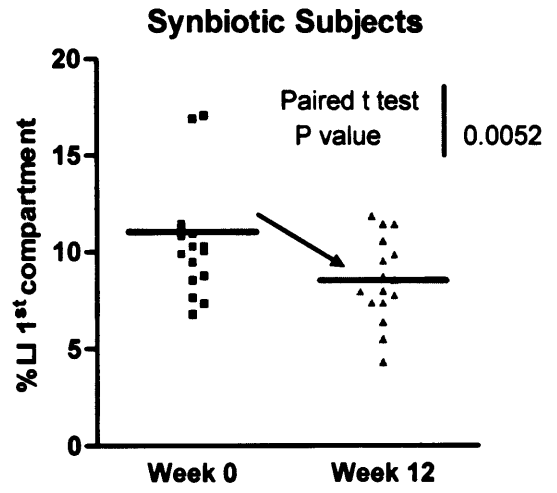


Figure 7 (a)

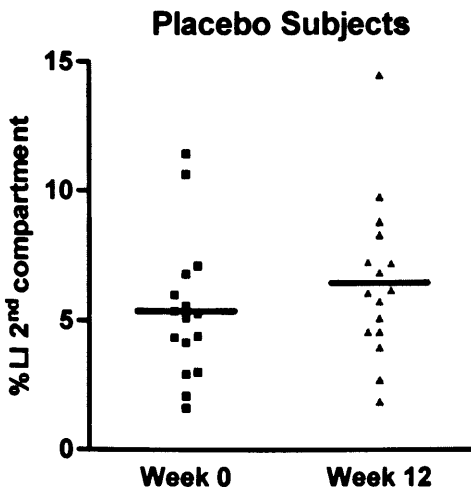


Figure 7 (b)

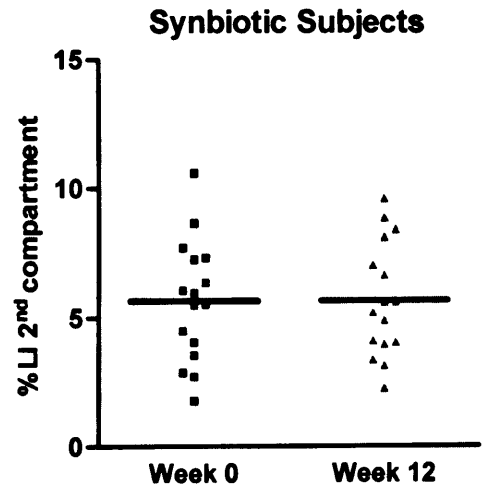


Figure 8 (a)

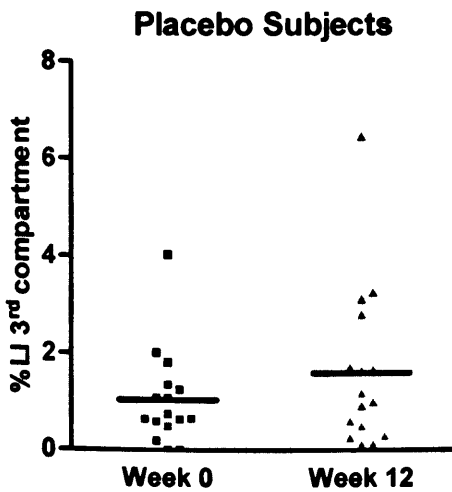
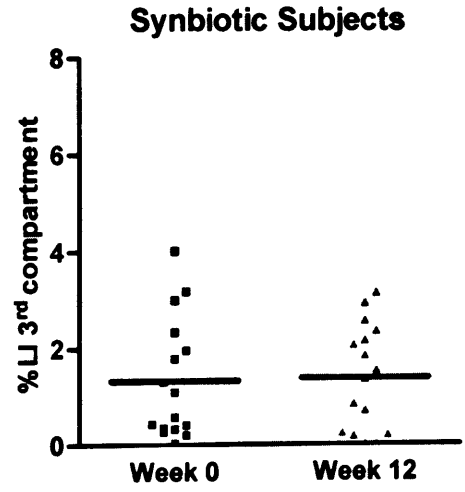


Figure 8 (b)



The Influence of Faecal water on Tumour Promotion

The influence of FW on tumour promotion was assessed by measuring a number of tumour promoting biomarkers;

1. trans-epithelial resistance (TER) in Caco-2 cells
2. ^{14}C mannitol flux in Caco-2 cells
3. Invasive potential of HT115 cells

Tight junction integrity

1. Trans-epithelial resistance

Changes in paracellular permeability across a Caco-2 monolayer induced by incubation with FW samples were measured as the resistance to the flow of an electric current (trans epithelial electrical resistance, TER). There was no significant difference in the tight junction integrity of Caco-2 cells measured by trans-epithelial resistance following incubation with FW derived from both cancer and polyp subjects irrespective of intervention at all time points (*Table 1*):

The influence of FW derived from cancer (synbiotic and placebo) and polyp (synbiotic and placebo) subjects at the beginning of intervention (week 0) on tight junction integrity was measured by TER (*Table 2*). FW derived from polyp subjects significantly improved tight junction integrity compared to FW derived from cancer subjects at the beginning of intervention. In fact TER was significantly higher following treatment of cells with FW derived from polyp subjects compared to FW from cancer subjects at all three time points. However FW derived from cancer subjects did not have an adverse effect on tight junction integrity, which was generally greater than in cells treated with tissue culture medium alone (100%).

2. ^{14}C mannitol flux

The influence of FW on paracellular permeability across a Caco-2 monolayer was also determined by measuring the paracellular transport of ^{14}C mannitol across that monolayer. The tight junction integrity measured by ^{14}C mannitol flux in Caco-2 cells was unaltered at week 6 and week 12 compared to week 0 for both cancer and polyp subjects irrespective of intervention (*Table 3*).

The influence of FW derived from cancer (synbiotic and placebo) and polyp (synbiotic and placebo) subjects at the beginning of intervention (week 0) on tight junction integrity was measured by assessing the paracellular transport of ^{14}C mannitol (*Table 2*). The paracellular transport of ^{14}C mannitol was not significantly different in cells treated with FW derived from polyp subjects compared to cells treated with FW derived from cancer subjects.

3. HT115 cell invasion

HT115 cells were grown on matrigel coated cell culture inserts and treated with FW. The number of cells on both sides of the insert, (i.e. non invasive and invasive cells) were counted microscopically and the number of invading cells was expressed as a percentage of the total cells (non invasive + invasive) counted. The percentage of invading cells at week 6 and week 12 were not significantly different than at week 0 for all groups of subjects whether consuming the synbiotic or the placebo product (*Table 4*).

The influence of FW derived from cancer (synbiotic and placebo) and polyp (synbiotic and placebo) subjects at the beginning of intervention (week 0) on the invasive potential of HT115 cells was analysed by determining the percentage of

invasive cells passing through the matrigel (*Table 2*). There was no significant difference in invasive potential in cells treated with FW derived from polyp subjects compared to FW derived from cancer subjects at the beginning of intervention or at all three time-points.

Table 1

There was no significant difference in the tight junction integrity of Caco-2 cells measured by trans-epithelial resistance following incubation with FW derived from both cancer and polyp subjects irrespective of intervention at all time points. Results are expressed as mean $\pm$ standard error.

Table 2

Comparison of biomarkers in all cancer (synbiotic and placebo) subjects compared to all polyp (synbiotic and placebo) subjects. Tight junction integrity (TER) in Caco-2 cells was significantly higher in cells following incubation with FW derived from polyp subjects compared to cancer subjects at the beginning of intervention and at all three time points. Mannitol flux (activity of ^{14}C mannitol transported across monolayer expressed as a percentage of initial activity applied to cells) was not significantly different at the beginning of intervention or at all time points. The percentage of invasive cells [number of invading cells expressed as a percentage of total cells (non invasive + invasive)] passing through the matrigel was not significantly different in cells treated with FW derived from polyp subjects compared to cells treated with FW derived from cancer subjects. Results are expressed as mean $\pm$ standard error. Statistical significance was assessed by unpaired t-test.

Table 1: Transepithelial electrical resistance in all subject groups
(% change in TER following treatment with FW)

Cancer subjects		Week 0	Week 6	Week 12	P value
Synbiotic	(n=16)	113.1 ± 7.019	114.8 ± 10.55	110.2 ± 8.144	n.s.
Placebo	(n=12)	101.6 ± 9.063	105.1 ± 9.240	101.8 ± 12.52	n.s.
Polyp subjects		Week 0	Week 6	Week 12	P value
Synbiotic	(n=14)	126.8 ± 5.433	132.7 ± 7.804	133.5 ± 5.466	n.s.
Placebo	(n=20)	133.5 ± 5.239	127.8 ± 7.432	125.2 ± 6.642	n.s.

Table 2: Biomarker levels in cancer vs. polyp subjects at the beginning of intervention and at all three time points

Biomarker (units)	Cancer Subjects Week 0	Polyp Subjects Week 0	P value	Cancer Subjects All times	Polyp Subjects All times	P value
Tight Junction Integrity						
TER (% of control)	108.8 ± 5.1 (n=31)	130.5 ± 3.7 (n=35)	0.0009	108.3 ± 5.2 (n=31)	127.7 ± 3.9 (n=35)	0.0036
Mannitol flux (% transported)	6.42 ± 0.83 (n=31)	6.17 ± 0.39 (n=35)	n.s.	6.21 ± 0.70 (n=31)	5.96 ± 0.39 (n=35)	n.s.
Invasive Potential						
Invasive cells (% of total cells)	6.37 ± 0.65 (n=25)	5.50 ± 0.71 (n= 34)	n.s.	7.10 ± 0.64 (n=31)	6.26 ± 0.60 (n=35)	n.s.

Table 3

The tight junction integrity measured by ^{14}C mannitol flux in Caco-2 (activity of ^{14}C mannitol transported across the monolayer expressed as a percentage of initial activity applied to cells) cells was unaltered during the twelve-week intervention for both cancer and polyp subjects irrespective of intervention. Results are expressed as mean $\pm$ standard error.

Table 4

The percentage of HT115 cells invading a matrigel coated cell insert following treatment with FW was not significantly different for FWs derived from all subjects (cancer and polyp) whether consuming the synbiotic or the placebo product. Results are expressed as mean $\pm$ standard error.

Table 3: Mannitol flux in all subject groups (% transported)

Cancer subjects		Week 0	Week 6	Week 12	P value
Synbiotic	(n=16)	7.356 ± 1.348	6.386 ± 1.123	6.379 ± 1.277	n.s.
Placebo	(n=12)	6.190 ± 0.999	6.949 ± 1.541	6.897 ± 1.696	n.s.
Polyp subjects		Week 0	Week 6	Week 12	P value
Synbiotic	(n=14)	6.749 ± 0.573	6.886 ± 0.605	6.640 ± 0.669	n.s.
Placebo	(n=20)	5.913 ± 0.531	6.065 ± 0.568	5.656 ± 0.574	n.s.

Table 4: HT115 Invasion in all subject groups [number of invading cells expressed as a percentage of total cells (non invasive + invasive)]

Cancer subjects		Week 0	Week 6	Week 12	P value
Synbiotic	(n=13)	6.564 ± 1.025	7.020 ± 1.086	9.475 ± 2.312	n.s.
Placebo	(n=9)	6.446 ± 1.091	8.636 ± 2.105	5.849 ± 1.719	n.s.
Polyp subjects		Week 0	Week 6	Week 12	P value
Synbiotic	(n=8)	5.689 ± 1.430	8.875 ± 2.155	9.043 ± 2.888	n.s.
Placebo	(n=7)	5.419 ± 1.046	5.981 ± 1.110	6.608 ± 1.473	n.s.

Glutathione –S- Transferase (GST) Activity in PBMCs

Total GST activity in PBMCs was measured spectrophoretically and expressed as nmoles GST / min per 10^6 cells and nmoles GST / min per μg total protein.

Cancer subjects

Total GST activity was significantly increased by synbiotic consumption in cancer subjects. GST activity per 10^6 cells was significantly increased ($p=0.0282$) at week 6 compared to week 0 in synbiotic cancer subjects ($n=18$) (*Figure 9(a)*). GST activity per 10^6 cells was unaltered in placebo cancer subjects ($n=14$) (*Figure 9(b)*).

In synbiotic cancer subjects GST activity per μg of protein was unaltered during the twelve week intervention period (*Figure 10(a)*). Again there was no change in the placebo group of cancer subjects (*Figure 10(b)*).

Polyp subjects

Total GST activity per 10^6 cells was unaltered in polyp subjects consuming synbiotic ($n=18$) (*Figure 11(a)*). However in polyp placebo subjects ($n=19$) GST activity per 10^6 cells was significantly increased ($p=0.0034$) at week 12 compared to week 0 (*Figure 11(b)*).

GST activity per μg of protein was increased significantly ($p=0.0176$) at week 12 compared to week 0 in the synbiotic polyp group (*Figure 12(a)*). GST activity per 10^6 cells was unaltered during the twelve week intervention period in the polyp placebo group (*Figure 12(b)*).

GST activity of lymphocytes from cancer (synbiotic and placebo) and polyp (synbiotic and placebo) subjects at the beginning of intervention was assessed. There was no significant difference in GST activity in cancer subjects compared to polyp subjects. GST activity per 10^6 cells was 1.06 ± 0.11 (cancer) and 0.88 ± 0.09 (polyp) and GST activity per μg of protein was 5.64 ± 0.74 (cancer) and 6.23 ± 0.86 (polyp).

Figure 9(a)

GST activity per 10^6 cells in synbiotic cancer subjects (n=18) was significantly increased at week 6 compared to week 0.

Figure 9(b)

GST activity per 10^6 cells in cancer subjects consuming placebo (n=14) was unaltered during the twelve-week intervention period.

Figure 10(a)

GST activity per μg of protein in synbiotic cancer subjects (n=18) was unaltered during the twelve-week intervention period.

Figure 10(b)

GST activity per μg of protein in cancer subjects consuming placebo (n=14) was unaltered during the twelve-week intervention period.

Figure 9(a)

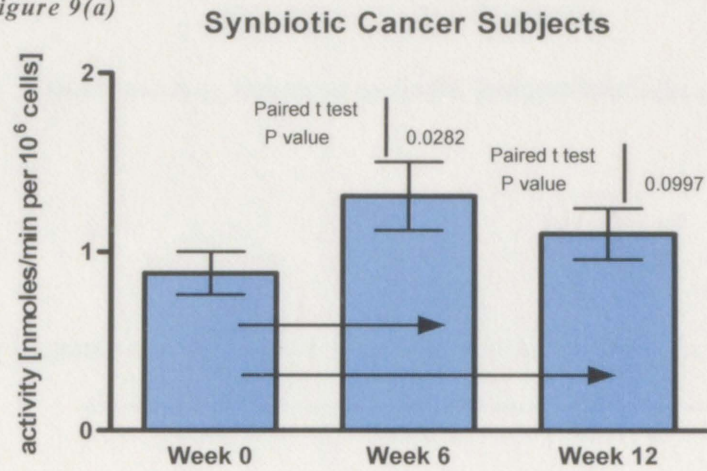


Figure 9(b)

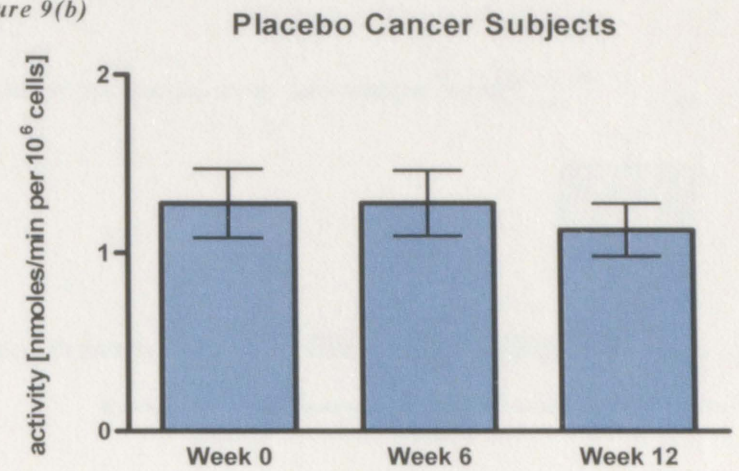


Figure 10(a)

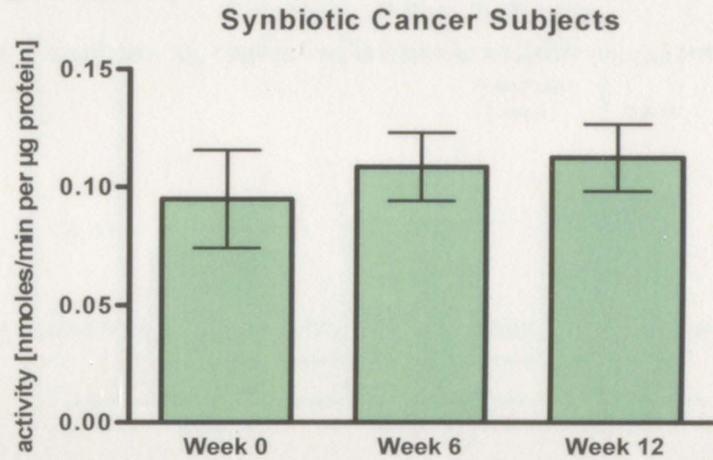


Figure 10(b)

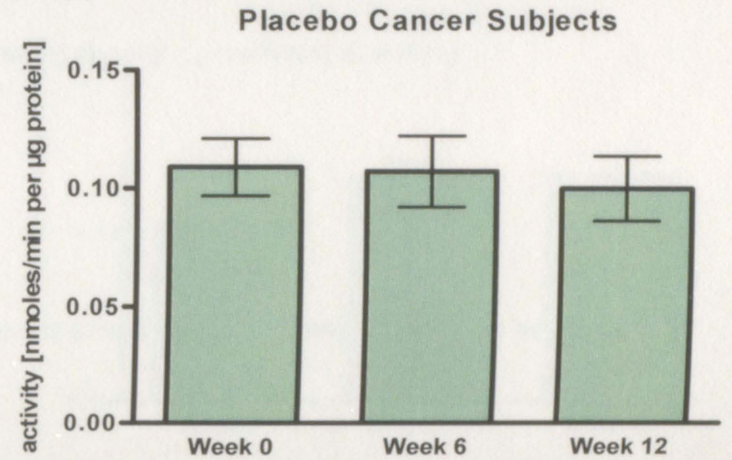


Figure 11(a)

GST activity per 10^6 cells in polyp subjects consuming synbiotic (n=18) was unaltered during the twelve-week intervention period

Figure 11(b)

GST activity per 10^6 cells in polyp placebo subjects (n=19) was significantly increased at week 12 compared to week 0.

Figure 12(a)

GST activity per μg of protein in the synbiotic polyp group (n=18) was significantly increased at week 12 compared to week 0.

Figure 12(b)

GST activity per μg of protein in the polyp placebo group (n=19) was unaltered during the twelve-week intervention period.

Figure 11(a)

Synbiotic Polyp Subjects

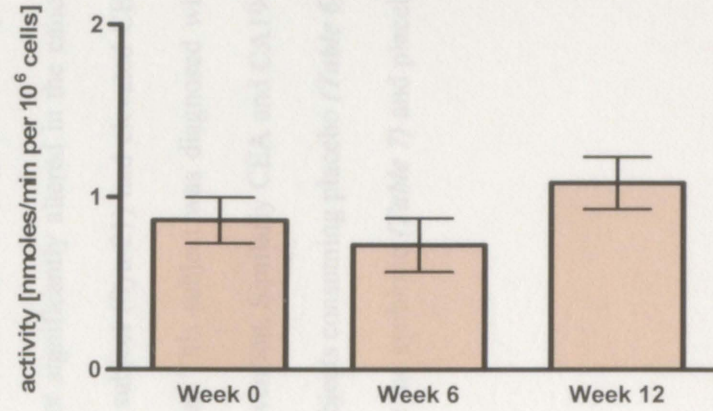


Figure 11(b)

Placebo Polyp Subjects

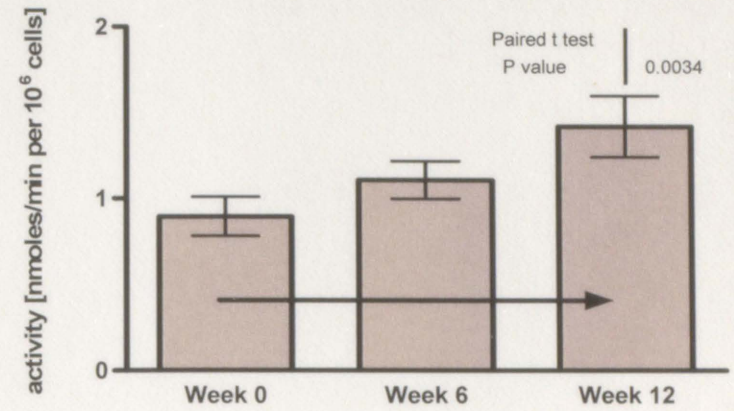


Figure 12(a)

Synbiotic Polyp Subjects

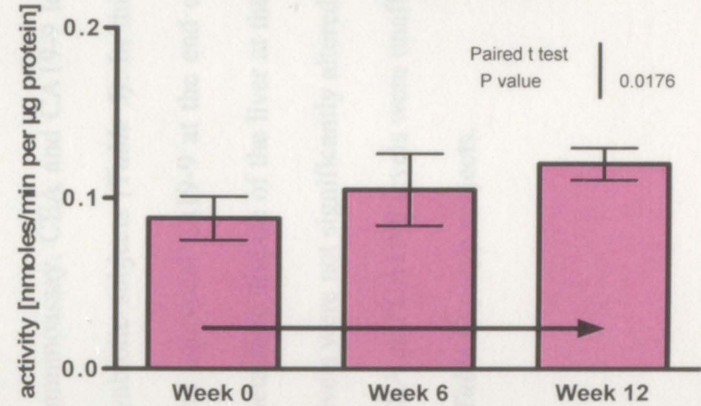
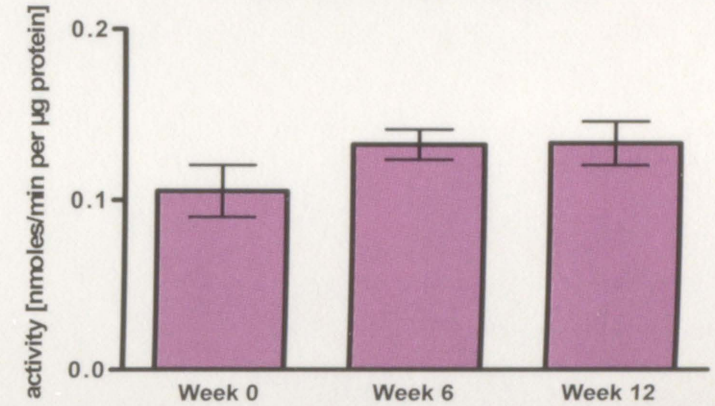


Figure 12(b)

Placebo Polyp Subjects



Biochemical Tumour Markers:

Carcinoembryonic antigen (CEA) & Carbohydrate antigen 19-9 (CA19-9)

Serum CEA and CA19-9 levels were determined by microparticle enzyme immunoassay. CEA and CA19-9 levels were not significantly altered in the cancer synbiotic subjects (*Table 5*). In this group one subject (Syn-21) had elevated CEA and equivocal Ca19-9 at the end of intervention. This subject was diagnosed with metastatic disease of the liver at the end of intervention. Similarly CEA and CA19-9 levels were not significantly altered in cancer subjects consuming placebo (*Table 6*). CEA and CA19-9 levels were unaffected in both the synbiotic (*Table 7*) and placebo (*Table 8*) polyp subjects.

Table 5

Serum CEA and Ca19-9 levels in cancer subjects consuming synbiotic.

CEA			CA19-9		
Normal range	0-3	ng/ml	Normal range	0-37	U/ml
Equivocal	3-10	ng/ml	Equivocal	37-60	U/ml
Elevated	>10	ng/ml	Elevated	>60	U/ml
ND = not determined.			WD=withdrew from intervention		

Table 6

Serum CEA and Ca19-9 levels in cancer subjects consuming placebo.

CEA			CA19-9		
Normal range	0-3	ng/ml	Normal range	0-37	U/ml
Equivocal	3-10	ng/ml	Equivocal	37-60	U/ml
Elevated	>10	ng/ml	Elevated	>60	U/ml
ND = not determined.			WD=withdrew from intervention		

Table 5

Subject no	CEA (week 0)	CEA (week 6)	CEA (week 12)	Ca19.9 (week 0)	Ca19.9 (week 6)	Ca19.9 (week 12)
Syn-01	ND	0.6	0.6	ND	9.1	8.2
Syn-03	ND	0.1	0.4	ND	4.7	3.9
Syn-04	0.4	0.4	0.5	6.6	10.0	7.4
Syn-05	3.8	3.0	4.2	2.0	1.5	1.6
Syn-08	0.7	0.9	1.1	0.5	0.8	0.6
Syn-09	0.7	0.6	0.5	10.4	5.8	6.4
Syn-13	1.0	1.0	1.1	0.0	0.0	0.0
Syn-14	0.8	0.8	0.7	5.9	6.0	5.9
Syn-20	1.8	2.0	2.3	19.9	18.0	20.2
Syn-21	ND	ND	11.8	ND	ND	42.8
Syn-25	0.6	0.6	0.3	19.2	21.2	22.7
Syn-26	0.8	1.0	0.8	11.8	10.9	13.5
Syn-39	2.5	2.0	2.5	4.1	3.3	5.0
Syn-43	1.6	1.6	1.8	11.8	14.6	11.7
Syn-51	2.2	1.9	1.9	4.4	3.4	11.9
Syn-58	0.5	0.5	0.6	11.5	9.1	10.4
Syn-71	3.1	2.9	2.6	0.0	0.0	0.0
Syn-75	2.3	1.9	2.0	0.0	0.0	0.0
Syn-76	1.5	2.0	1.7	6.4	4.0	43.0

Table 6

Subject no	CEA (week 0)	CEA (week 6)	CEA (week 12)	Ca19.9 (week 0)	Ca19.9 (week 6)	Ca19.9 (week 12)
Syn-02	ND	3.1	3.5	ND	19.3	19.3
Syn-06	2.3	2.3	2.8	ND	ND	ND
Syn-07	1.6	1.9	2.2	1.8	2.7	3.1
Syn-10	1.4	1.6	1.5	3.9	4.2	3.0
Syn-11	1.6	WD	WD	ND	WD	WD
Syn-12	0.7	1.0	0.9	9.2	6.8	7.7
Syn-15	0.2	0.5	0.2	1.2	1.4	1.0
Syn-16	0.4	0.1	0.4	0.9	0.5	0.7
Syn-17	1.7	1.4	1.8	27.4	27.9	25.9
Syn-18	1.0	1.2	1.2	14.5	14.1	12.8
Syn-19	0.7	0.7	1.0	0.7	0.0	1.0
Syn-30	1.0	1.0	1.2	1.0	1.4	6.4
Syn-38	2.7	2.7	3.1	13.5	15.9	14.8
Syn-44	1.3	WD	WD	21.7	WD	WD
Syn-47	0.3	WD	WD	9.5	WD	WD
Syn-52	1.7	1.5	1.7	12.9	11.3	13.0
Syn-73	0.7	0.7	0.8	3.2	3.8	3.7
Syn-77	1.9	2.4	2.4	12.7	22.0	19.9

Table 7

Serum CEA and Ca19-9 levels in polyp subjects consuming synbiotic.

CEA			CA19-9		
Normal range	0-3	ng/ml	Normal range	0-37	U/ml
Equivocal	3-10	ng/ml	Equivocal	37-60	U/ml
Elevated	>10	ng/ml	Elevated	>60	U/ml
ND = not determined.			WD=withdrew from intervention		

Table 8

Serum CEA and Ca19-9 levels in polyp subjects consuming placebo.

CEA			CA19-9		
Normal range	0-3	ng/ml	Normal range	0-37	U/ml
Equivocal	3-10	ng/ml	Equivocal	37-60	U/ml
Elevated	>10	ng/ml	Elevated	>60	U/ml
ND = not determined.			WD=withdrew from intervention		

Table 7

Subject no	CEA (week 0)	CEA (week 6)	CEA (week 12)	Ca19.9 (week 0)	Ca19.9 (week 6)	Ca19.9 (week 12)
Syn-23	1.4	1.5	1.4	21.2	26.6	29.8
Syn-27	ND	1.4	0.8	ND	18.2	15.6
Syn-29	1.7	1.6	2.1	2.9	2.3	2.6
Syn-33	0.7	0.8	0.8	2.8	3.0	2.0
Syn-34	0.1	0.2	0.1	3.1	3.6	4.2
Syn-36	1.7	1.5	1.5	2.7	3.0	3.4
Syn-37	3.5	3.3	WD	52.9	42.2	WD
Syn-40	3.1	3.0	2.4	79.7	80.5	81.1
Syn-45	1.8	WD	WD	14.0	WD	WD
Syn-48	0.9	0.8	0.9	6.9	6.2	6.3
Syn-53	3.0	2.3	2.6	9.5	8.2	8.2
Syn-54	0.4	0.5	0.6	7.9	5.2	4.1
Syn-57	2.1	2.1	2.3	1.3	0.1	0.0
Syn-60	1.3	1.0	0.9	5.7	4.5	3.5
Syn-61	1.4	1.3	1.3	26.8	24.0	25.9
Syn-62	1.6	1.4	1.3	4.7	4.9	5.2
Syn-65	0.4	0.5	0.6	5.6	6.0	5.9
Syn-67	0.9	0.9	1.0	10.9	10.6	16.9
Syn-68	1.8	1.7	1.8	10.6	16.9	10.9
Syn-70	2.4	2.5	2.1	2.1	2.5	1.3
Syn-79	ND	2.0	2.4	ND	32.2	32.6

Table 8

Subject no	CEA (week 0)	CEA (week 6)	CEA (week 12)	Ca19.9 (week 0)	Ca19.9 (week 6)	Ca19.9 (week 12)
Syn-22	1.4	WD	WD	0.3	WD	WD
Syn-24	2.5	3.3	3.0	2.5	2.7	3.4
Syn-28	0.9	0.5	0.8	12.5	11.5	22.2
Syn-31	0.4	0.7	0.7	14.1	19.9	27.9
Syn-32	0.5	0.6	0.6	3.9	3.6	3.0
Syn-35	1.1	1.3	1.3	4.5	5.9	3.9
Syn-41	1.9	1.6	1.7	41.0	36.0	38.7
Syn-42	3.8	4.7	4.2	0.0	0.0	0.0
Syn-46	0.6	0.6	0.8	22.7	20.5	20.8
Syn-49	0.7	0.9	0.9	0.0	0.0	0.0
Syn-50	2.0	1.8	1.9	15.8	15.8	14.9
Syn-55	5.5	5.0	5.5	12.7	12.2	15.4
Syn-56	2.3	1.7	2.1	3.9	5.1	4.3
Syn-59	1.7	1.6	1.5	2.6	1.5	1.7
Syn-63	0.9	1.1	1.1	34.0	31.8	35.6
Syn-64	0.7	0.8	0.8	1.1	1.7	1.7
Syn-66	0.9	0.9	1.0	3.5	2.5	2.1
Syn-69	0.8	0.8	0.9	0.8	0.6	0.9
Syn-70	0.9	0.8	0.8	2.2	1.9	2.1
Syn-72	3.2	3.6	4.2	2.4	1.8	4.1
Syn-74	2.5	2.2	1.7	13.6	12.7	10.8
Syn-80	1.9	1.9	1.9	0.0	0.0	0.0

Discussion

In the current study we evaluated the ability of a synbiotic combination of *Lactobacillus rhamnosus* GG, *Bifidobacterium lactis* Bb12 and Synergy[®]1 to modulate the proliferative activity of the colonic mucosa in polypectomised subjects and to influence the expression of tumour markers and tumour promoting activities of faecal water in polypectomised subjects and in cancer subjects who had previously undergone 'curative resection'.

Epidemiological and experimental studies suggest that a high proliferative activity or a deregulation of the normal pattern of proliferation along the crypt are risk factors for colon carcinogenesis. De Leon *et al.* (1988) reported that proliferation in the upper crypt was significantly increased in patients with polyps and colorectal cancer compared to healthy controls. Many studies have shown that dietary intervention can reduce colorectal mucosal proliferation (Caderni *et al.* 1988; Holt *et al.* 1988). In the present study proliferative activity in the colonic mucosa of polypectomised subjects was significantly decreased following a twelve-week intervention with the synbiotic. The distribution of proliferating cells along the colonic crypt however was not altered, and the reduction in proliferative activity was found to be most prominent in the lower compartment of the crypt.

These results correlate with previous work by our group which showed that consumption of this synbiotic by azoxymethane treated rats reduced the proliferative activity as well as the number of colonic tumours in these rats but did not influence the distribution of proliferating cells in the colon (Femia *et al.* 2002). These results suggest a protective effect of synbiotics in colon carcinogenesis. However, further

work is required to elucidate the mechanisms by which synbiotics mediate their protective effects.

Within the gastro-intestinal tract tight junctions (TJs) control paracellular transport between the luminal and the baso-lateral fluid compartments. Disruption of TJs leads to a 'leaky gut' with an increase in intestinal paracellular permeability, (Sugi *et al.* 2001). Soler *et al.* (1999) demonstrated the association between increased tight junction permeability and colonic tumours. Tight junction integrity is influenced by the components of the gut lumen, with which it is in intimate contact. Human faeces represents the end products of diet, digestive and excretory processes as well as the metabolic activities of the gut flora. Therefore investigation of faecal contents provides a non-invasive method of studying the stresses to which the colorectal mucosa is exposed.

The influence of faecal water on the integrity of the epithelial barrier function was assessed by transepithelial electrical resistance (TER) and paracellular transport of radiolabelled mannitol. There was no significant difference in the trans-epithelial resistance or mannitol flux in Caco-2 cells following incubation with FW derived from both the synbiotic and placebo groups of polyp and cancer subjects at the end of intervention compared to the beginning. Interestingly TER was always significantly higher in cells treated with FW derived from polyp subjects compared to cells treated with FW from cancer subjects although there was no detrimental effect of FW from cancer subjects on tight junction integrity, which was generally greater than in cells treated with tissue culture medium alone (100%). Therefore although FW derived from cancer subjects does not adversely affect tight junction

integrity it does not have the positive influence on integrity observed with FW derived from polyp subjects. It may be that FW from polyp subjects contains substances, which promote tight junction integrity and these substances are either absent, inactive or present at lower concentrations in FW of cancer subjects. Further work identifying the substances present in FW of healthy subjects and subjects with disease e.g. by mass spectrometry, and analysis of their effects, either individually or in combination, on tight junction integrity will help in designing strategies to alter the metabolic activity in the colon, to reduce the production of damaging substances, and increase the production of cancer protective substances.

In contrast to the TER results the paracellular transport of ^{14}C mannitol, which is another method to assess tight junction integrity was not significantly different in cells treated with FW derived from polyp subjects or cancer subjects. TER and mannitol flux are generally inversely related and this was not observed in this study. This may be explained by the fact that treatment with FW generally resulted in TER measurements of the same magnitude or greater than control (100%) indicating that tight junction integrity was maximal and therefore the paracellular transport of mannitol is unaltered in this situation.

The influence of FW on the invasion of HT115 cells across a matrigel barrier was not significantly different at the end of intervention compared to the beginning of intervention in any of the study groups. It is interesting however that over the 24 hour incubation period, 20-25% of HT115 cells treated with serum free growth medium (negative control) typically invade the matrigel coated inserts, but in this study the majority of FW samples reduced invasion below that of the negative

control (ranging from 0-19%). Exactly why this occurred is not known but other factors such as cell loss due to sample toxicity or the induction of expression of MMPs/TIMPs, COX-2, prostaglandin E₂ etc., by FW may play a role in modulating the invasive ability of these cells.

Glutathione-S-transferases (GSTs) are important detoxifying enzymes, which inactivate a number of genotoxic and carcinogenic compounds by catalysing their conjugation with glutathione. GST induction in normal tissue is considered protective against cancer. In this study GST activity was significantly increased in PBMCs from cancer and polyp subjects consuming synbiotic. Interestingly PBMCs derived from placebo polyp subjects also had significantly increased GST activity (per 10⁶ cells) at the end of the twelve-week period compared to the beginning of intervention. Szarka *et al.* (1995) reported that GST activity ranged from 0.0258-0.1387 nmoles/min per µg protein in healthy volunteers. In this study there was a large inter-individual variability in GST activity with values ranging from 0.009-0.390 nmoles/min per µg protein. Therefore, although there is an indication that synbiotic consumption induces GST activity, the large inter-individual variation in activity may prove difficult when employing GST activity as a biomarker in intervention studies. Large scale studies with a number of baseline sampling times would be of use in determining if GST activity is suitable as an intervention biomarker.

Studies have shown that GST activity of blood lymphocytes from high cancer risk individuals is significantly lower than that of blood lymphocytes from control individuals (Szarka *et al.* 1995). In this study we found that GST activity in PBMCs

from cancer subjects was not significantly different from that in PBMCs derived from polyp subjects. Although we consider that polyp subjects are at a lower cancer risk than cancer subjects and therefore would expect polyp subjects to have lower GST activity it would be interesting to compare GST levels in polyp, cancer and healthy subjects to determine if this parameter is useful as a biomarker of cancer risk.

Carcinoembryonic antigen (CEA) is a high molecular weight glycoprotein belonging to the immunoglobulin superfamily of molecules. It has been postulated to play a role in a number of biological processes including cell adhesion, immunity and apoptosis. After CEA, CA 19-9 is the most widely used tumour marker. It is the best available marker for pancreatic cancer but it is less sensitive than CEA for detecting colorectal carcinoma. Because of their widespread use as diagnostic tools for colon cancer we investigated if synbiotic intervention influenced the levels of CEA and CA19-9.

The levels of CEA and CA19-9 were within the normal range for the majority of subjects. In the synbiotic cancer group SYN-21 had an elevated CEA and equivocal CA19-9 level at T3, however this is to be expected as this subject was diagnosed with metastatic disease during the intervention. There were no significant effects of synbiotic intervention on the levels of CEA and CA19-9 in this study. In fact the individual with metastatic disease was identified by CEA and CA19-9 indicating that synbiotic intervention did not influence the usefulness of these markers as diagnostic tools.

In conclusion, twelve-week consumption of a synbiotic combination of *Lactobacillus rhamnosus* GG, *Bifidobacterium lactis* Bb12 and Synergy[®]1 significantly reduced the proliferative activity of the colorectal mucosa of polyp subjects, indicating a potential reduction in cancer risk. GST activity in PBMCs from synbiotic fed cancer subjects increased, indicating the induction of a putative cancer protective state due to synbiotic consumption. Synbiotic consumption did not influence the tumour promoting activities (tight junction integrity and invasive potential) of FW in either cancer or polyp subjects. However FW derived from polyp subjects significantly increased tight junction integrity compared to FW derived from cancer subjects indicating that the composition and or activity of FW from polyp subjects is significantly different from that of cancer subjects. CEA and CA19-9 were unaltered in this study showing that synbiotic intervention does not adversely affect these commonly used diagnostic tools.

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Chapter 5

The genotoxic potential of faecal water and *in vivo* genotoxic damage in cancer and polyp subjects: can synbiotics modulate genotoxic damage?

Abstract

Increased genotoxic damage is associated with increased cancer risk. Animal and human studies have shown that pre-, pro- and synbiotics can protect against genotoxic damage *in vivo* and reduce the genotoxic potential of faecal water (FW). Studies have also shown that dietary intervention (e.g. vegetables) can modulate the level of DNA damage in peripheral blood mononuclear cells (PBMCs). In azoxymethane treated rats synbiotic consumption reduced the genotoxic potential of FW with a concomitant reduction in the number of colonic tumours. The aim of this study was to determine if synbiotic consumption could influence the level of *in vivo* DNA damage (colonic mucosa and PBMCs) and the DNA damaging potential of FW. We also investigated if the level of genotoxic damage was different in cancer and polyp subjects. We found that synbiotic consumption protects against genotoxic damage in the colon and modulates the genotoxic potential of FW in a cancer protective and health promoting manner. There was no difference in the genotoxic potential of FW of cancer and polyp subjects but the levels of DNA damage in the mucosa and in PBMCs of these two groups were different. We also found that gender and smoking status play a role in susceptibility to genotoxic damage.

Introduction

The human colon is continually exposed to a complex mixture of compounds, which are either of direct dietary origin or the result of digestive, microbial and excretory processes. Cells of the colonic mucosa are in intimate contact with the contents of the gut lumen, which contains compounds that may be either damaging to or protective of the mucosa. Damage to cells of the mucosa leads to increased proliferative activity to replace damaged cells and therefore increased risk of tumour development. It is therefore useful to develop methods to analyse the mutagenic burden to which the colonic mucosa is exposed, identify components of the luminal contents, which may protect the mucosa and employ this information to develop strategies to reduce the mutagenic burden in the colon.

Traditionally the Ames test, where mutagenicity is determined based on the ability of the test substance to induce mutations in *Salmonella typhimurium*, was used to investigate the mutagenic potential of compounds associated with cancer (Bruce *et al.* 1977). However, the ability of this test to identify human carcinogens is limited as the mutagenic potential of substances is evaluated using prokaryotic cells. In recent years, the use of methods, which measure genotoxic damage in eukaryotic cells, has proven to be more promising as an indicator of cancer risk.

Genotoxic agents cause cellular DNA damage but are not cytotoxic to the cell. One of the most commonly used methods to assess genotoxic damage is the microgelelectrophoresis assay or comet assay, which was developed by Ostling & Johanson (1984), and modified by Singh *et al.* (1988) as the alkaline comet assay.

This method has been used to study the genotoxic potential of many substances by determining the extent of DNA damage in cell lines following *in vitro* incubation with the substance of interest (Pool-Zobel & Leucht 1997; Pool-Zobel *et al.* 2000). *In vivo* genotoxic damage can be determined in cells derived from tissues of interest. Pool-Zobel *et al.* (1999) demonstrated that the comet assay is sufficiently sensitive to detect genotoxic damage in small numbers of cells derived from human biopsy material.

The level of genotoxic damage is associated with the risk of colon cancer. Pool-Zobel & Leucht (1997) reported that substances associated with cancer risk such as N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and dinitrosocaffeidine (DNC) induced genotoxic damage in freshly derived colon cells from healthy subjects. Hambly *et al.* (1997) fed DMH treated, human flora-associated (HFA) rats, diets containing high and low risk factors for colon cancer. DNA damage was significantly higher in colon cells from animals on the high-risk diet.

Pre-, pro and synbiotic consumption has been associated with reduced genotoxicity. Studies have shown that some lactic acid bacteria inhibit MNNG and DMH induced genotoxicity in colon cells isolated from rats (Pool-Zobel *et al.* 1996; Wollowski *et al.* 1999). Zsivkovits *et al.* (2003) reported that bacterial strains commonly used in yoghurt production protect against hetero-cyclic amine induced DNA damage in colon and liver cells of rats.

Faecal water (FW), which is the aqueous phase of faeces, contains most of the free reactive and soluble factors, which likely interact with the mucosa *in vivo* (Rafter *et al.* 1987). The genotoxic potential of FW is considered a measure of the potential of

the dissolved factors to induce damage in the colonic mucosa. Many colonic tumours arise from damage to the colonic mucosa, which is a result of the interactions with gut luminal contents. Studies have shown that there is an inter- and intra-individual variation of faecal water genotoxicity even in subjects consuming the same diet (Oßwald *et al.* 2000; Woods *et al.* 2002). However, Oßwald *et al.* (2000) conclude that despite this variation FW genotoxicity is suitable as a biomarker for application in intervention studies when each subject is analysed as his or her own control.

Diets considered high risk for colon cancer have been reported to increase the genotoxic potential of FW in healthy volunteers (Rieger *et al.* 1999). Lactic acid bacteria have been shown to modulate the genotoxic potential of FW. The genotoxic potential of FW from rats treated with AOM was significantly reduced in animals fed a synbiotic (Synergy[®]1, Bb12 and LGG) compared to controls and this correlated with a reduction in tumour incidence in the synbiotic fed group (Klinder *et al.* 2004). The genotoxic potential of FW derived from tumour bearing rats was significantly higher than the genotoxic potential of FW derived from non tumour bearing rats. Burns & Rowland (2004) investigated the effect of incubating a genotoxic FW sample from a human subject with six different strains of lactic acid bacteria on the DNA damaging potential of the FW. They found that DNA damage induced by FW in HT-29 cells was significantly reduced by all strains except *Strep. thermophilus* and that *Lactobacillus plantarum* and Bb12 showed the greatest protective effect.

Another putative non-invasive biomarker of cancer risk is genotoxic damage in lymphocytes. Duthie *et al.* (1996) reported that vitamin supplementation reduced DNA damage in human lymphocytes. The first demonstration that consumption of

whole foods (in this case vegetable juices) influenced lymphocyte DNA damage was shown by Pool-Zobel *et al.* (1997) who reported that consumption of carotenoid-containing vegetable juices which are considered cancer protective by 23 healthy male subjects reduced genotoxic damage in lymphocytes. Other studies have confirmed that dietary intervention can modulate genotoxic damage in lymphocytes. Reduced DNA damage was reported after intervention with tomato puree (Riso *et al.* 1999), soy milk (Mitchell & Collins 1999) and with fruit juices (Bub *et al.* 2003). However other investigators using bread (Pool-Zobel *et al.* 2000) or vegetables and fruits (Moller *et al.* 2003) have not observed the same protective effect.

This could indicate that differences in the bioavailability of the active ingredients may limit the sensitivity of the comet assay biomarker when using only lymphocytes as the surrogate tissue. Further studies comparing DNA damage in both lymphocytes and colon cells will determine the usefulness of lymphocytes as surrogate tissues.

The aims of this study were:

- 1) To investigate the influence of twelve-week consumption of a synbiotic combination of *Lactobacillus rhamnosus* GG, *Bifidobacterium lactis* Bb12 and Synergy[®]1 on *in vivo* genotoxic damage by measuring DNA damage in biopsies and lymphocytes.
- 2) To investigate the influence of synbiotic intervention on the genotoxic potential of faecal water and to assess if the genotoxic potential of FW is associated with *in vivo* DNA damage as measured in biopsies and PBMCs.

- 3) To assess if exposure to genotoxins in cancer subjects who had undergone previous curative resection is significantly different from that of polypectomised subjects.

Methods

Work described in this chapter was conducted in collaboration with Dr Annett Klinder, Dr Michaela May and Prof. Beatrice L. Pool-Zobel of the Department of Nutritional Toxicology, Friedrich-Schiller University of Jena, Germany.

DNA Damage in Biopsies

2 rectal biopsies from each subject were obtained by sigmoidoscopy for each of two time points one at the beginning of intervention (week 0) and the other at the cessation of feeding (week 12). Biopsies were immediately placed in a collection tube containing Hanks Balanced Salt Solution, (HBSS; 8.0 g/l NaCl; 0.4 g/l KCl; 0.06 g/l Na₂HPO₄·2H₂O; 0.06 g/l K₂HPO₄; 1 g/l glucose; 0.35 g/l; NaHCO₃; 4.8 g/l HEPES; pH 7.4) and kept on ice until further processing.

Biopsies were washed several times in HBSS, minced using a fine edge scissors and incubated in 3mls HBSS supplemented with 2 mg/ml proteinase K (Sigma, Steinheim, Germany) and 1 mg/ml collagenase P (Boehringer, Mannheim, Germany) for 30 mins in a water-bath at 37°C with agitation. Following incubation the suspension was supplemented with 12ml fresh HBSS, centrifuged at 400 x g for 5mins, and the pellet resuspended in 200µl of HBSS. Viability and yield of cells were determined by trypan blue exclusion.

Cell concentration was adjusted to 0.2x10⁶ cells/ml and 50µl of cells were mixed with 350µl of 0.7% low melting point agarose. 50µl of this agarose/cell suspension was then pipetted to each well of a trevigen slide (Trevigen, U.S.) and spread with

Figure 1(a)

Migration of damaged DNA in an electric field gives rise to a 'comet'. The head contains intact DNA, and the tail contains damaged DNA.

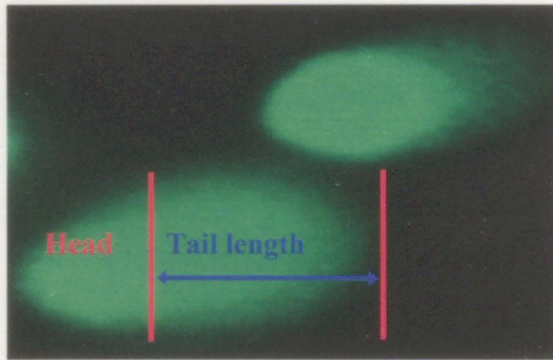


Figure 1(b)

A cell with moderately damaged DNA gives rise to a comet with most DNA in the head and a small amount of DNA in the tail.

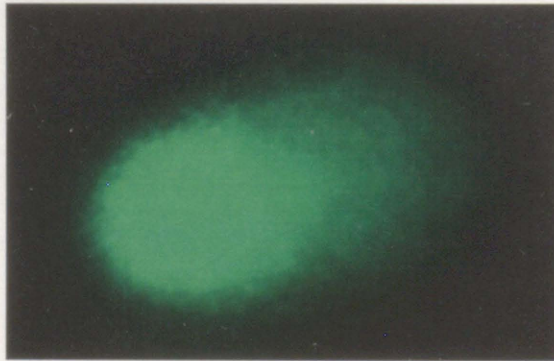
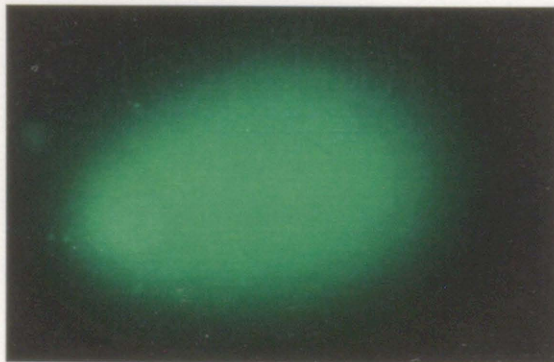


Figure 1(c)

A cell with severely damaged DNA gives rise to a comet with the majority of DNA in the tail and a small amount of DNA in the head.



the pipette tip. Slides were allowed to dry for 10 mins on a cool box (5°C – 10°C) and treated with lysis solution (100 mM Na₂EDTA, 1 % Triton X- 100, 2.5 M NaCl; 1 % N-lauroyl sarcosin sodium salt, 10 % DMSO, 10 mM Tris, pH 10) for 1hr at 4°C in the dark.

Slides were then placed in an electrophoresis chamber and soaked in electrophoresis buffer (1mM Na₂EDTA, 300mM NaOH pH13) for 20 mins at 4°C to allow alkaline unwinding. Electrophoresis was performed for 20 mins at 300mA, 25V at 4°C. Slides were then fixed in absolute ethanol for 5 mins and allowed to dry for 10 mins at room temperature. Slides were stored at 4°C in the dark until analysed.

Slides were stained with SYBR Green 1 (Sigma) and microscopical analysis revealed images of more or less damaged DNA ("comets") (*Figures 1(a-c)*). The comets are a consequence of DNA migration within the electrical field (Singh *et al.* 1988). DNA damage was quantified by measuring the proportion and extent of DNA migration using the image analysis system of Perceptive Instruments (Halstead; UK). 50 DNA spots (individual biopsy cells) were evaluated per slide and six slides were analysed for each sample. DNA damage was expressed as tail intensity (% DNA in the tail) and tail moment (% DNA in the tail x tail length). The percentage of undamaged DNA (intact cells) on each slide was also measured.

DNA Damaging Potential of Faecal Water

Microscope slides were coated with 50µl of 0.7% low melting point agarose (LMP) followed by 85µl of 0.5% normal melting point (NMP) agarose and kept overnight at 4°C. The following day HT29clone19a cells were harvested and adjusted to a

concentration of 2×10^6 cells/ml. The cell line HT29clone19a is a subclone of the HT29 human colon cancer cell line and was produced by permanent differentiation with 5mM sodium butyrate (Augeron & Labois 1984). Cells were incubated with either 50% FW or with DMEM as control at 37°C for 30 minutes with agitation.

Following FW incubation cell numbers and viabilities were determined with trypan blue in 20µl aliquots of the suspension. The remaining cells were pelleted by centrifuging for 5 mins at 400 x g. The supernatant was discarded and the cell pellet was mixed with 75µl of LMP agarose and distributed onto the pre-coated microscope slides. Further steps were performed as originally described by Singh *et al.* (1988) and modified by Pool-Zobel & Leucht (1997). Briefly slides were placed in lysis solution (100 mM Na₂EDTA, 1 % Triton X 100, 2.5 M NaCl; 1 % N-lauroyl sarcosine sodium salt, 10 % DMSO, 10 mM Tris, pH 10) for at least 60 minutes at 4°C. Following this slides were placed in the microgel electrophoresis chamber containing alkaline solution (1 mM Na₂EDTA, 300 mM NaOH, pH 13) to allow alkaline unwinding. After 20 minutes of DNA unwinding, electrophoresis was performed at 25 V, 300 mA for 20 minutes. Afterwards slides were washed with neutralisation buffer (0.4M Tris, pH 7.5) and stained with ethidium bromide (Sigma).

DNA damage was quantified using the same method as used to assess DNA damage in biopsies. 50 DNA spots were evaluated per slide and three slides were analysed for each sample.

DNA Damage in PBMCs

PBMCs were isolated by density gradient centrifugation and cell number was adjusted to 1×10^6 cells/ml in cryoprotective medium (90% FBS, 10% DMSO), and stored under liquid nitrogen until analysed.

Microscope slides were pre-coated with agarose as described for faecal water samples. Cryopreserved PBMCs were thawed quickly at 37°C , centrifuged at $400 \times g$ for 8 mins to remove cryoprotective medium and resuspended in 1ml PBS. Cell numbers and viabilities were determined with trypan blue exclusion. The remaining cells were pelleted by centrifuging at $400 \times g$ for 8 mins, the supernatant discarded and the PBMCs were mixed with (0.5%) LMP agarose. The volume of LMP agarose used was dependant on the number of viable cells recovered from the cryoprotective medium. If there were less than 3×10^5 cells the pellet was resuspended in $150\mu\text{l}$ agarose. At higher cell numbers cell concentration was adjusted to 2×10^5 cells/ml and then resuspended in $150\mu\text{l}$. $50\mu\text{l}$ of agarose/cell suspension was distributed in triplicate onto pre-coated microscopes slides. Lysis, electrophoresis, neutralisation and analysis steps were performed as described for faecal water samples.

Strategy for Analysis of Genotoxic Data Taking Account of Gender and Whether or not Subjects Smoked

Initial data analysis included all subjects and there were no exclusions based on smoking status or gender. However increased DNA damage in subjects who smoke compared to non-smokers has consistently been reported. Piperakis *et al.* (1998) reported that smoking was associated with a higher level of DNA damage in lymphocytes in Greek males. A Chinese study found that both smokers and

employees in a cigarette factory had increased DNA damage in whole blood compared to control subjects who were non-smokers and not occupationally exposed to tobacco dust (Zhu *et al.* 1999). Oßwald *et al.* (2003) reported that buccal leucocytes isolated from smokers had more DNA damage than the same cells isolated from non-smokers. Based on these reports we excluded smokers from our analysis.

Data from studies investigating basal DNA damage in men compared to women has yielded conflicting results. Some studies have shown no effect of gender on DNA damage while others have reported that DNA damage is higher in men than in women (Bajpayee *et al.* 2002). It is probable that hormonal influences or sex-related differences in metabolic activation or inactivation may be contributing factors which may affect endogenous genotoxin production and thus mask effects of simultaneous intervention. Therefore we further analysed our data excluding smokers and females.

Results

DNA Damage in Biopsies

Colorectal biopsies were obtained from cancer and polyp subjects at the beginning (week 0) and the end of intervention (week 12). The level of DNA damage in biopsies was assessed in single cells derived from colorectal biopsies using the comet assay.

Following enzymatic digestion the yield and viability of cells derived from colorectal biopsies was determined using trypan blue exclusion. The yield (mean $\pm$ SD) was 10.38 ± 15.14 million cells per 2 biopsies and viability (mean $\pm$ SD) was $93.42\% \pm 3.591$.

Cancer subjects

Synbiotic consumption influenced genotoxic damage in biopsies from cancer subjects (including smokers). Tail intensity (TI) (*Figure 2(b)*) and tail moment (TM) (*Figure 2(d)*) in cancer subjects consuming placebo (n=12) were significantly increased at week 12 compared to week 0 (TI $p=0.0488$, TM $p=0.0396$). This increase in DNA damage was not observed in cancer subjects consuming the synbiotic product (n=17) suggesting a protective effect of synbiotic consumption (*Figures 2(a) & 2(c)*). The percentage of intact cells in both groups was unaltered (*Figures 2(e) & 2(f)*).

Exclusion of smokers from analysis yielded the same results as when smokers were included. Tail intensity (TI) (*Figure 3(b)*) and tail moment (TM) (*Figure 3(d)*) were significantly increased at week 12 compared to week 0 (TI $p=0.0488$, TM $p=0.0396$)

in the placebo group of non-smoking cancer subjects (n=12) and this increase was not observed in non-smoking cancer subjects consuming the synbiotic product (n=15) (*Figures 3(a) & 3(c)*). The percentage of intact cells in both groups was unaltered (*Figures 3(e) & 3(f)*).

In contrast when smokers and females were excluded from analysis there was a trend towards increase in DNA damage in biopsies from male non-smoking cancer subjects consuming placebo (n=10) however this increase did not reach significance (TI, $p=0.772$ TM, $p=0.0716$) (*Figures 4(b) & 4(d)*). The percentage of intact cells was unaltered (*Figures 4(f)*). In biopsies from the synbiotic group of male non-smoking cancer subjects (n=7) tail intensity, tail moment and the percentage of intact cells was unaltered during the twelve-week intervention period (*Figures 4(a), 4(c) & 4(e)*).

Polyp subjects

There was a significant reduction in tail intensity ($p=0.0476$) (*Figure 5(a)*) and tail moment ($p=0.0135$) (*Figure 5(c)*) in biopsies from polyp subjects (including smokers) consuming synbiotics (n=17) at week 12 compared to week 0. In polyp subjects consuming placebo (n=15) tail moment ($p=0.0388$) but not tail intensity was significantly reduced at week 12 compared to week 0 (*Figure 5(b) & 5(d)*). The reduction was not as pronounced as in the synbiotic consuming subjects (*Figures 5(a) & 5(c)*). The percentage of intact cells in both groups was unaltered (*Figures 5(e) & 5(f)*).

Similar results were observed when smokers were excluded from analysis. Tail intensity ($p=0.0496$) and tail moment ($p=0.0164$) were significantly reduced in biopsies from non-smoking subjects consuming synbiotic ($n=15$) at week 12 compared to week 0 (*Figures 6(a) & 6(c)*). In placebo non-smoking polyp subjects ($n=14$) tail moment ($p=0.0312$) but not tail intensity was significantly reduced at week 12 compared to week 0 (*Figures 6(b) & 6(d)*). The reduction was not as pronounced as in the synbiotic consuming subjects (*Figures 6(a) & 6(c)*). The percentage of intact cells in both groups was unaltered (*Figures 6(e) & 6(f)*).

Male non-smoking polyp subjects

In biopsies from male non-smoking polyp subjects consuming placebo ($n=12$) tail intensity (*Figure 7(b)*) tail moment (*Figure 7(d)*) and the percentage of intact cells (*Figure 7(f)*) were not altered during the twelve-week intervention period. In biopsies from synbiotic non-smoking polyp subjects ($n=6$) tail intensity (*Figure 7(a)*) and the percentage of intact cells (*Figure 7(e)*) were not significantly different at week 12 compared to week 0. Tail moment however was reduced ($p=0.0803$) at week 12 compared to week 0 (*Figure 7(c)*).

The initial level of genotoxic damage in biopsies from cancer and polyp subjects was analysed. Tail intensity, tail moment and the percentage of intact cells was not significantly different in biopsies from cancer subjects compared to polyp subjects at week 0 (*Table 1*). Similar results were observed in non-smokers (male and female) (*Table 2*) and in non-smoking males (*Table 3*). Interestingly DNA damage in biopsies from cancer subjects at all time points (weeks 0 and 12) was significantly

higher than in polyp subjects at all times (*Table 1*). The same result was observed when smokers were excluded from the analysis (*Table 2*). However when smoking subjects and females were excluded there was no difference in DNA damage in biopsies in male non-smoking cancer and polyp subjects (*Table 3*).

Figure 2(a)

Tail intensity in biopsies from cancer subjects consuming synbiotic (n=17) at the beginning (week 0) and the end of intervention (week 12). Tail intensity was unchanged at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 2(b)

Tail intensity in biopsies from cancer subjects consuming placebo (n=12) at the beginning (week 0) and the end of intervention (week 12). Tail intensity was significantly increased at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 2(c)

Tail moment in biopsies from cancer subjects consuming synbiotic (n=17) at the beginning (week 0) and the end of intervention (week 12). Tail moment was unchanged at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 2(d)

Tail moment in biopsies from cancer subjects consuming placebo (n=12) at the beginning (week 0) and the end of intervention (week 12). Tail moment was significantly increased at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 2(e)

The percentage of intact cells (non comet images) in biopsies from cancer subjects consuming synbiotic (n=17) at the beginning (week 0) and the end of intervention (week 12). There was no change at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 2(f)

The percentage of intact cells (non comet images) in biopsies from cancer subjects consuming placebo (n=12) at the beginning (week 0) and the end of intervention (week 12). There was no change at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 2 (a)

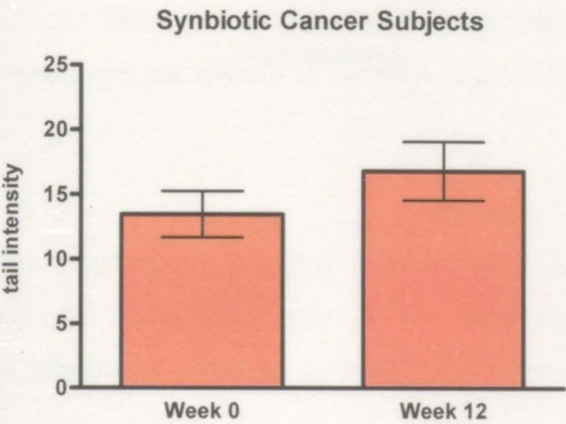


Figure 2 (b)

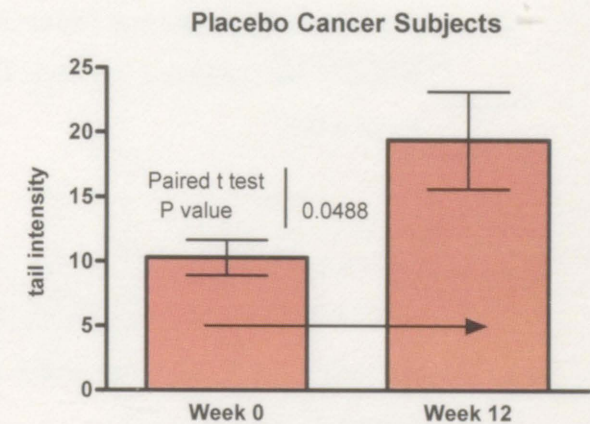


Figure 2 (c)

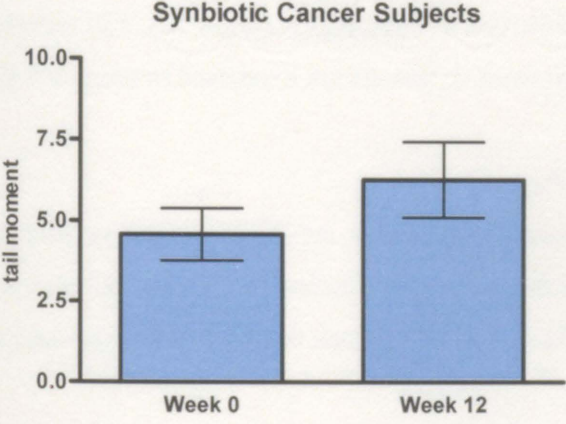


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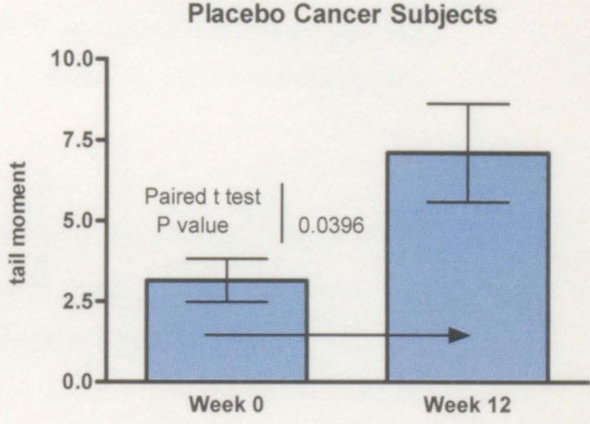


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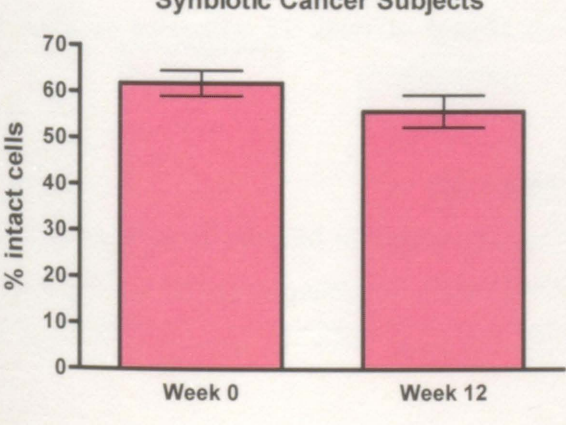


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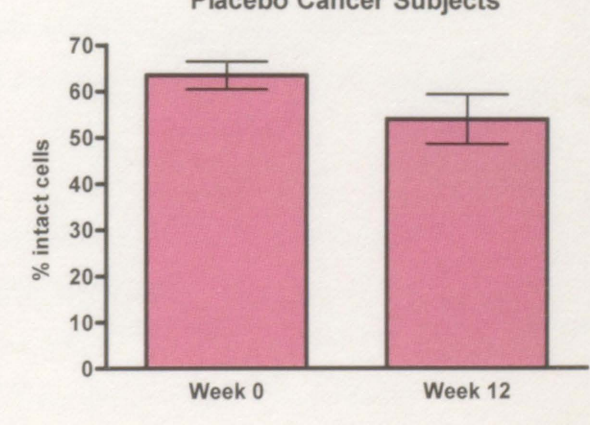


Figure 3(a)

Tail intensity in biopsies from non-smoking cancer subjects consuming synbiotic (n=15) at the beginning (week 0) and the end of intervention (week 12). Tail intensity was unaltered at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 3(b)

Tail intensity in biopsies from non-smoking cancer subjects consuming placebo (n=12) at the beginning (week 0) and the end of intervention (week 12). Tail intensity significantly increased at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 3(c)

Tail moment in biopsies from non-smoking cancer subjects consuming synbiotic (n=15) at the beginning (week 0) and the end of intervention (week 12). Tail moment was unaltered at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 3(d)

Tail moment in biopsies from non-smoking cancer subjects consuming placebo (n=12) at the beginning (week 0) and the end of intervention (week 12). Tail moment was significantly increased at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 3(e)

The percentage of intact cells (non comet images) in biopsies from non-smoking cancer subjects consuming synbiotic (n=15) at the beginning (week 0) and the end of intervention (week 12). There was no change at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 3(f)

The percentage of intact cells (non comet images) in biopsies from non-smoking cancer subjects consuming placebo (n=12) at the beginning (week 0) and the end of intervention (week 12). There was no change at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 3 (a)

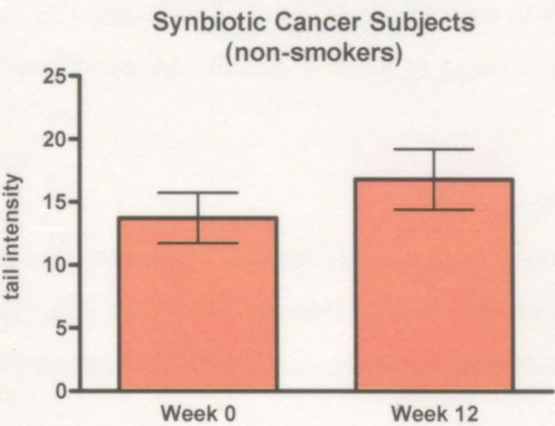


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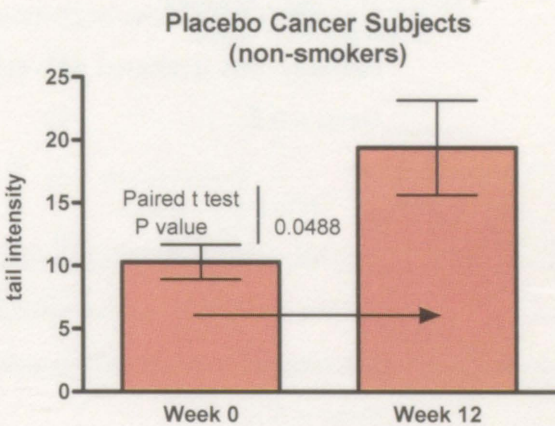


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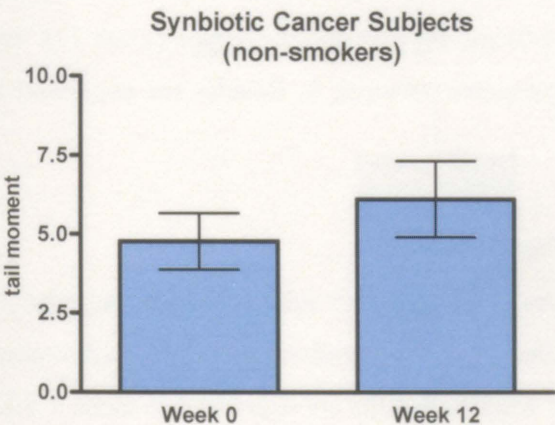


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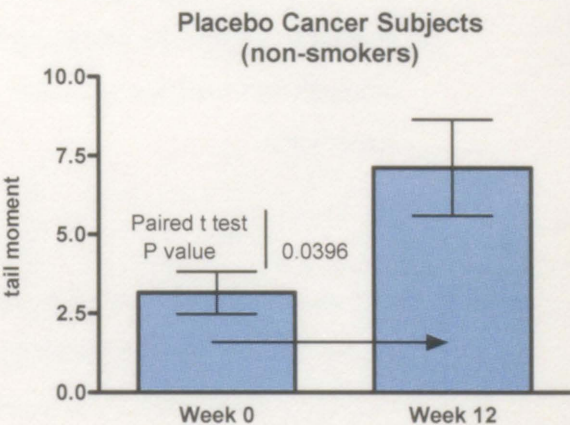


Figure 3 (e)

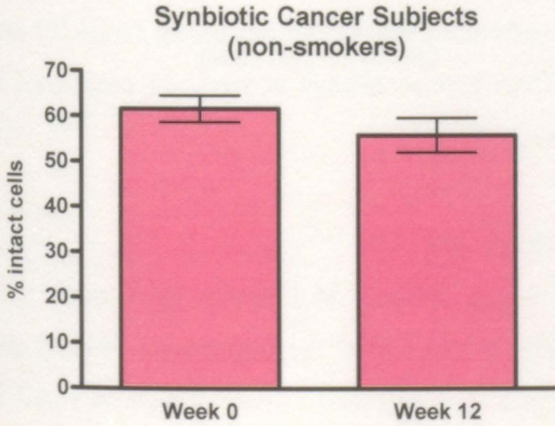


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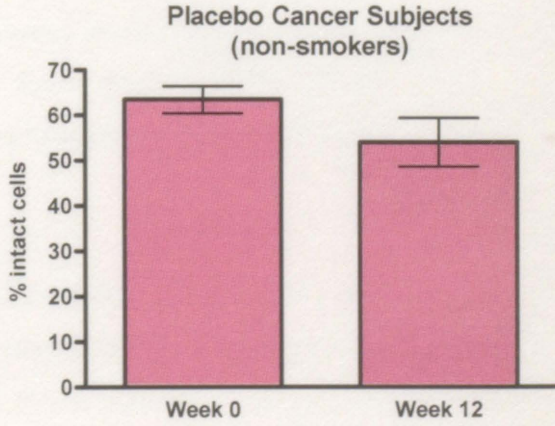


Figure 4(a)

Tail intensity in biopsies from male non-smoking cancer subjects consuming synbiotic (n=7) at the beginning (week 0) and the end of intervention (week 12). Tail intensity was unaltered at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 4(b)

Tail intensity in biopsies from male non-smoking cancer subjects consuming placebo (n=10) at the beginning (week 0) and the end of intervention (week 12). Tail intensity was increased at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 4(c)

Tail moment in biopsies from male non-smoking cancer subjects consuming synbiotic (n=7) at the beginning (week 0) and the end of intervention (week 12). Tail moment was unaltered at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 4(d)

Tail moment in biopsies from male non-smoking cancer subjects consuming placebo (n=10) at the beginning (week 0) and the end of intervention (week 12). Tail moment was increased at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 4(e)

The percentage of intact cells (non comet images) in biopsies from male non-smoking cancer subjects consuming synbiotic (n=7) at the beginning (week 0) and the end of intervention (week 12). There was no change at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 4(f)

The percentage of intact cells (non comet images) in biopsies from male non-smoking cancer subjects consuming placebo (n=10) at the beginning (week 0) and the end of intervention (week 12). There was no change at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 4 (a)

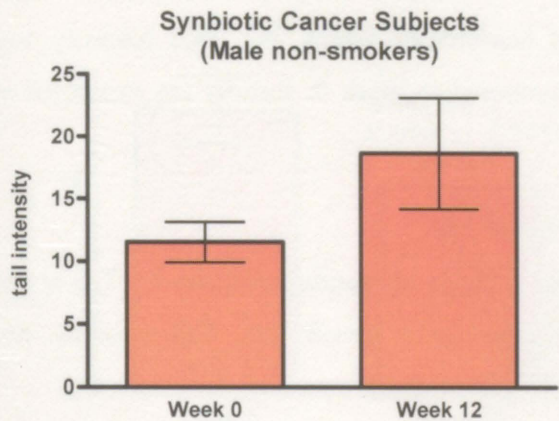


Figure 4(b)

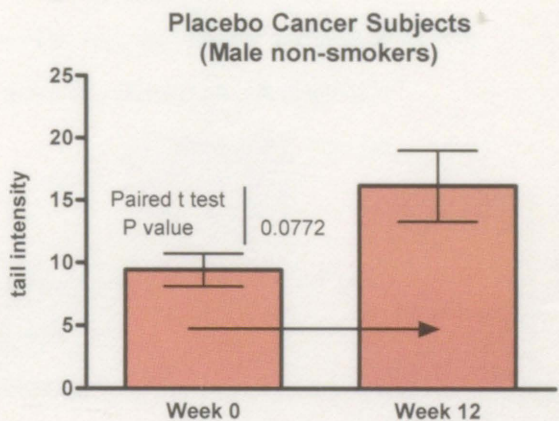


Figure 4 (c)

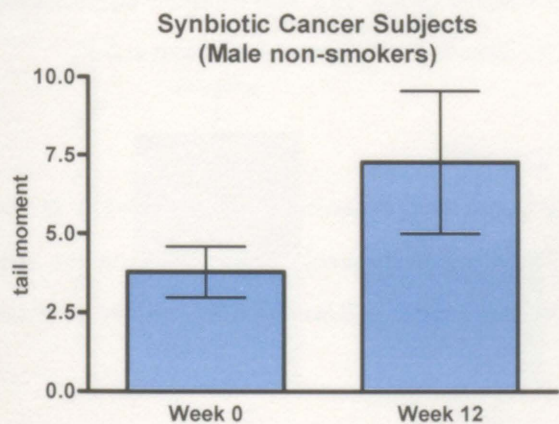


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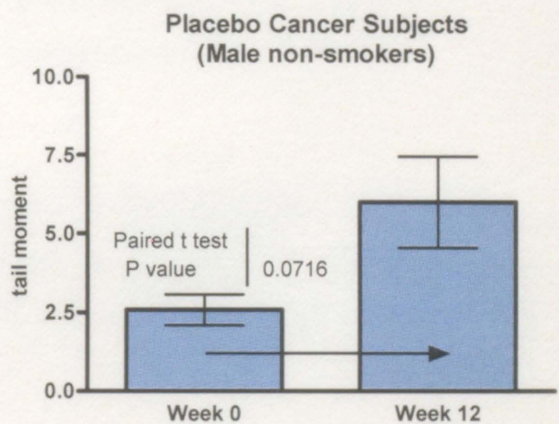


Figure 4 (e)

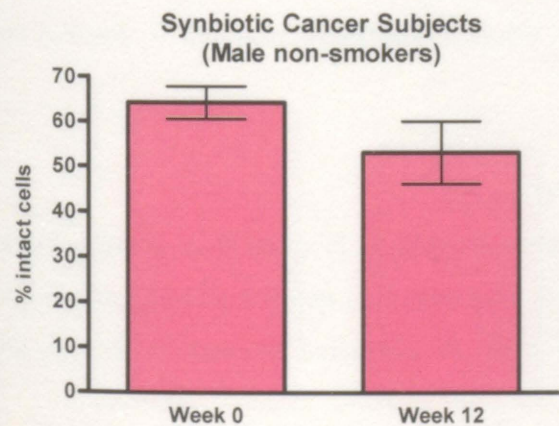


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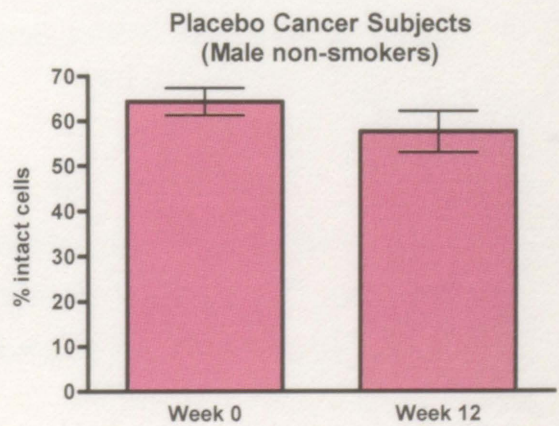


Figure 5(a)

Tail intensity in biopsies from polyp subjects consuming synbiotic (n=17) at the beginning (week 0) and the end of intervention (week 12). Tail intensity was significantly decreased at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 5(b)

Tail intensity in biopsies from polyp subjects consuming placebo (n=15) at the beginning (week 0) and the end of intervention (week 12). Tail intensity was unaltered at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 5(c)

Tail moment in biopsies from polyp subjects consuming synbiotic (n=17) at the beginning (week 0) and the end of intervention (week 12). Tail moment significantly reduced at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 5(d)

Tail moment in biopsies from polyp subjects consuming placebo (n=15) at the beginning (week 0) and the end of intervention (week 12). Tail moment was significantly reduced at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 5(e)

The percentage of intact cells (non comet images) in biopsies from polyp subjects consuming synbiotic (n=17) at the beginning (week 0) and the end of intervention (week 12). There was no change at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 5(f)

The percentage of intact cells (non comet images) in biopsies from polyp subjects consuming placebo (n=15) at the beginning (week 0) and the end of intervention (week 12). There was no change at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 5 (a)

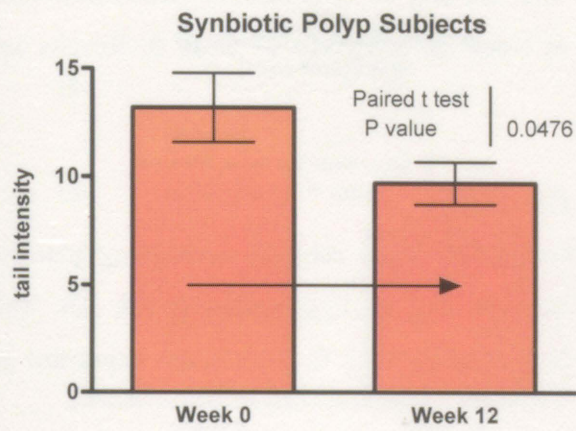


Figure 5 (b)

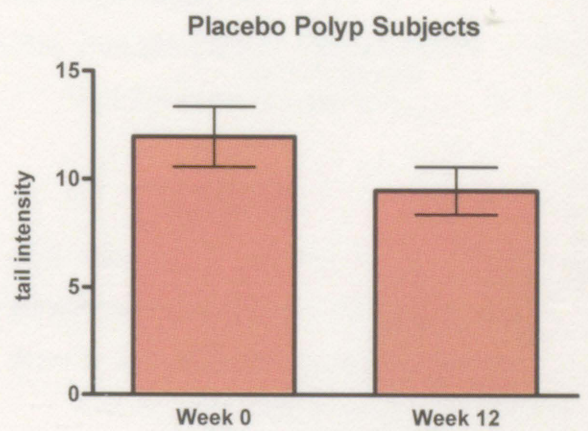


Figure 5 (c)

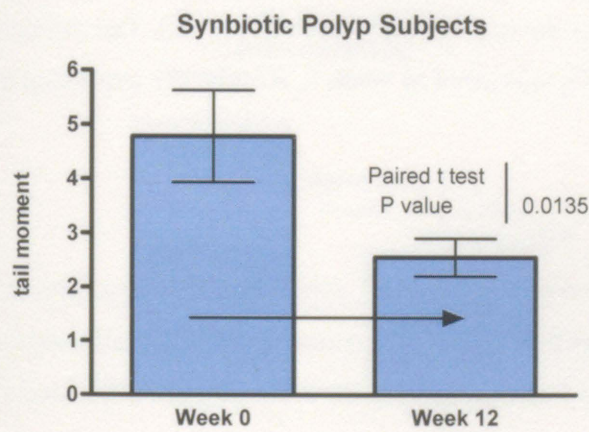


Figure 5 (d)

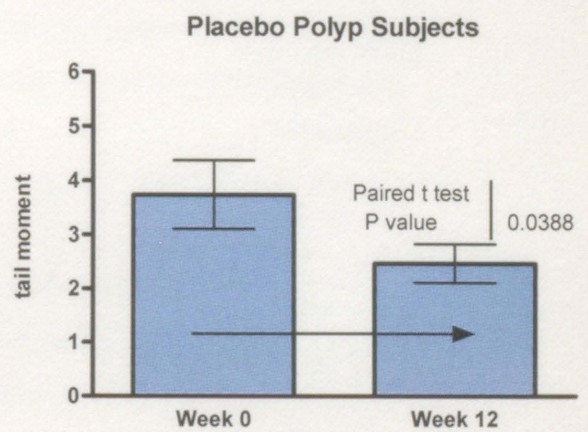


Figure 5 (e)

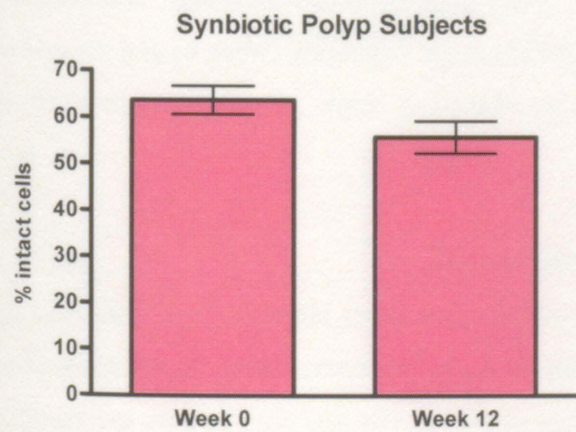


Figure 5 (f)

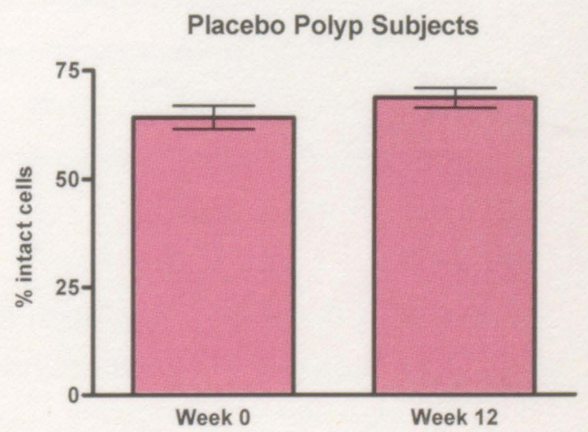


Figure 6(a)

Tail intensity in biopsies from non-smoking polyp subjects consuming synbiotic (n=15) at the beginning (week 0) and the end of intervention (week 12). Tail intensity was significantly reduced at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 6(b)

Tail intensity in biopsies from non-smoking polyp subjects consuming placebo (n=14) at the beginning (week 0) and the end of intervention (week 12). Tail intensity was unaltered at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 6(c)

Tail moment in biopsies from non-smoking polyp subjects consuming synbiotic (n=15) at the beginning (week 0) and the end of intervention (week 12). Tail moment was significantly reduced at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 6(d)

Tail moment in biopsies from non-smoking polyp subjects consuming placebo (n=14) at the beginning (week 0) and the end of intervention (week 12). Tail moment was significantly reduced at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 6(e)

The percentage of intact cells (non comet images) in biopsies from non-smoking polyp subjects consuming synbiotic (n=15) at the beginning (week 0) and the end of intervention (week 12). There was no change at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 6(f)

The percentage of intact cells (non comet images) in biopsies from non-smoking polyp subjects consuming placebo (n=14) at the beginning (week 0) and the end of intervention (week 12). The percentage of intact cells was significantly increased at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 6 (a)

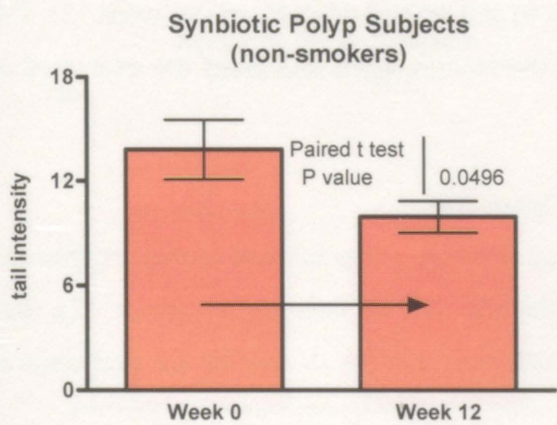


Figure 6 (b)

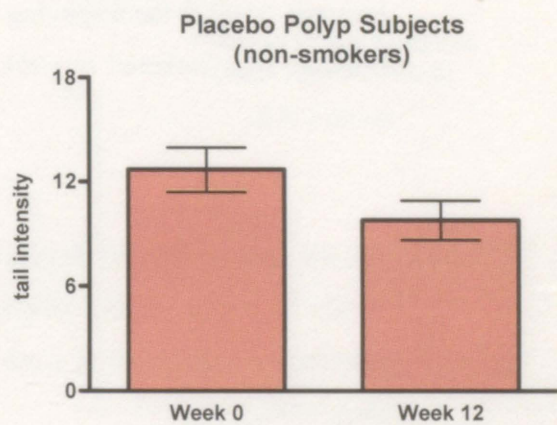


Figure 6 (c)

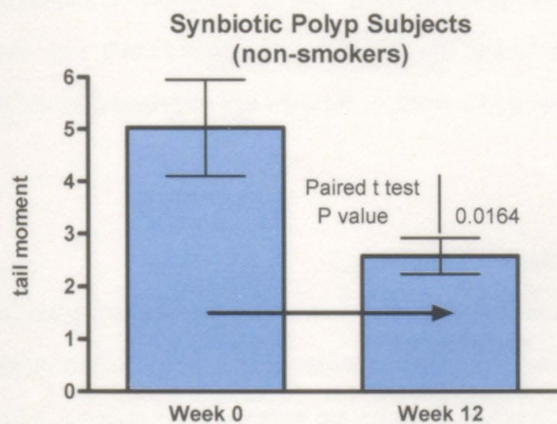


Figure 6 (d)

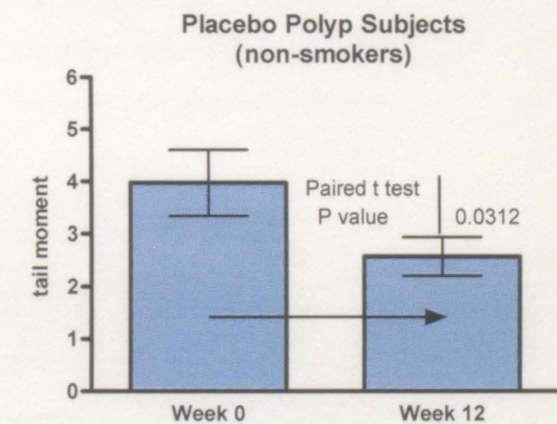


Figure 6 (e)

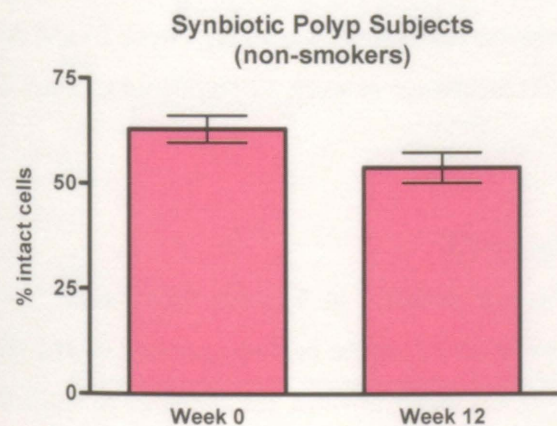


Figure 6 (f)

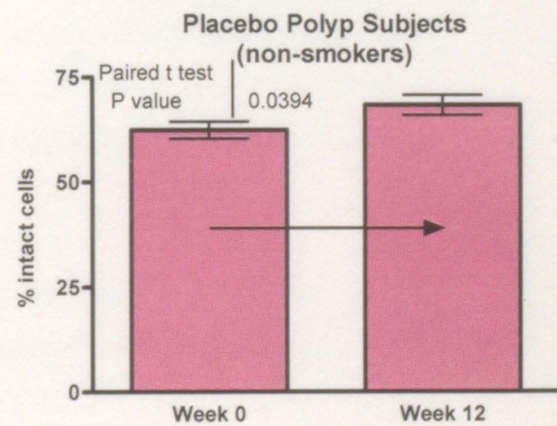


Figure 7(a)

Tail intensity in biopsies from male non-smoking polyp subjects consuming synbiotic (n=6) at the beginning (week 0) and the end of intervention (week 12). Tail intensity was unaltered at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 7(b)

Tail intensity in biopsies from male non-smoking polyp subjects consuming placebo (n=12) at the beginning (week 0) and the end of intervention (week 12). Tail intensity was unaltered at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 7(c)

Tail moment in biopsies from male non-smoking polyp subjects consuming synbiotic (n=6) at the beginning (week 0) and the end of intervention (week 12). Tail moment was reduced at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 7(d)

Tail moment in biopsies from male non-smoking polyp subjects consuming placebo (n=12) at the beginning (week 0) and the end of intervention (week 12). Tail moment was unaltered at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 7(e)

The percentage of intact cells (non comet images) in biopsies from male non-smoking polyp subjects consuming synbiotic (n=6) at the beginning (week 0) and the end of intervention (week 12). There was no change at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 7(f)

The percentage of intact cells (non comet images) in biopsies from male non-smoking polyp subjects consuming placebo (n=12) at the beginning (week 0) and the end of intervention (week 12). There was no change at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 7 (a)

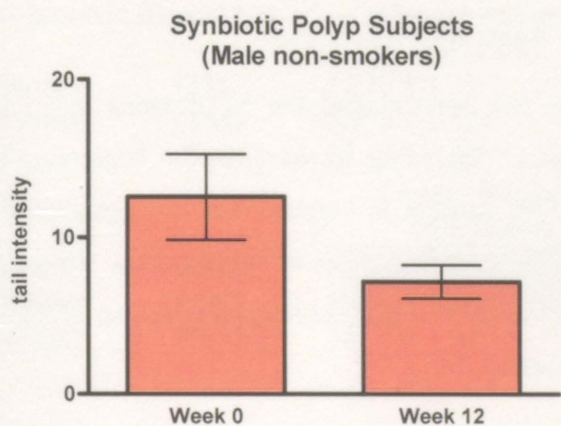


Figure 7 (b)

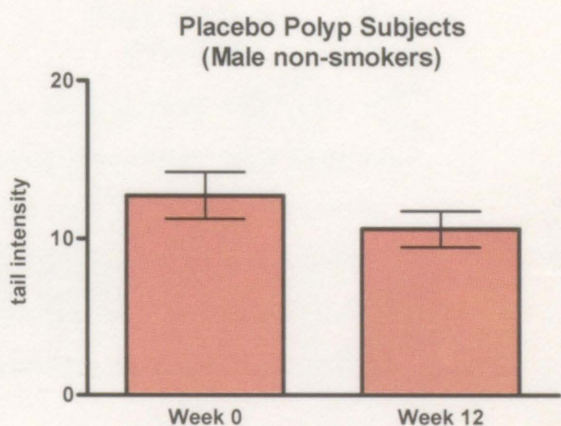


Figure 7 (c)

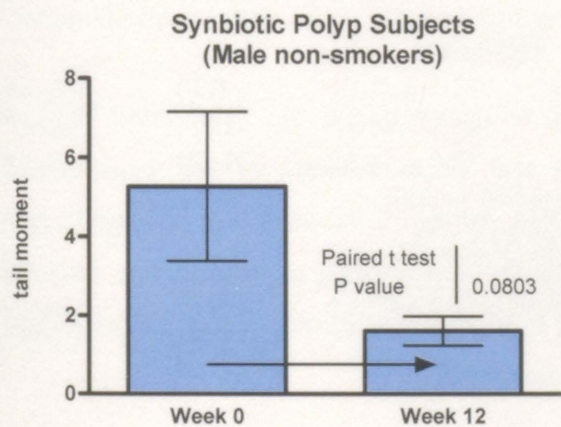


Figure 7 (d)

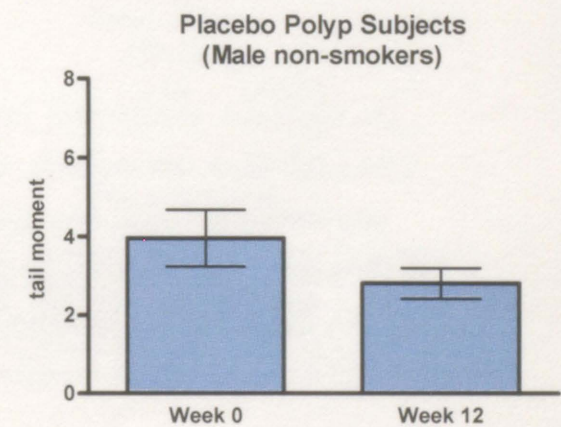


Figure 7 (e)

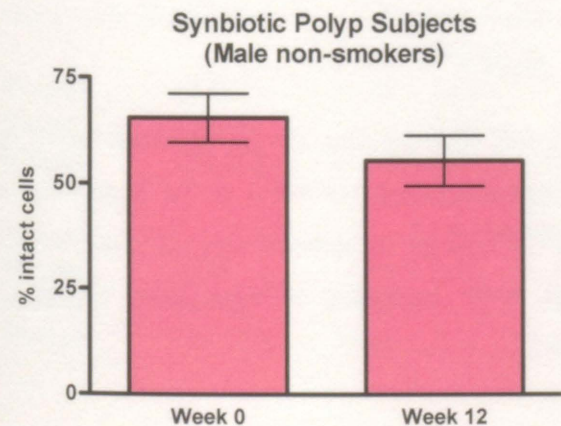


Figure 7 (f)

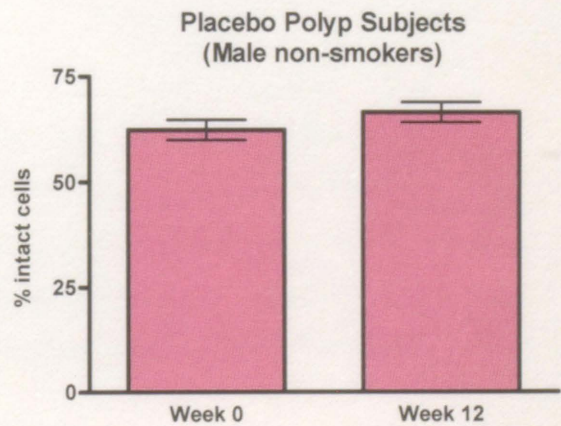


Table 1

In vivo DNA damage (tail intensity, tail moment and the % of intact cells) in biopsies from cancer and polyp subjects (including smokers) at the beginning of intervention and at all time points. DNA damage in biopsies was not significantly different in cancer and polyp subjects at the beginning of intervention. However DNA damage was significantly lower in polyp subjects compared to cancer subjects at all times. Results are expressed as mean $\pm$ S.E.

Table 2

In vivo DNA damage (tail intensity, tail moment and the % of intact cells) in biopsies from non-smoking cancer and polyp subjects at the beginning of intervention and at all time points. DNA damage in biopsies was not significantly different in cancer and polyp subjects at the beginning of intervention. However DNA damage was significantly lower in polyp subjects compared to cancer subjects at all times. Results are expressed as mean $\pm$ S.E.

Table 3

In vivo DNA damage (tail intensity, tail moment and the % of intact cells) in biopsies from cancer and polyp subjects (including smokers) at the beginning of intervention and at all time points. DNA damage in biopsies was not significantly different in cancer and polyp subjects at the beginning of intervention and at all times. Results are expressed as mean $\pm$ S.E.

Table 1:

All subjects (incl smokers)

Genotoxic Biomarker		Cancer Subjects (Week 0)		Polyp Subjects (Week 0)		P value
Biopsy	(TI)	12.16 ± 1.21	(n=29)	12.62 ± 1.06	(n=32)	n.s.
Biopsy	(TM)	3.988 ± 0.554	(n=29)	4.296 ± 0.536	(n=32)	n.s.
Biopsy	(% IC)	62.49 ± 2.00	(n=29)	63.97 ± 2.01	(n=32)	n.s.
		Cancer Subjects (All Times)		Polyp Subjects (All Times)		
Biopsy	(TI)	15.03 ± 1.27	(n=29)	11.11 ± 0.66	(n=32)	0.0047
Biopsy	(TM)	5.304 ± 0.557	(n=29)	3.407 ± 0.313	(n=32)	0.0029
Biopsy	(% IC)	58.78 ± 1.83	(n=29)	62.54 ± 1.96	(n=32)	0.0845

Table 2:

Non-smokers

Genotoxic Biomarker		Cancer Subjects (Week 0)		Polyp Subjects (Week 0)		P value
Biopsy	(TI)	12.21 ± 1.28	(n=27)	11.25 ± 0.80	(n=29)	n.s.
Biopsy	(TM)	4.049 ± 0.585	(n=27)	3.256 ± 0.370	(n=29)	n.s.
Biopsy	(% IC)	62.43 ± 2.08	(n=27)	57.93 ± 2.22	(n=29)	n.s.
		Cancer Subjects (All Times)		Polyp Subjects (All Times)		
Biopsy	(TI)	15.09 ± 1.28	(n=27)	11.55 ± 0.67	(n=29)	0.0143
Biopsy	(TM)	5.304 ± 0.573	(n=27)	3.550 ± 0.332	(n=29)	0.0082
Biopsy	(% IC)	58.73 ± 1.92	(n=27)	61.75 ± 1.58	(n=29)	n.s.

Table 3:

Male non-smokers

Genotoxic Biomarker		Cancer Subjects (Week 0)		Polyp Subjects (Week 0)		P value
Biopsy	(TI)	10.29 ± 1.02	(n=17)	12.69 ± 1.29	(n=18)	n.s.
Biopsy	(TM)	3.077 ± 0.447	(n=17)	4.398 ± 0.774	(n=18)	n.s.
Biopsy	(% IC)	64.29 ± 2.23	(n=17)	63.42 ± 2.41	(n=18)	n.s.
		Cancer Subjects (All Times)		Polyp Subjects (All Times)		
Biopsy	(TI)	13.74 ± 1.43	(n=17)	11.08 ± 0.82	(n=18)	n.s.
Biopsy	(TM)	4.803 ± 0.712	(n=17)	3.406 ± 0.444	(n=18)	n.s.
Biopsy	(% IC)	60.06 ± 2.31	(n=17)	63.15 ± 1.80	(n=18)	n.s.

DNA Damaging Potential of Faecal Water (FW)

Faecal samples were obtained from cancer and polyp subjects at the beginning of (week 0), mid-way through (week 6) and the end of intervention (week 12). FW was prepared by ultracentrifugation and sterile filtration as described previously. HT29clone19a cells were treated with FW and the DNA damaging potential of FW was assessed using the comet assay. Following incubation with FW the viability of HT29clone19a cells was assessed by trypan blue. Cell viability was $97.01\% \pm 4.031$ (mean $\pm$ SD) well within the accepted range of $>75\%$ (Henderson *et al.* 1998) indicating that the FW samples tested were not cytotoxic to HT29clone19a cells.

Cancer subjects

Synbiotic consumption did not influence the genotoxic potential of FW derived from cancer subjects. Tail intensity, tail moment and the percentage of intact cells in HT29clone19a cells following incubation with FW derived from both synbiotic (n=17) and placebo cancer subjects (n=14) were not significantly altered during the twelve week intervention period (*Figures 8(a-f)*). Similar results were observed with FW derived from synbiotic (n=15) and placebo (n=14) non-smoking cancer subjects (*Figures 9(a-f)*) and placebo (n=9) and synbiotic (n=8) male non-smoking cancer subjects (*Figures 10(a-f)*).

Polyp subjects

FW derived from the placebo group of polyp subjects (n=20) increased DNA damage in HT29clone19a cells. The percentage of intact cells was significantly reduced at week 6 ($p=0.0185$) and week 12 ($p=0.0351$) compared to week 0 (*Figure 11(f)*). There was a significant increase in tail intensity ($p=0.0354$) at week 6 and an

increase although not reaching significance at week 12 ($p=0.0547$) compared to week 0 (*Figure 11(b)*). There was a trend towards increased tail moment at week 6 ($p=0.0662$) and week 12 ($p=0.735$) but this increase did not reach significance (*Figure 11(d)*). FW derived from polyp subjects consuming the synbiotic ($n=17$) did not influence DNA damage in HT29clone19a cells at weeks 6 and 12 compared to week 0 (*Figure 11(a), 11(c), 11(e)*).

Similar but more pronounced results were observed in non-smoking synbiotic ($n=15$) and placebo ($n=19$) polyp subjects (*Figures 12(a-f)*). Interestingly the increase in DNA damage in HT29clone19a cells was most pronounced following incubation with FW derived from male non-smoking placebo polyp subjects ($n=14$). Tail intensity ($p=0.0025$) and tail moment ($p=0.0067$) were significantly increased at week 12 compared to week 0 *Figures (13(b) & 13(d))* and the percentage of intact cells was significantly decreased at week 6 ($p=0.0308$) and week 12 ($p=0.0053$) compared to week 0 *Figure (13(f))*. In contrast FW derived from male non-smoking synbiotic polyp subjects ($n=7$) did not influence DNA damage.

The genotoxic potential of FW derived from cancer and polyp subjects at the beginning of intervention and at all time points was analysed. There was no significant difference in the genotoxic potential of FW derived from cancer subjects compared to polyp subjects at the beginning of intervention or at all time points (*Table 4*). Similar results were observed in non-smokers (male and female) (*Table 5*) and in non-smoking males (*Table 6*).

Figure 8(a)

Tail intensity in HT29clone19a cells induced by FW from cancer subjects consuming synbiotic (n=17) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 8(b)

Tail intensity in HT29clone19a cells induced by FW from cancer subjects consuming placebo (n=14) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 8(c)

Tail moment in HT29clone19a cells induced by FW from cancer subjects consuming synbiotic (n=17) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 8(d)

Tail moment in HT29clone19a cells induced by FW from cancer subjects consuming placebo (n=14) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 8(e)

The percentage of intact cells (non comet images) in HT29clone19a cells induced by FW from cancer subjects consuming synbiotic (n=17) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). There was no change at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 8(f)

The percentage of intact cells (non comet images) in HT29clone19a cells induced by FW from cancer subjects consuming placebo (n=14) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). There was no change at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 8 (a)

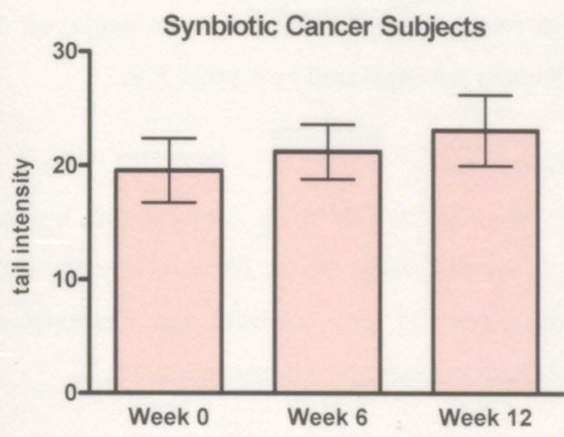


Figure 8 (b)

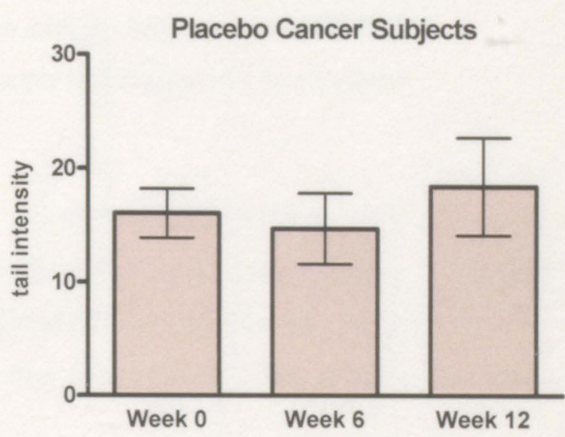


Figure 8 (c)

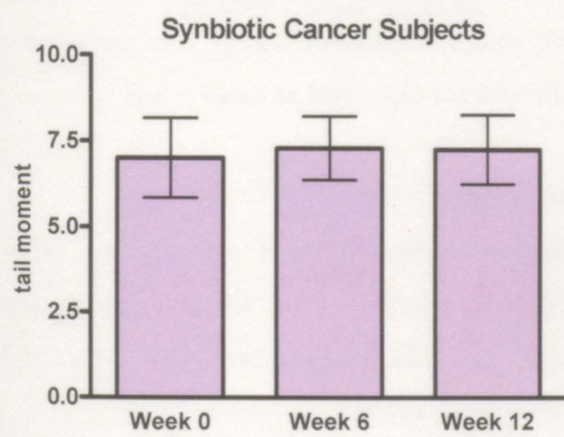


Figure 8 (d)

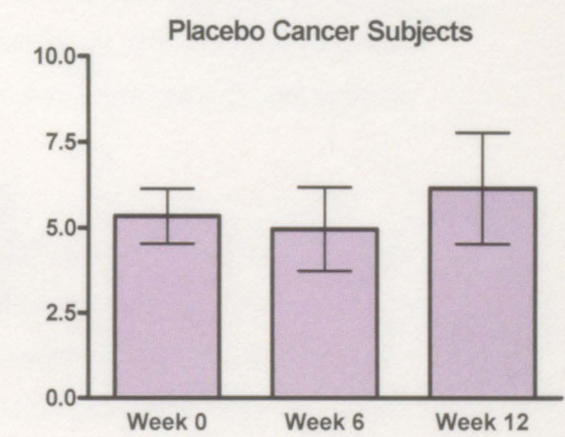


Figure 8 (e)

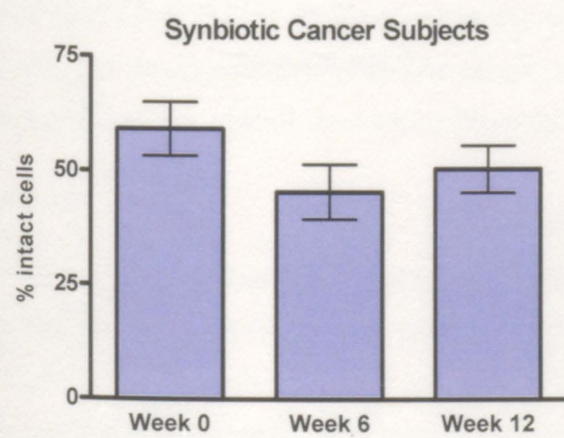


Figure 8 (f)

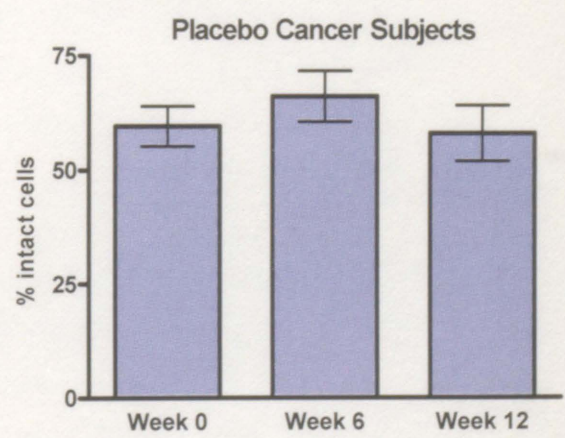


Figure 9(a)

Tail intensity in HT29clone19a cells induced by FW from non-smoking cancer subjects consuming synbiotic (n=15) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 9(b)

Tail intensity in HT29clone19a cells induced by FW from non-smoking cancer subjects consuming placebo (n=14) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 9(c)

Tail moment in HT29clone19a cells induced by FW from non-smoking cancer subjects consuming synbiotic (n=15) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 9(d)

Tail moment in HT29clone19a cells induced by FW from non-smoking cancer subjects consuming placebo (n=14) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 9(e)

The percentage of intact cells (non comet images) in HT29clone19a cells induced by FW from non-smoking cancer subjects consuming synbiotic (n=15) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). There was no change at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 9(f)

The percentage of intact cells (non comet images) in HT29clone19a cells induced by FW from non-smoking cancer subjects consuming placebo (n=14) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). There was no change at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 9 (a)

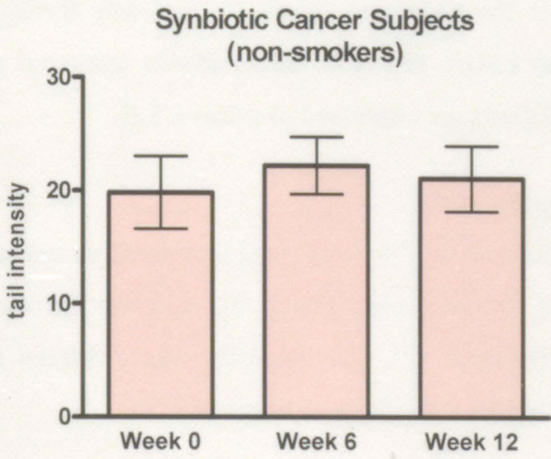


Figure 9 (b)

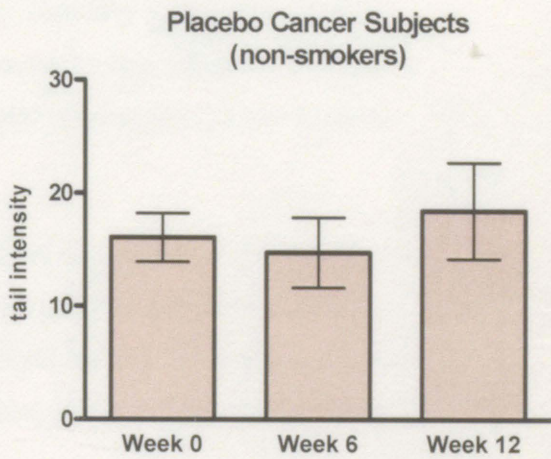


Figure 9 (c)

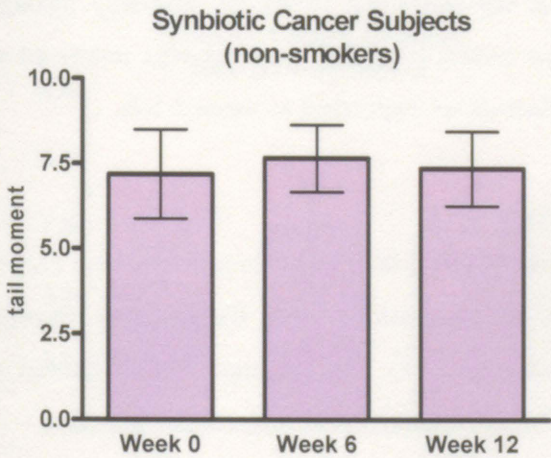


Figure 9 (d)

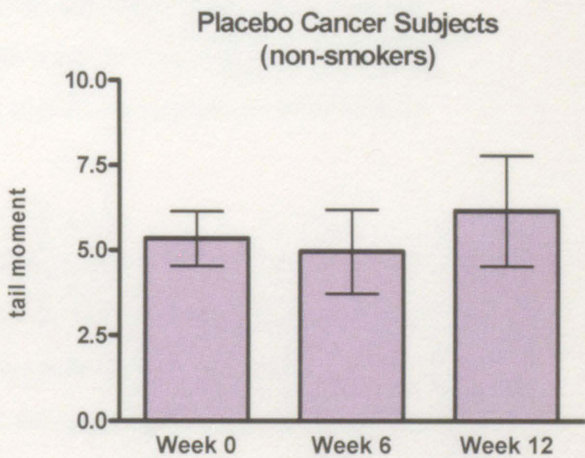


Figure 9 (e)

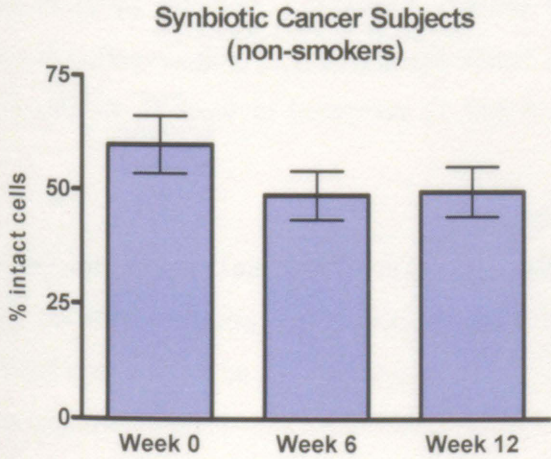


Figure 9 (f)

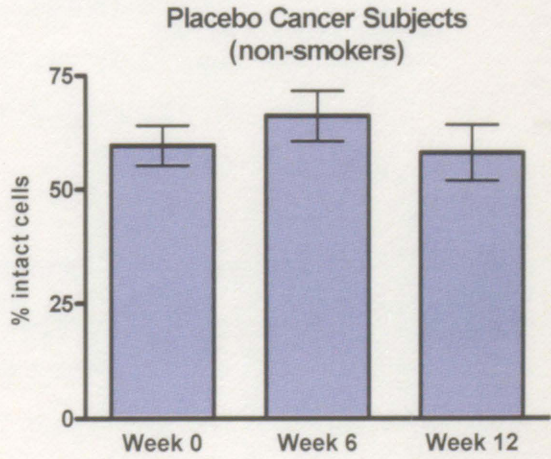


Figure 10(a)

Tail intensity in HT29clone19a cells induced by FW from male non-smoking cancer subjects consuming synbiotic (n=8) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 10(b)

Tail intensity in HT29clone19a cells induced by FW from male non-smoking cancer subjects consuming placebo (n=9) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 10(c)

Tail moment in HT29clone19a cells induced by FW from male non-smoking cancer subjects consuming synbiotic (n=8) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 10(d)

Tail moment in HT29clone19a cells induced by FW from male non-smoking cancer subjects consuming placebo (n=9) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 10(e)

The percentage of intact cells (non comet images) in HT29clone19a cells induced by FW from male non-smoking cancer subjects consuming synbiotic (n=8) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). There was no change at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 10(f)

The percentage of intact cells (non comet images) in HT29clone19a cells induced by FW from male non-smoking cancer subjects consuming placebo (n=9) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). There was no change at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 10 (a)

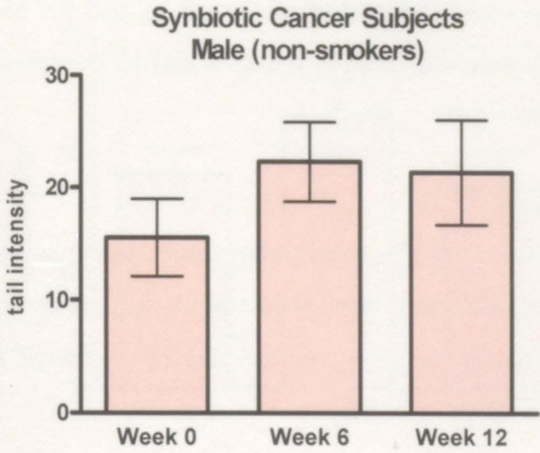


Figure 10 (b)

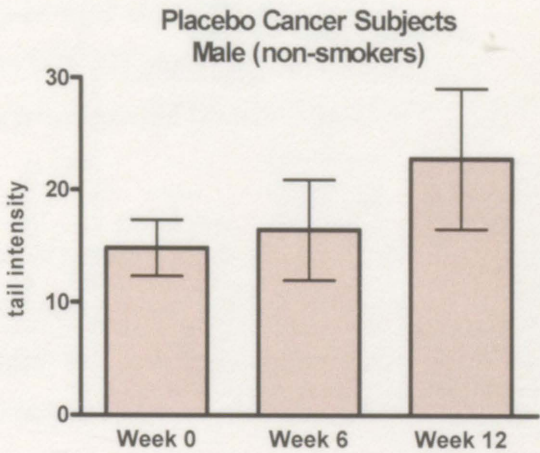


Figure 10 (c)

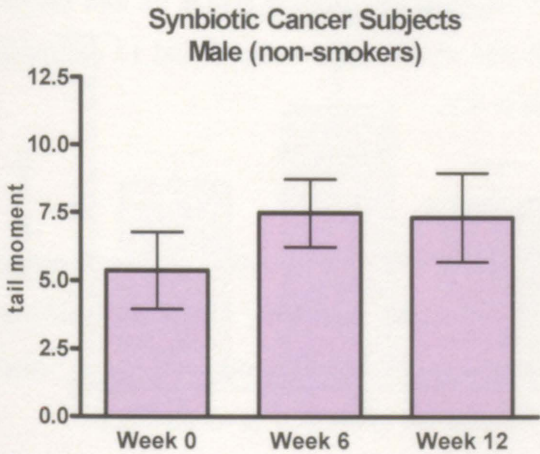


Figure 10 (d)

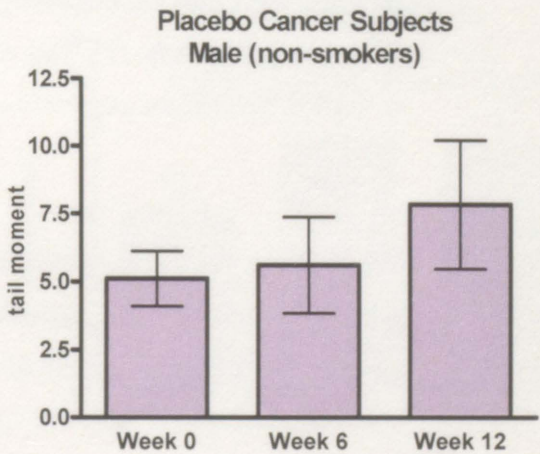


Figure 10 (e)

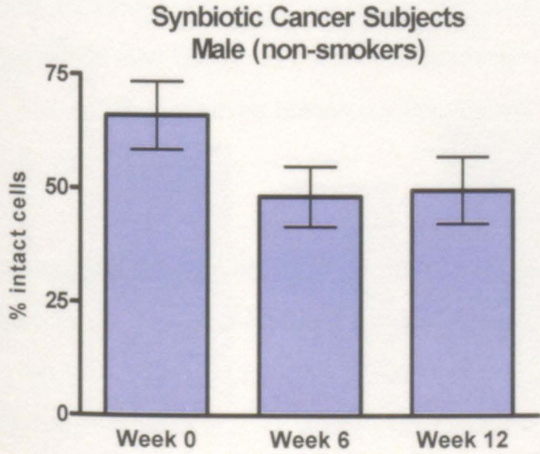


Figure 10 (f)

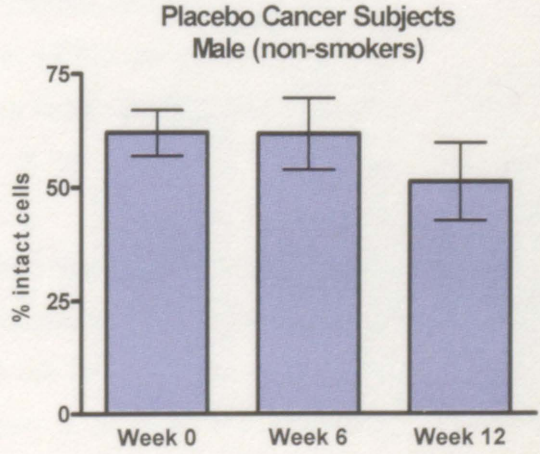


Figure 11(a)

Tail intensity in HT29clone19a cells induced by FW from polyp subjects consuming synbiotic (n=17) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 11(b)

Tail intensity in HT29clone19a cells induced by FW from polyp subjects consuming placebo (n=20) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was increased at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 11(c)

Tail moment in HT29clone19a cells induced by FW from polyp subjects consuming synbiotic (n=17) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 11(d)

Tail moment in HT29clone19a cells induced by FW from polyp subjects consuming placebo (n=20) at the beginning (week 0) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was increased at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 11(e)

The percentage of intact cells (non comet images) in HT29clone19a cells induced by FW from polyp subjects consuming synbiotic (n=17) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). There was no change at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 11(f)

The percentage of intact cells (non comet images) in HT29clone19a cells induced by FW from polyp subjects consuming placebo (n=20) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). The percentage of intact cells was reduced at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 11 (a)

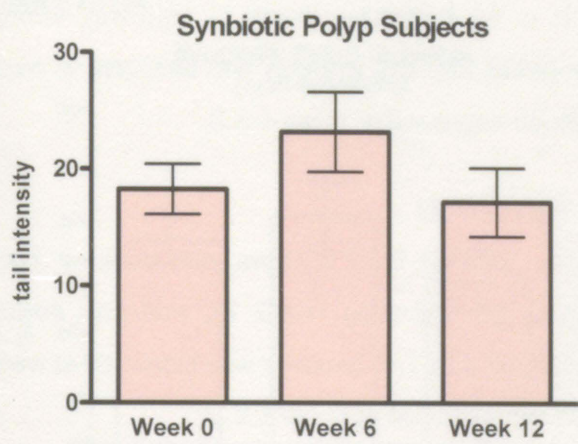


Figure 11 (b)

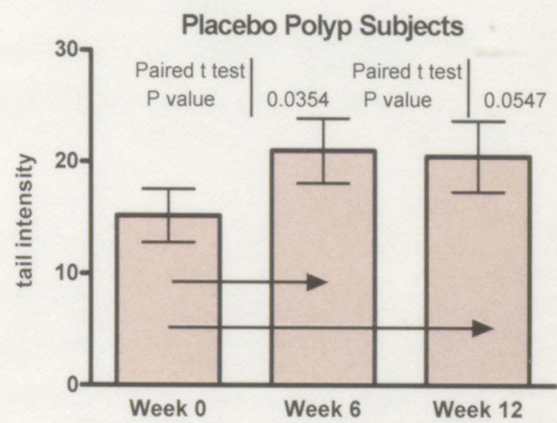


Figure 11 (c)

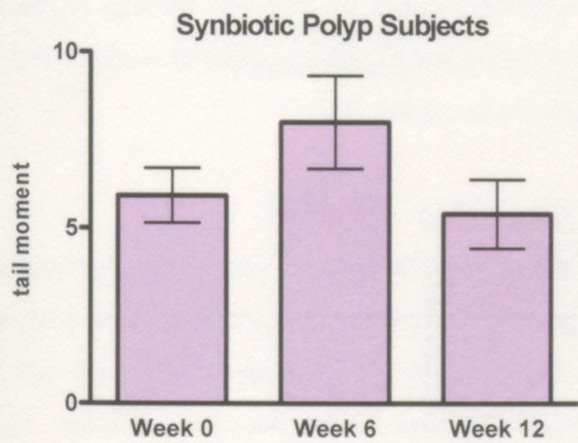


Figure 11 (d)

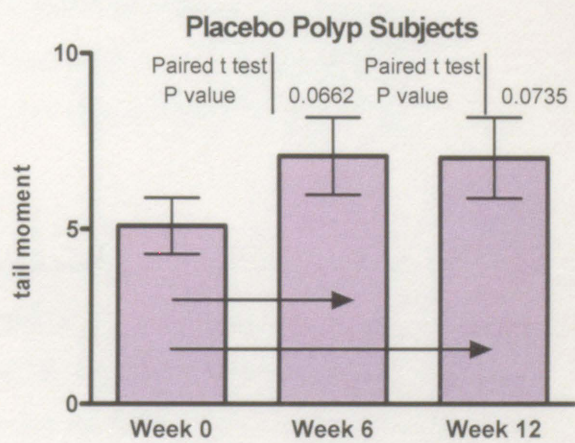


Figure 11 (e)

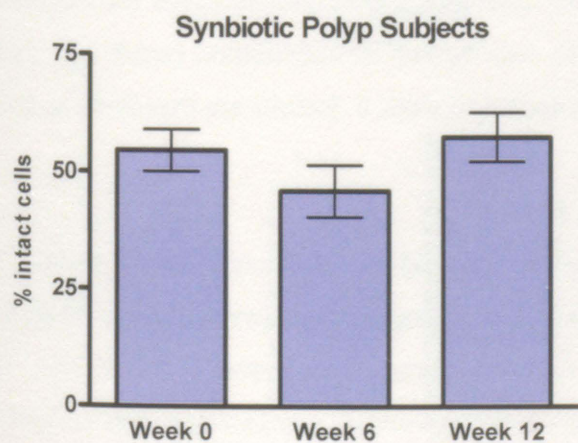


Figure 11 (f)

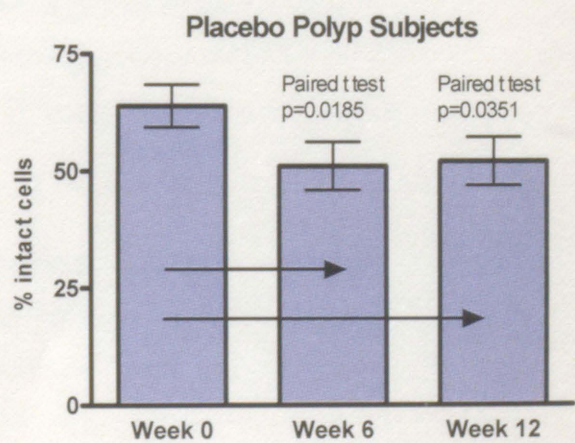


Figure 12(a)

Tail intensity in HT29clone19a cells induced by FW from non-smoking polyp subjects consuming synbiotic (n=15) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 12(b)

Tail intensity in HT29clone19a cells induced by FW from non-smoking polyp subjects consuming placebo (n=19) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was increased at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 12(c)

Tail moment in HT29clone19a cells induced by FW from non-smoking polyp subjects consuming synbiotic (n=15) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 12(d)

Tail moment in HT29clone19a cells induced by FW from non-smoking polyp subjects consuming placebo (n=19) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was increased at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 12(e)

The percentage of intact cells (non comet images) in HT29clone19a cells induced by FW from non-smoking polyp subjects consuming synbiotic (n=15) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). There was no change at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 12(f)

The percentage of intact cells (non comet images) in HT29clone19a cells induced by FW from non-smoking polyp subjects consuming placebo (n=19) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). The percentage of intact cells was reduced at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 12 (a)

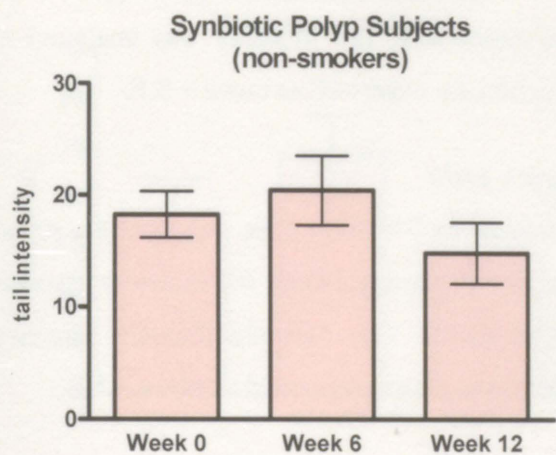


Figure 12 (b)

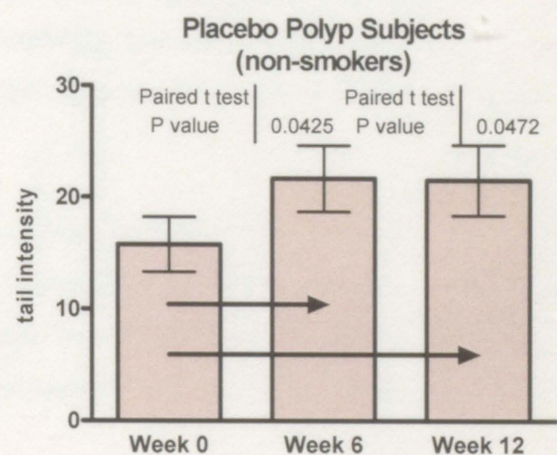


Figure 12 (c)

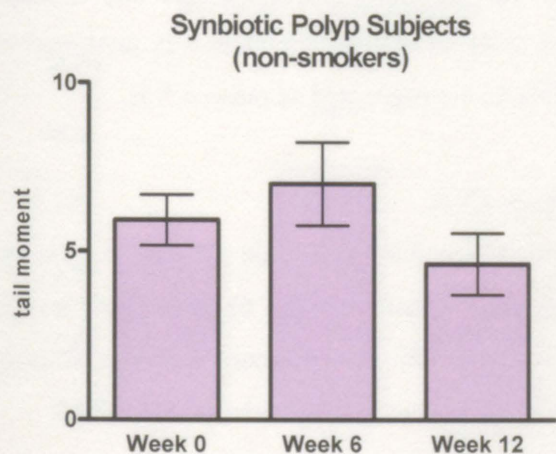


Figure 12 (d)

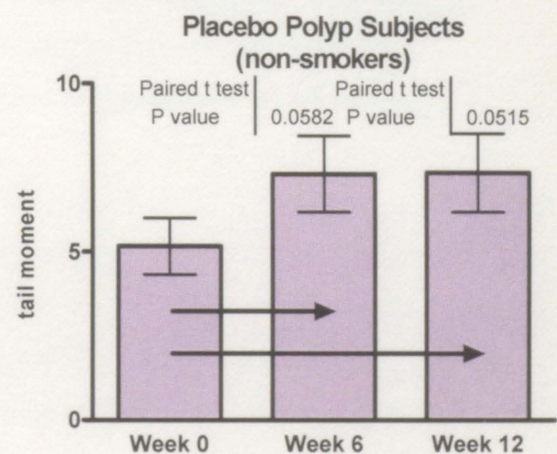


Figure 12 (e)

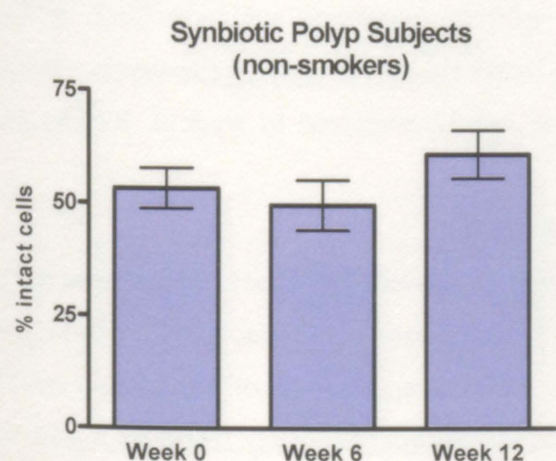


Figure 12 (f)

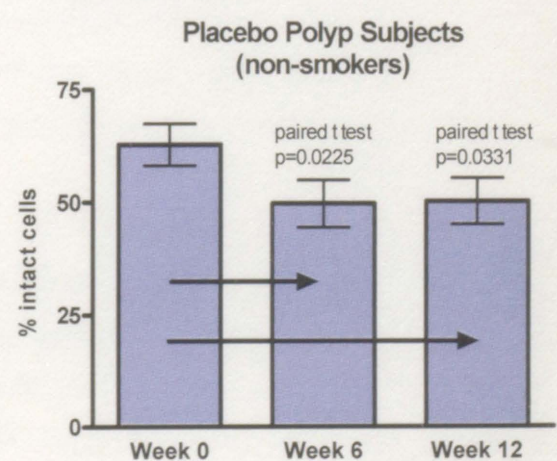


Figure 13(a)

Tail intensity in HT29clone19a cells induced by FW from male non-smoking polyp subjects consuming synbiotic (n=7) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 13(b)

Tail intensity in HT29clone19a cells induced by FW from male non-smoking polyp subjects consuming placebo (n=14) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail significantly intensity increased at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 13(c)

Tail moment in HT29clone19a cells induced by FW from male non-smoking polyp subjects consuming synbiotic (n=7) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 13(d)

Tail moment in HT29clone19a cells induced by FW from male non-smoking polyp subjects consuming placebo (n=14) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was significantly increased at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 13(e)

The percentage of intact cells (non comet images) in HT29clone19a cells induced by FW from male non-smoking polyp subjects consuming synbiotic (n=7) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). There was no change at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 13(f)

The percentage of intact cells (non comet images) in HT29clone19a cells induced by FW from male non-smoking polyp subjects consuming placebo (n=14) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). The percentage of intact cells was significantly decreased at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 13 (a)

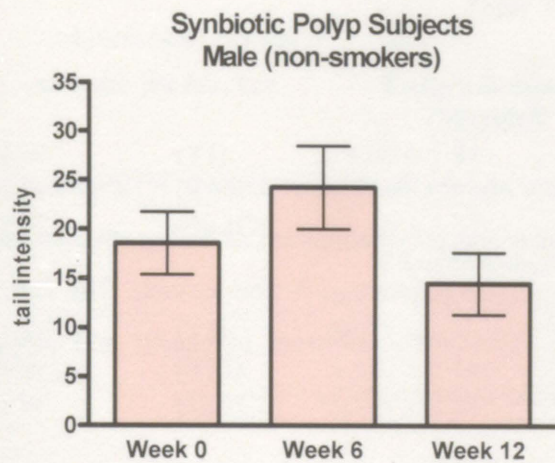


Figure 13 (b)

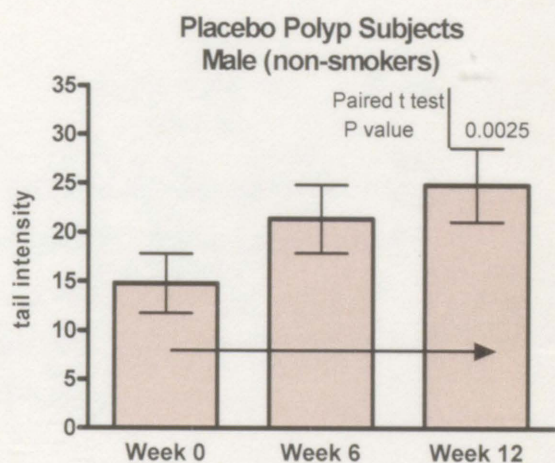


Figure 13 (c)

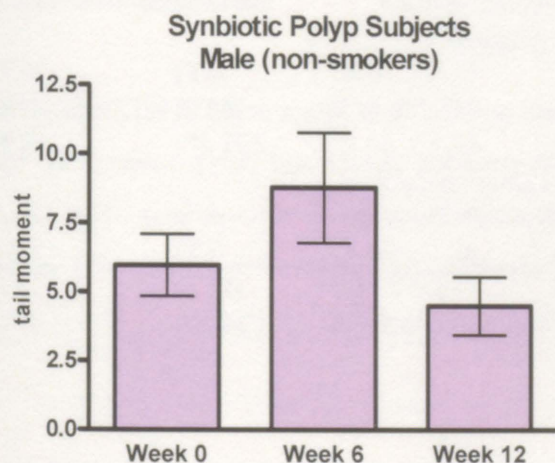


Figure 13 (d)

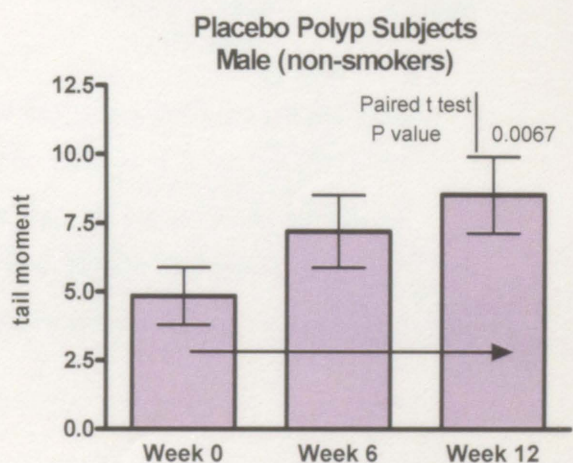


Figure 13 (e)

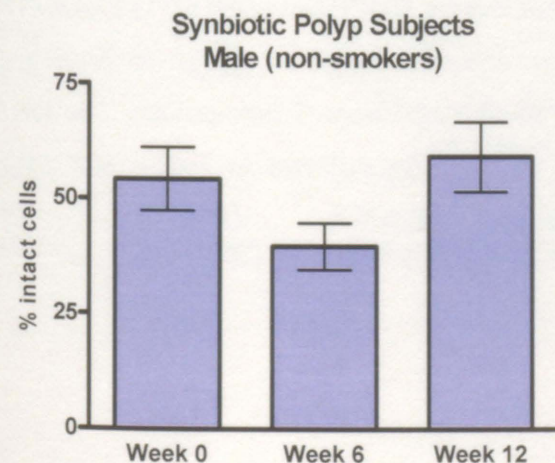


Figure 13 (f)

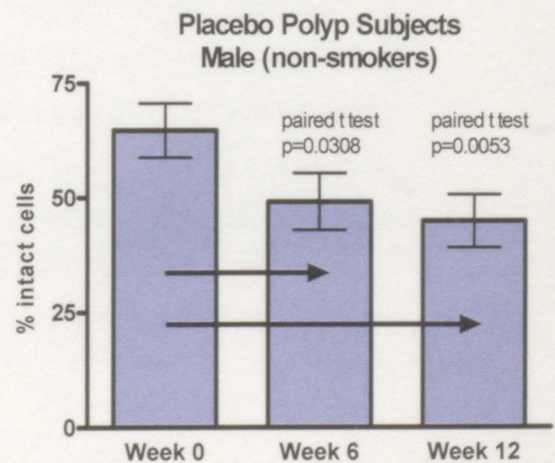


Table 4

DNA damage (tail intensity, tail moment and the % of intact cells) in HT29clone19a cells induced by FW derived from cancer and polyp subjects (including smokers) at the beginning of intervention and at all times (average 3 time-points). The DNA damaging potential of FW was not significantly different in cancer and polyp subjects at all times. Results are expressed as mean $\pm$ S.E.

Table 5

DNA damage (tail intensity, tail moment and the % of intact cells) in HT29clone19a cells induced by FW derived from non-smoking cancer and polyp subjects at the beginning of intervention and at all times (average 3 time-points). The DNA damaging potential of FW was not significantly different in cancer and polyp subjects at all times. Results are expressed as mean $\pm$ S.E.

Table 6

DNA damage (tail intensity, tail moment and the % of intact cells) in HT29clone19a cells induced by FW derived from male non-smoking cancer and polyp subjects at the beginning of intervention and at all times (average 3 time-points). The DNA damaging potential of FW was not significantly different in cancer and polyp subjects at all times. Results are expressed as mean $\pm$ S.E.

Table 4:

All subjects (incl smokers)

Genotoxic Biomarker		Cancer Subjects (Week 0)		Polyp Subjects (Week 0)		P value
FW	(TI)	18.00 ± 1.82	(n=31)	16.59 ± 1.63	(n=37)	n.s.
FW	(TM)	6.254 ± 0.736	(n=31)	5.476 ± 0.554	(n=37)	n.s.
FW	(% IC)	59.26 ± 3.42	(n=31)	59.64 ± 3.23	(n=37)	n.s.
		Cancer Subjects (All Times)		Polyp Subjects (All Times)		
FW	(TI)	19.11 ± 1.23	(n=31)	19.20 ± 1.17	(n=37)	n.s.
FW	(TM)	6.420 ± 0.461	(n=31)	6.424 ± 0.426	(n=37)	n.s.
FW	(% IC)	56.67 ± 2.28	(n=31)	54.31 ± 2.08	(n=37)	n.s.

Table 5:

Non-smokers

Genotoxic Biomarker		Cancer Subjects (Week 0)		Polyp Subjects (Week 0)		P value
FW	(TI)	18.03 ± 1.95	(n=29)	16.86 ± 1.63	(n=34)	n.s.
FW	(TM)	6.30 ± 0.79	(n=29)	5.50 ± 0.57	(n=34)	n.s.
FW	(% IC)	59.56 ± 3.54	(n=29)	58.61 ± 3.30	(n=34)	n.s.
		Cancer Subjects (All Times)		Polyp Subjects (All Times)		
FW	(TI)	18.82 ± 1.26	(n=29)	18.86 ± 1.56	(n=34)	n.s.
FW	(TM)	6.48 ± 0.49	(n=29)	6.28 ± 0.43	(n=34)	n.s.
FW	(% IC)	57.32 ± 2.27	(n=29)	54.41 ± 2.10	(n=34)	n.s.

Table 6:

Male non-smokers

Genotoxic Biomarker		Cancer Subjects (Week 0)		Polyp Subjects (Week 0)		P value
FW	(TI)	15.17 ± 2.02	(n=17)	16.05 ± 2.27	(n=21)	n.s.
FW	(TM)	5.24 ± 0.83	(n=17)	5.22 ± 0.78	(n=21)	n.s.
FW	(% IC)	63.22 ± 3.97	(n=17)	61.30 ± 4.56	(n=21)	n.s.
		Cancer Subjects (All Times)		Polyp Subjects (All Times)		
FW	(TI)	18.83 ± 1.76	(n=17)	19.94 ± 1.53	(n=21)	n.s.
FW	(TM)	6.44 ± 0.66	(n=17)	6.72 ± 0.58	(n=21)	n.s.
FW	(% IC)	56.69 ± 2.93	(n=17)	52.36 ± 2.75	(n=21)	n.s.

DNA Damage in PBMCs

DNA damage in PBMCs isolated from whole blood at week 0, week 6 and week 12 was assessed by comet assay. PBMCs were cryopreserved following isolation and stored under liquid nitrogen until analysed. PBMCs were thawed at 37°C and viability assessed by trypan blue exclusion. Average viability was $94.73\% \pm 7.426$ within accepted standards. Although the majority of samples contained greater than 90% viable cells a small number contained less viable cells and overall viabilities ranged from 29.3% to 100% and this must be remembered when interpreting the data.

Cancer subjects

Genotoxic damage in PBMCs was unaltered during the twelve-week intervention period in cancer subjects. Tail intensity and tail moment in PBMCs derived from cancer subjects (synbiotic n=18, placebo n=15) showed no difference at weeks 6 and 12 compared to week 0 (*Figures 14 (a-d)*). The same outcome was observed in non-smoking cancer subjects (synbiotic n=16, placebo n=15) (*Figures 15 (a-d)*) and male non-smoking cancer subjects (synbiotic n=18, placebo n=10) (*Figures 16 (a-d)*).

Polyp subjects

Genotoxic damage in PBMCs was significantly reduced in both the synbiotic and placebo groups of polyp subjects at the end of the twelve-week intervention period. Tail intensity ($p=0.0306$) and tail moment ($p=0.0246$) were significantly decreased at week 12 compared to week 6 in the synbiotic group (n=18) of polyp subjects (*Figures 17(a) & 17(c)*). In the placebo group (n=19) tail intensity ($p=0.0049$) and

tail moment ($p=0.0085$) were significantly decreased at week 12 compared to week 0 (*Figures 17(b) & 17(d)*).

Similarly for non-smoking polyp subjects in the synbiotic group ($n=15$) tail intensity ($p=0.0509$) and tail moment ($p=0.0429$) in PBMCs were decreased at week 12 compared to week 6 (*Figures 18(a) & 18(c)*). Similarly in the non-smoking placebo group ($n=17$) there was a significant reduction in tail intensity ($p=0.0202$) and tail moment ($p=0.0329$) at week 12 compared to week 0 (*Figures 18(b) & 18(d)*). In PBMCs from male non-smoking polyp subjects tail intensity ($p=0.0212$) and tail moment ($p=0.388$) were significantly reduced at week 12 compared to week 0 in the synbiotic group of polyp subjects ($n=7$). In the placebo polyp subjects ($n=12$) there was a trend towards lower DNA damage at week 12 compared to week 0 but this reduction was not statistically significant (tail intensity $p=0.0567$, tail moment $p=0.0708$) (*Figures 19 (a-d)*).

DNA damage in PBMCs from cancer and polyp subjects at the beginning of intervention and at all three time points was analysed. DNA damage in PBMCs was significantly higher ($p<0.05$) in polyp subjects compared to cancer subjects at week 0 and at all three time points (*Table 7*). Similar results were observed in non-smokers (male and female) (*Table 8*) and in non-smoking males (*Table 9*).

Figure 14(a)

Tail intensity in PBMCs from cancer subjects consuming synbiotic (n=18) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 14(b)

Tail intensity in PBMCs from cancer subjects consuming placebo (n=15) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 14(c)

Tail moment in PBMCs from cancer subjects consuming synbiotic (n=18) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 14(d)

Tail moment in PBMCs from cancer subjects consuming placebo (n=15) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 14 (a)

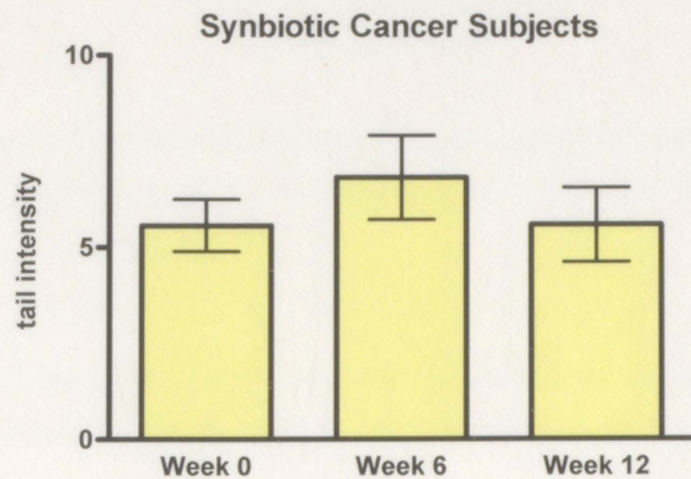


Figure 14 (b)

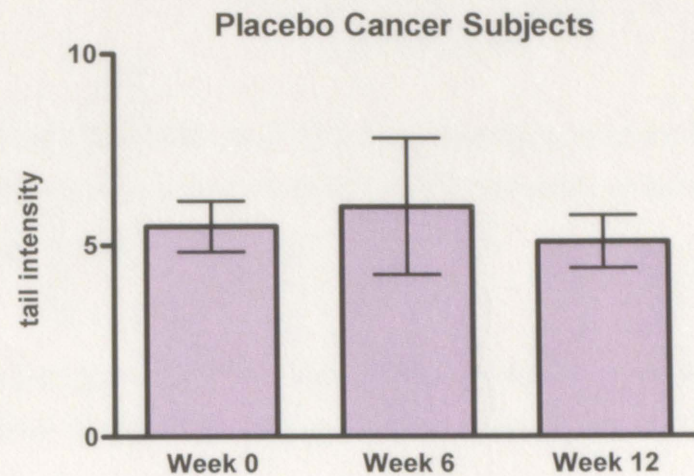


Figure 14 (c)

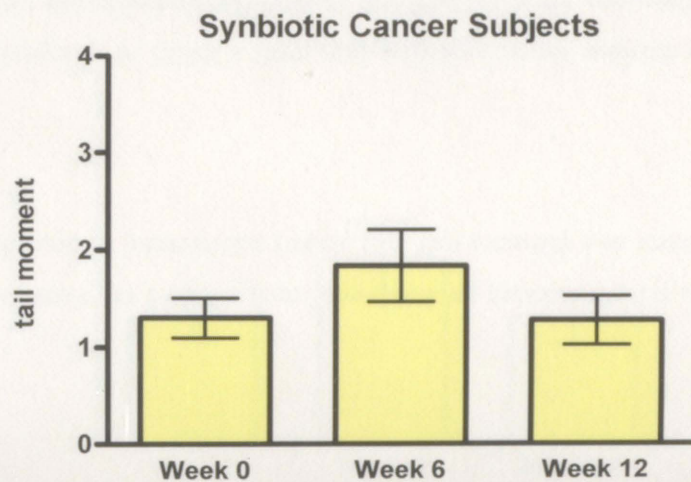


Figure 14 (d)

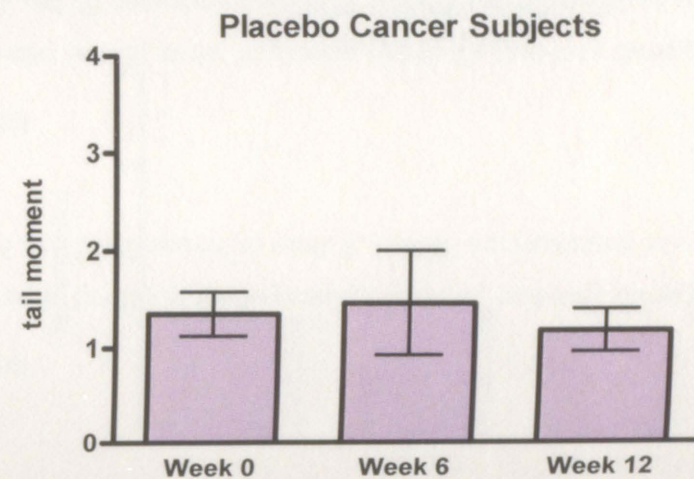


Figure 15(a)

Tail intensity in PBMCs from non-smoking cancer subjects consuming synbiotic (n=18) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 15(b)

Tail intensity in PBMCs from non-smoking cancer subjects consuming placebo (n=15) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 15(c)

Tail moment in PBMCs from non-smoking cancer subjects consuming synbiotic (n=18) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 15(d)

Tail moment in PBMCs from non-smoking cancer subjects consuming placebo (n=15) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 15(a)

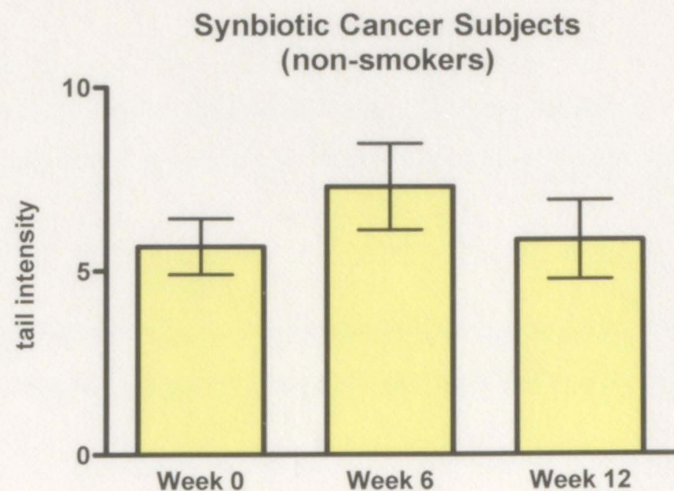


Figure 15 (b)

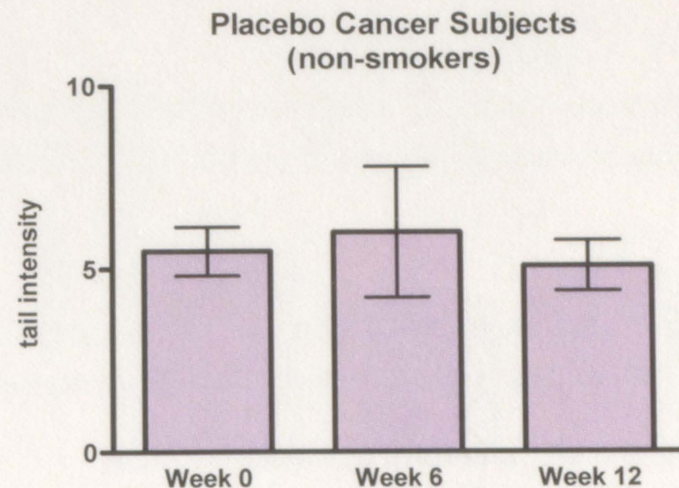


Figure 15 (c)

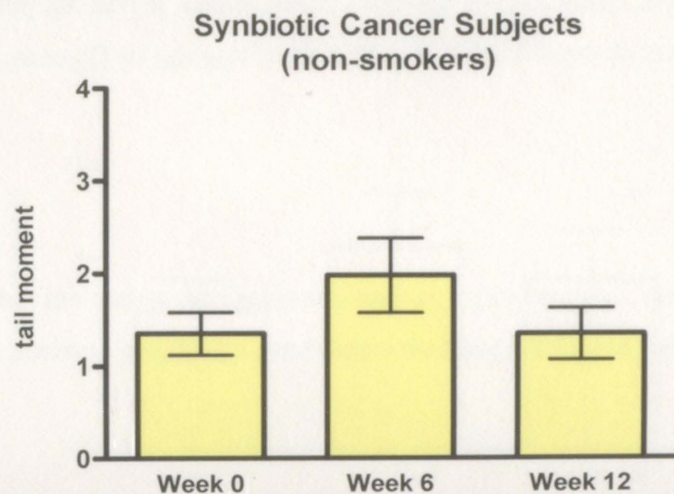


Figure 15 (d)

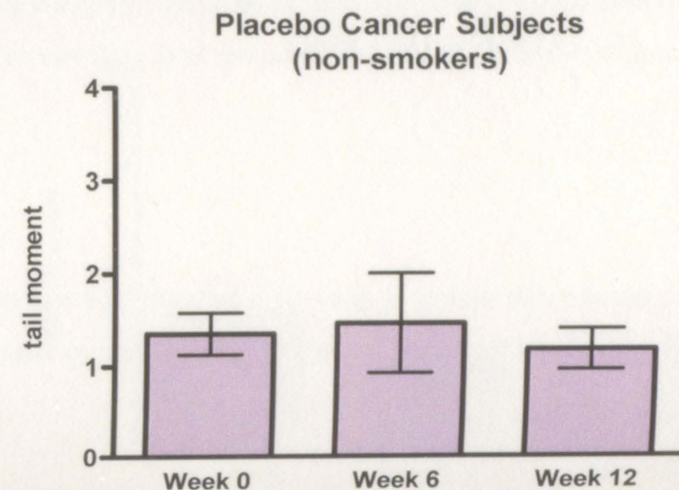


Figure 16(a)

Tail intensity in PBMCs from male non-smoking cancer subjects consuming synbiotic (n=18) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 16(b)

Tail intensity in PBMCs from male non-smoking cancer subjects consuming placebo (n=15) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 16(c)

Tail moment in PBMCs from male non-smoking cancer subjects consuming synbiotic (n=18) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 16(d)

Tail moment in PBMCs from male non-smoking cancer subjects consuming placebo (n=15) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was unaltered at weeks 6 and 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 16 (a)

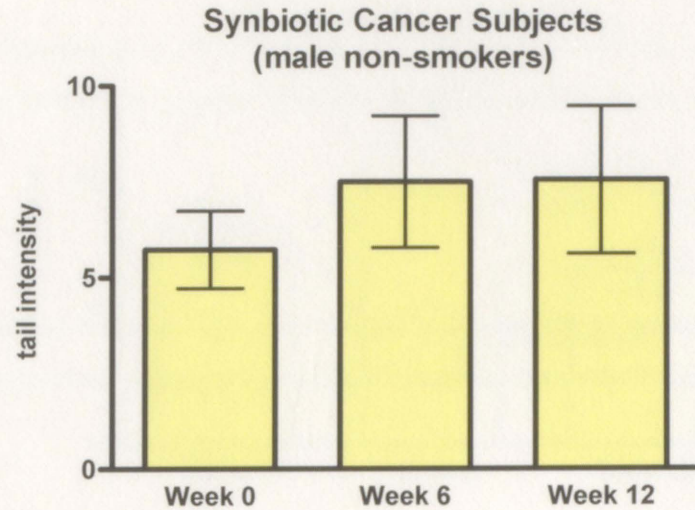


Figure 16 (b)

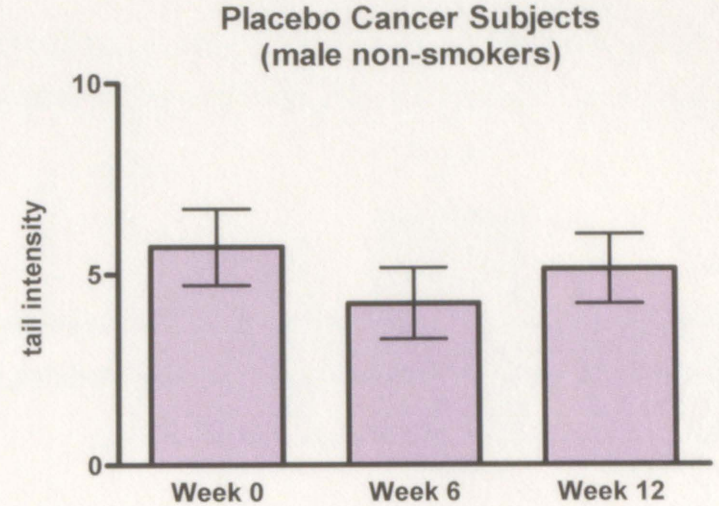


Figure 16 (c)

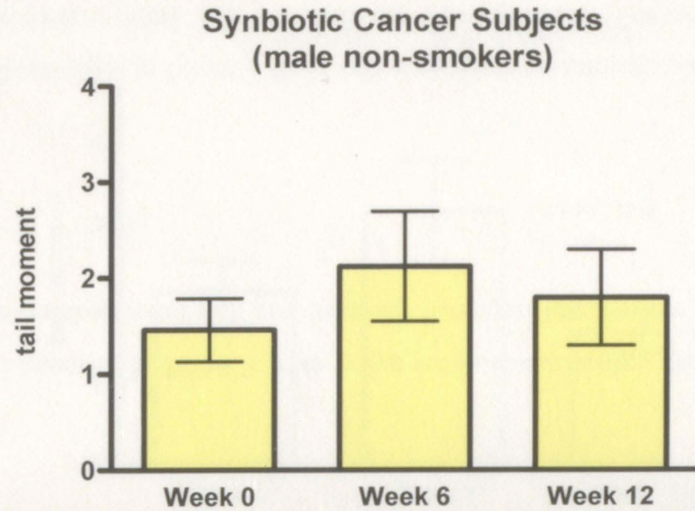


Figure 16(d)

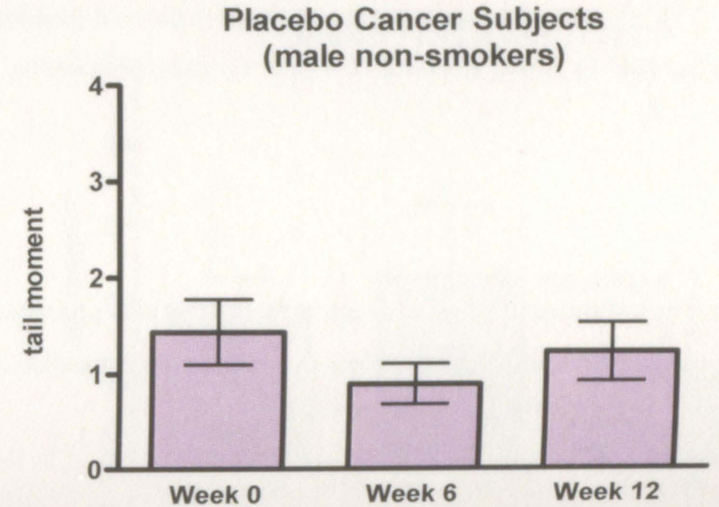


Figure 17(a)

Tail intensity in PBMCs from polyp subjects consuming synbiotic (n=18) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was significantly decreased at week 12 compared to week 6. Results are expressed as mean $\pm$ S.E.

Figure 17(b)

Tail intensity in PBMCs from polyp subjects consuming placebo (n=19) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was significantly decreased at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 17(c)

Tail moment in PBMCs from polyp subjects consuming synbiotic (n=18) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was significantly decreased at week 12 compared to week 6. Results are expressed as mean $\pm$ S.E.

Figure 17(d)

Tail moment in PBMCs from polyp subjects consuming placebo (n=19) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was significantly decreased at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 17 (a)

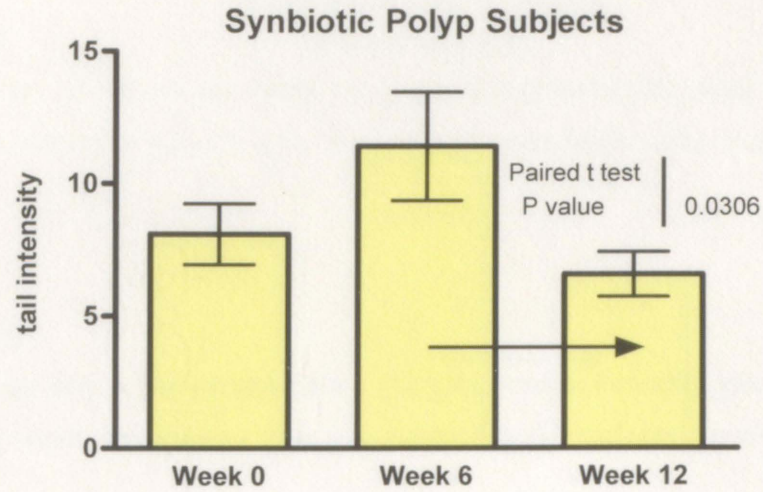


Figure 17 (b)

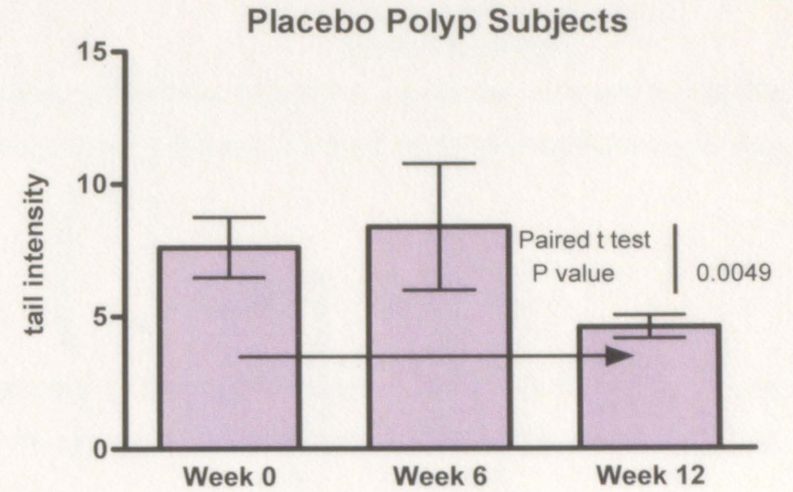


Figure 17 (c)

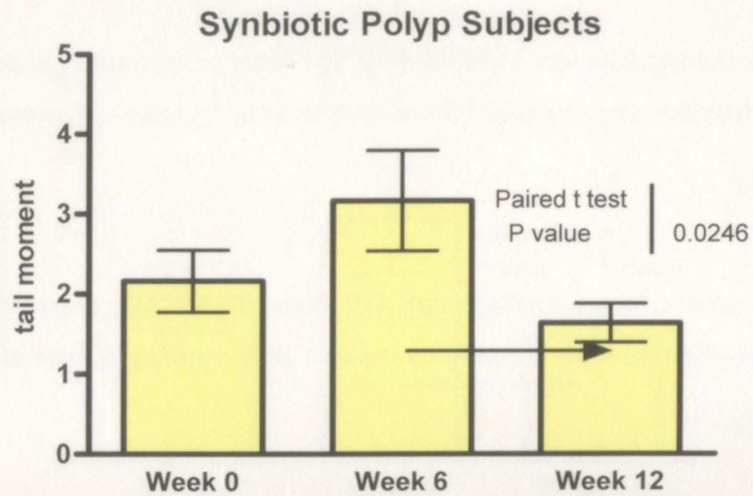


Figure 17 (d)

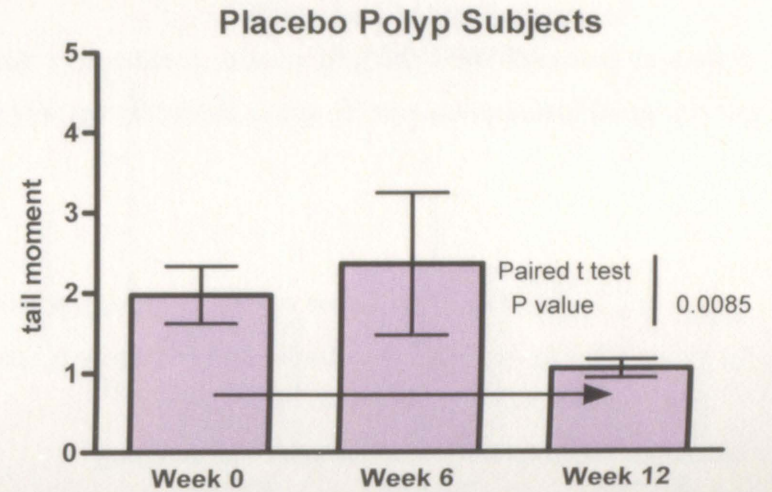


Figure 18(a)

Tail intensity in PBMCs from non-smoking polyp subjects consuming synbiotic (n=15) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was decreased at week 12 compared to week 6. Results are expressed as mean $\pm$ S.E.

Figure 18(b)

Tail intensity in PBMCs from non-smoking polyp subjects consuming placebo (n=17) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was significantly decreased at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 18(c)

Tail moment in PBMCs from non-smoking polyp subjects consuming synbiotic (n=15) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was significantly decreased at week 12 compared to week 6. Results are expressed as mean $\pm$ S.E.

Figure 18(d)

Tail moment in PBMCs from non-smoking polyp subjects consuming placebo (n=17) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was significantly decreased at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 18 (a)

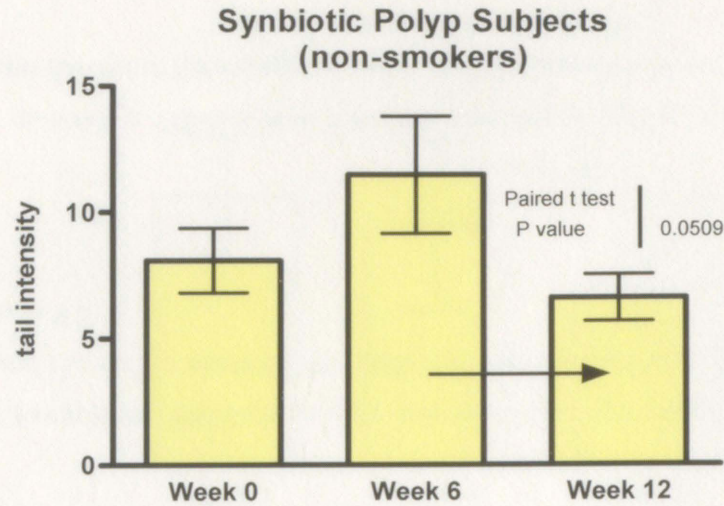


Figure 18 (b)

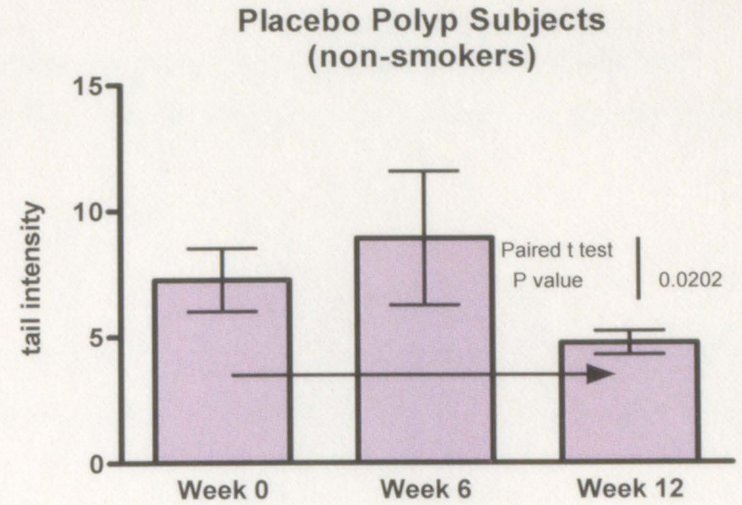


Figure 18 (c)

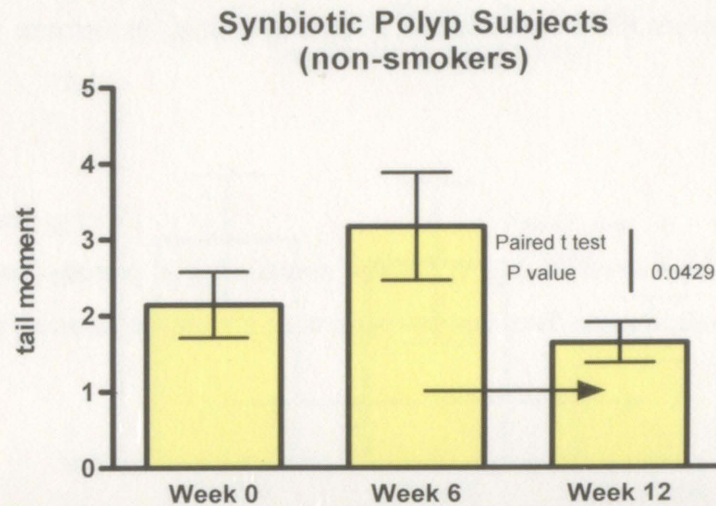


Figure 18 (d)

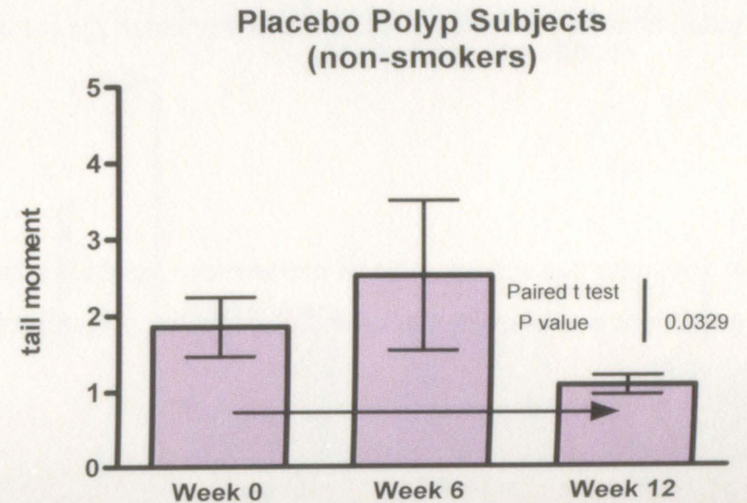


Figure 19(a)

Tail intensity in PBMCs from male non-smoking polyp subjects consuming synbiotic (n=7) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was significantly decreased at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 19(b)

Tail intensity in PBMCs from male non-smoking polyp subjects consuming placebo (n=12) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail intensity was decreased at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 19(c)

Tail moment in PBMCs from male non-smoking polyp subjects consuming synbiotic (n=7) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was significantly decreased at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 19(d)

Tail moment in PBMCs from male non-smoking polyp subjects consuming placebo (n=12) at the beginning (week 0), mid-way through (week 6), and the end of intervention (week 12). Tail moment was decreased at week 12 compared to week 0. Results are expressed as mean $\pm$ S.E.

Figure 19 (a)

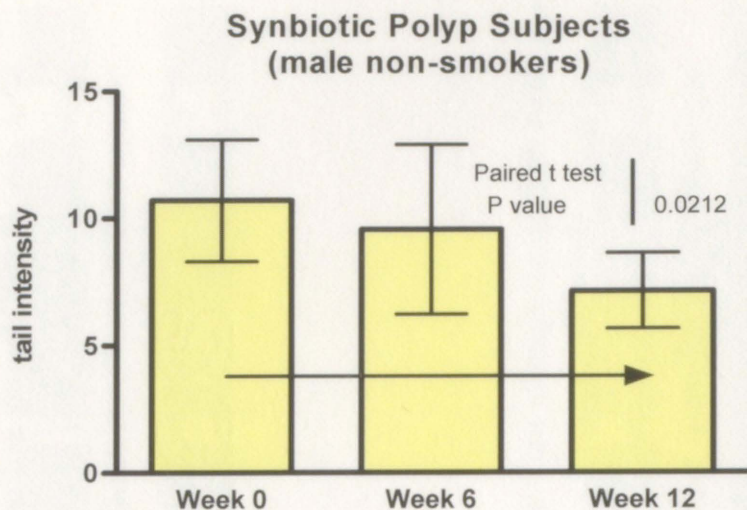


Figure 19 (b)

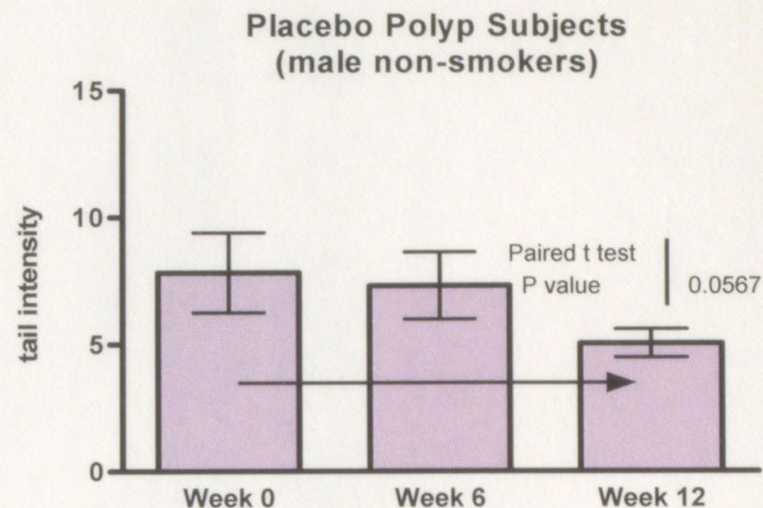


Figure 19 (c)

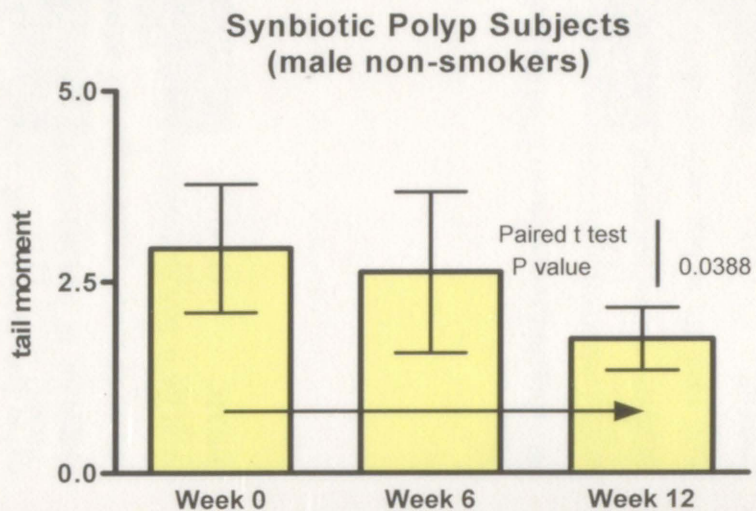


Figure 19 (d)

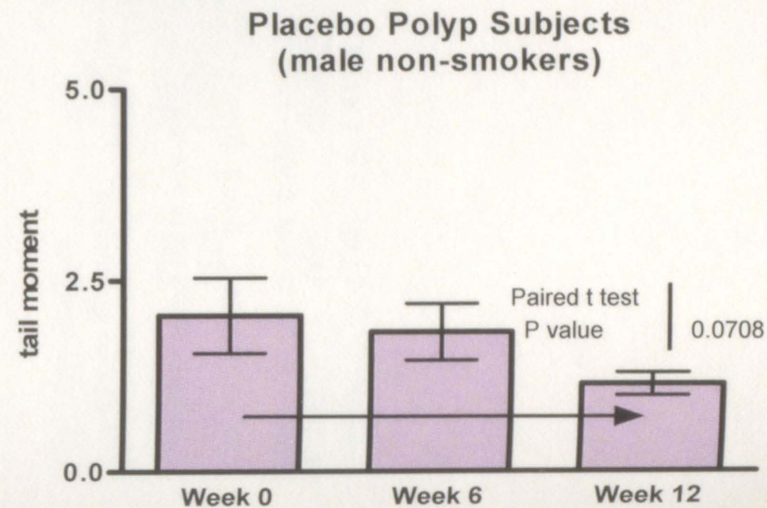


Table 7

DNA damage (tail intensity and tail moment) in PBMCs from cancer and polyp subjects (including smokers) at the beginning of intervention and at all times (average 3 time-points). DNA damage in PBMCS was significantly lower in cancer subjects compared to polyp subjects at the beginning of intervention and at all three time points. Results are expressed as mean $\pm$ S.E.

Table 8

DNA damage (tail intensity and tail moment) in PBMCs from non-smoking cancer and polyp subjects at the beginning of intervention and at all times (average 3 time-points). DNA damage in PBMCS was significantly lower in cancer subjects compared to polyp subjects at the beginning of intervention and at all three time points. Results are expressed as mean $\pm$ S.E.

Table 9

DNA damage (tail intensity and tail moment) in PBMCs from male non-smoking cancer and polyp subjects at the beginning of intervention and at all times (average 3 time-points). DNA damage in PBMCS was significantly lower in cancer subjects compared to polyp subjects at the beginning of intervention and at all three time points. Results are expressed as mean $\pm$ S.E.

Table 7:

All subjects (incl smokers)

Genotoxic Biomarker		Cancer Subjects (Week 0)		Polyp Subjects (Week 0)		P value
PBMC	(TI)	5.530 ± 0.470	(n=33)	7.843 ± 0.800	(n=37)	0.0184
PBMC	(TM)	1.327 ± 0.150	(n=33)	2.068 ± 0.259	(n=37)	0.0192
		Cancer Subjects (All Times)		Polyp Subjects (All Times)		
PBMC	(TI)	5.780 ± 0.418	(n=33)	7.760 ± 0.630	(n=37)	0.0114
PBMC	(TM)	1.413 ± 0.128	(n=33)	2.056 ± 0.211	(n=37)	0.0123

Table 8:

Non-smokers

Genotoxic Biomarker		Cancer Subjects (Week 0)		Polyp Subjects (Week 0)		P value
PBMC	(TI)	5.580 ± 0.500	(n=31)	7.662 ± 0.884	(n=33)	0.0483
PBMC	(TM)	1.353 ± 0.158	(n=31)	1.995 ± 0.286	(n=33)	0.0585
		Cancer Subjects (All Times)		Polyp Subjects (All Times)		
PBMC	(TI)	5.911 ± 0.441	(n=31)	7.815 ± 0.710	(n=33)	0.0253
PBMC	(TM)	1.453 ± 0.136	(n=31)	2.067 ± 0.239	(n=33)	0.0281

Table 9:

Male non-smokers

Genotoxic Biomarker		Cancer Subjects (Week 0)		Polyp Subjects (Week 0)		P value
PBMC	(TI)	5.631 ± 0.662	(n=19)	8.890 ± 1.328	(n=19)	0.0346
PBMC	(TM)	1.414 ± 0.222	(n=19)	2.376 ± 0.435	(n=19)	0.0566
		Cancer Subjects (All Times)		Polyp Subjects (All Times)		
PBMC	(TI)	5.840 ± 0.504	(n=19)	7.600 ± 0.725	(n=19)	0.0490
PBMC	(TM)	1.447 ± 0.153	(n=19)	1.964 ± 0.227	(n=19)	0.0615

Discussion

Cancer arises from an accumulation of mutations in a single cell and is often referred to as a disease of DNA. Mutations may arise as a result of errors in DNA replication or as a result of exposure to environmental mutagens such as ultra violet radiation and dietary mutagens. The colonic mucosa is constantly exposed to the contents of the gut lumen, which contains compounds that may be either damaging to or protective of the mucosa. DNA damage followed by ineffective repair leads to permanent mutations altering gene expression and activity.

Studies have shown that DNA damage is higher in the colon of rats fed diets containing high risk factors for colon cancer than in the colons of rats fed low risk factors and that lactic acid bacteria can inhibit DNA damage, (Pool-Zobel *et al.* 1996; Hambly *et al.* 1997; Zsivkovits *et al.* 2003).

In the current study we evaluated the influence of a synbiotic combination of *Lactobacillus rhamnosus* GG, *Bifidobacterium lactis* Bb12 and Synergy[®]1 on *in vivo* genotoxic damage by measuring DNA damage in biopsies and lymphocytes. In placebo cancer subjects DNA damage in biopsies was significantly increased after the twelve-week intervention period but this increase was not observed in the synbiotic consuming cancer subjects. Similar results were seen when subjects who smoke were excluded from the analysis. These results indicate that in cancer subjects synbiotic consumption is protective against DNA damage in the colonic mucosa. This protection may be due to binding of genotoxic substances in the gut lumen by probiotic bacteria preventing these substances from coming in contact with the colonic mucosa. Interestingly when both smokers and females were excluded from

analysis there was a trend towards increased DNA damage in the placebo cancer group but this increase did not reach significance. This suggests that the colonic mucosa of females is more susceptible to genotoxic damage as the most significant increase was observed when females were included in the analysis. This gender difference is not unexpected and studies have shown gender related differences in genotoxic damage. The number of females in this study, however, was small and further work is needed to confirm this observation.

In polyp subjects genotoxic damage was significantly decreased following synbiotic consumption, however there was also a decrease although less pronounced in the placebo group. It may be that *in vivo* genotoxic damage in polypectomised subjects is reduced over time and intervention with synbiotics may hasten this reduction. In contrast when females and smokers were excluded from analysis there was no change in DNA damage in placebo subjects but there was a trend towards reduction in the synbiotic subjects but this reduction was not as significant as when smokers and females were included in the analysis. This suggests that the level of DNA damage decreases over time at a greater rate in female polyp subjects than in male polyp subjects. This result is in contrast to the cancer subjects where female subjects were more susceptible to DNA damage. It may be that the level or activity of genotoxins to which the colonic mucosa is exposed is different in female cancer and female polyp subjects, however a larger scale study would be required to investigate if this is true.

The initial level of genotoxic damage in biopsies from cancer and polyp subjects was also analysed. There was no difference in DNA damage in biopsies from cancer

subjects compared to polyp subjects at the beginning of intervention. Interestingly DNA damage in biopsies from cancer subjects was significantly higher than in polyp subjects at the end of intervention. This result indicates that the increase in DNA damage in the colonic mucosa of cancer subjects over time is significantly greater than that observed in polyp subjects. A similar result was observed when smokers were excluded from the analysis. However when smoking subjects and females were excluded there was no difference in DNA damage in biopsies from male non-smoking cancer and polyp subjects indicating the colonic mucosa of females is more susceptible to DNA damage than the mucosa of males.

We also investigated the influence of synbiotic consumption on the genotoxic potential of FW. Diets considered high risk for colon cancer have been reported to increase the genotoxic potential of FW in healthy volunteers (Rieger *et al.* 1999). In the current study there was no effect of synbiotic intervention on the genotoxic potential of FW during the twelve-week intervention period. However FW derived from the placebo group of polyp subjects increased DNA damage in HT29clone19a cells during the twelve-week intervention period and this increase was most pronounced in the male non-smoking subjects. In the synbiotic group of polyp subjects there was no change in the genotoxic potential of FW suggesting that synbiotic consumption prevented the increase in genotoxicity seen in the placebo group of subjects. This result suggests that synbiotic consumption in polyp subjects modulates the genotoxic potential of FW and that this effect is most pronounced in male non-smoking subjects.

The genotoxic potential of FW derived from cancer and polyp subjects at the beginning of intervention and at all time points was analysed. There was no significant difference in the genotoxic potential of FW derived from cancer subjects compared to polyp subjects. We would have expected FW genotoxicity to be higher in cancer subjects as these subjects had significantly more DNA damage in the colonic mucosa than polyp subjects. There are a number of possible explanations for this result. It may be that *in vivo* DNA damage in the cancer subjects was caused by factors other than those in the FW. It may also be that the colonic mucosa of cancer subjects is more susceptible to genotoxic damage than the mucosa of polyp subjects. Further large scale studies investigating the relationship between *in vivo* DNA damage and the genotoxic potential of FW are needed to determine if FW genotoxicity is useful as a biomarker of cancer risk.

Dietary intervention has been reported to modulate genotoxic damage in lymphocytes (Pool-Zobel *et al.* 1997). In the current study genotoxic damage in PBMCs was unaltered during the twelve-week intervention period in cancer subjects. In contrast, DNA damage in PBMCs was significantly reduced at week 12 compared to week 6 in the synbiotic groups of polyp subjects and in non-smoking polyp subjects. In male non-smoking polyp synbiotic subjects DNA damage was significantly decreased at week 12 compared to week 0. Interestingly, DNA damage was significantly reduced at the end of the intervention period in all placebo polyp and in non-smoking placebo polyp subjects. There was also a trend towards lower damage in the male non-smoking placebo polyp subjects but this decrease was not statistically significant. This reduction in DNA damage correlates with induction of GST activity (*chapter 4*). These results indicate that genotoxic damage in PBMCs

may normally decrease over time in polyp subjects, however in male non-smoking subjects synbiotic intervention hastens this decrease.

Comparison of DNA damage in PBMCs from cancer and polyp subjects revealed that there was more damage in PBMCs from polyp subjects than in PBMCs from cancer subjects. In contrast the level of DNA damage in the colonic mucosa of cancer subjects was significantly higher than in polyp subjects. Therefore in this study the level of DNA damage in the colon, was not mirrored by the level of damage in the PBMCs. It may be that there is a relationship between DNA damage in the colon and in the PBMCs and further studies to investigate this relationship should be performed.

In conclusion synbiotic consumption protects against *in vivo* genotoxic damage of the colonic mucosa in cancer and polyp subjects and reduces the genotoxic potential of FW derived from polyp subjects. In polyp subjects DNA damage in PBMCs decreases over time, however synbiotic consumption may positively influence this reduction in a subset of subjects e.g. male non-smokers. Cancer subjects have significantly higher DNA damage in the colonic mucosa and significantly lower damage in PBMCs than polyp subjects. These markers may prove useful as biomarkers of cancer risk, however large-scale studies comparing the levels of the markers in healthy and diseased states are needed. Gender, smoking status and genetic polymorphisms seem to play a role in susceptibility to genotoxic damage. Future studies should be designed and sufficient participants enrolled to control for such factors.

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Chapter 6

**Study of the effect(s) of synbiotic consumption
on other potential biomarkers for cancer**

Abstract

The normal gut flora has many beneficial effects on the host (e.g. vitamin synthesis) but is also involved in the metabolism of carcinogens ingested in the diet to both active and inactive compounds. Imbalance in the composition of the gastrointestinal flora has been associated with increased risk of colon cancer. Pre-, pro- and synbiotic consumption has been reported to alter the composition and the metabolic activity of the gut flora. Consumption of pre- and probiotics has also been reported to beneficially affect cholesterol levels in animals and humans. In this study we investigated the influence of synbiotic consumption on the composition of the faecal flora, the concentration of short chain fatty acids (SCFAs), faecal calprotectin, the cytotoxic potential of FW, and cholesterol levels. Lactobacilli, bifidobacteria and enterococci numbers were increased with decreases in the levels of *Clostridium perfringens* and coliforms being recorded following twelve weeks of synbiotic consumption. No effects on the levels of faecal calprotectin, FW cytotoxicity, SCFAs or cholesterol following synbiotic consumption were observed. Interestingly the initial numbers of faecal lactobacilli and enterococci were lower while the cytotoxic potential of FW and the concentration of propionic, isobutyric and isovaleric acids were significantly higher in cancer subjects than in polyp subjects at the beginning of the study. We conclude that there are differences in the composition and metabolic activity of the gastrointestinal flora of cancer and polyp subjects and that synbiotic consumption increases the numbers of beneficial bacteria. However in this study this increase in bacterial numbers was not associated with a change in SCFAs or the cytotoxic potential of FW. It may be that a longer period of intervention is required for such changes to occur.

Introduction

The gastrointestinal (GI) tract is home to a rich flora of greater than 500 different bacterial species the vast majority (<400 species) of which reside in the large intestine (colon). Obligate anaerobes predominate in the large intestine (99.9% of the total flora). Genera such as *Bacteroides*, *Eubacterium*, *Bifidobacterium* and *Peptostreptococcus* dominate at levels of approx. 10^{10} - 10^{11} cfu/g. Enterobacteriaceae, streptococci and lactobacilli are also present at levels of 10^8 cfu/g, (Naidu *et al.* 1999).

The normal gut flora has many beneficial effects on the host including vitamin synthesis, and improved absorption of minerals by the host e.g. calcium, magnesium and iron. The flora is also involved in the conversion of pro-drugs to their active form and the metabolism of carcinogens ingested in the diet to both active and inactive compounds. Imbalance in the composition of the GI flora has been associated with increased risk of colon cancer (Gorbach & Goldin 1990). Modulating the composition of the GI flora has been proposed as a possible mechanism for reduction of cancer risk.

The consumption of pre-, pro- and synbiotics has been reported to shift the composition of the gut flora to a more beneficial composition. Most studies in this area have shown that pre-, pro or synbiotic consumption increases bifidobacteria and lactobacilli numbers, (Kruse *et al.* 1999; Tuohy *et al.* 2001). However some studies have demonstrated that pre-, pro- or synbiotic consumption can also affect other members of the gut flora.

Spanhaak *et al.* (1998) demonstrated that consumption of a milk fermented by *Lactobacillus casei* strain *shirota* significantly increased lactobacilli and bifidobacteria and decreased the numbers of clostridium although not significantly. Gibson *et al.* (1995) demonstrated that consumption of the prebiotics, oligofructose and inulin, significantly increased the numbers of faecal bifidobacteria, while decreasing the numbers of bacteroides, clostridia, fusobacteria and Gram positive cocci. The protective role of pre-, pro-, and synbiotics against colon carcinogenesis has been demonstrated in animal studies (Femia *et al.* 2002; Gallaher & Khil 1999; Goldin *et al.* 1996).

The inflammatory protein calprotectin found in faeces is often used as a biomarker for inflammation. Elevated levels of faecal calprotectin have been found in patients with colonic polyps and colon cancer (Kristinsson *et al.* 1998; Roseth *et al.* 1993; Tibble *et al.* 2001) and active inflammatory bowel disease (Roseth *et al.* 1993; Tibble *et al.* 2000). Calprotectin is a calcium-binding protein found mainly in neutrophils. Immunohistochemical examination of colorectal cancer specimens has shown calprotectin reactivity confined to neutrophils with no reactivity in neoplastic cells, (Roseth *et al.* 1993; Tibble *et al.* 2000). The amount of calprotectin in faeces is dependent on the integrity of the colonic tight junctions. If tight junction integrity is impaired neutrophils containing calprotectin migrate from the blood stream through the leaky junctions and into the gut lumen. Berstad *et al.* (2000) demonstrated a correlation between increased intestinal permeability and calprotectin concentrations in gut lavage fluid of IBD patients.

Despite the growing acceptance of calprotectin as a marker of inflammation its' use as a risk marker for colon cancer is not yet fully proven and some studies have found no correlation between faecal calprotectin levels and carcinogenesis. A study performed by (Limburg *et al.* 2003) in colonoscopy patients with a personal history of colorectal neoplasia, family history of colorectal cancer, or iron deficiency anaemia found that faecal calprotectin levels did not differ significantly between subjects with, versus subjects without colorectal neoplasms. Poullis *et al.* (2004) reported a significant positive relationship between faecal calprotectin and cancer risk factors such as increasing age, obesity, physical inactivity, and an inverse relationship with fiber intake and vegetable consumption. Bruzzese *et al.* (2004) reported that, in children with either active inflammatory bowel disease or cystic fibrosis, consumption of *Lactobacillus rhamnosus* GG significantly reduced faecal calprotectin levels.

Many of the substances to which the colonic mucosa is exposed induce cytotoxic damage leading to cell death. Stimulation of proliferation in the colon is, in part, to compensate for the loss of cells due to cytotoxic mechanisms, an increase in mitotic activity could increase the risk of colon cancer. The cytotoxic potential of faecal water is now accepted as a risk marker for colon cancer (Lapre & Van der Meer 1992; Lipkin 1991) and *in vitro* faecal water cytotoxicity towards colon cells is often employed as a biomarker in intervention studies.

van Muster *et al.* (1994) demonstrated that the consumption of resistant starch (a prebiotic) significantly decreased proliferation in rectal biopsies and the cytotoxicity of faecal water in healthy volunteers. Glinghammar *et al.* (1997) demonstrated that a

shift from a dairy-product rich diet (low cancer risk) to a dairy product-free diet (high risk) increased cytotoxicity of FW from healthy volunteers; however the genotoxic potential of FW was unaltered. The exact mechanism by which dairy products lower cancer risk is unknown however it may be speculated that, in this study, the increase in cytotoxicity seen in volunteers on the dairy product free diet may have been due to the reduction of calcium in the diet.

Short-chain fatty acids (SCFAs) are the major end products of bacterial metabolism in the human large intestine. They are formed by bacterial fermentation of polysaccharides, oligosaccharides, proteins, peptides and glycoproteins (Cummings & Macfarlane 1991). SCFAs provide between 10-30% of basal metabolic requirements for liver cells, colonocytes and peripheral tissues (Dunne 2001). Acetate, propionate and butyrate are the principal SCFAs produced in the colon although formate, valerate, caproate, isobutyrate and isovalerate are produced in lesser amounts (Macfarlane & Macfarlane 2003). SCFAs contribute to normal large bowel function and prevent pathology through their actions in the lumen and on the colonic musculature and vasculature and through their metabolism by colonocytes (Topping & Clifton 2001). Segal *et al.* (1995) reported that total faecal SCFA concentration was significantly higher in healthy black South African subjects (lower CRC incidence) than in healthy white South African subjects (higher CRC incidence).

Butyrate has been associated with many biological properties in the colon and is thought to directly enhance cell proliferation in normal cells and suppress proliferation in transformed cells. In addition, apoptosis may be increased in transformed cells but inhibited in normal cells when butyrate is present (Hague *et al.*

1995; Hass *et al.* 1997; Marchetti *et al.* 1997). Butyrate has been reported to reduce hydrogen peroxide induced DNA damage in freshly isolated rat colon cells (Abrahamse *et al.* 1999) and human colonocytes (Rosignoli *et al.* 2001). Butyrate is known to reduce risk factors involved in the aetiology of colon cancer and adenoma development (Smith *et al.* 1998).

Emenaker *et al.* (2001) reported that incubation with acetate, propionate and butyrate reduced the invasiveness of cells isolated from surgical specimens of patients resected for invasive colorectal cancer and that this reduction seemed to be dependent on inhibition of urokinase plasminogen activator (uPA) and the stimulation of tissue inhibitor matrix metalloproteinase (TIMP)-1 and TIMP-2 protein activities. Acetate, propionate and butyrate also demonstrated the ability to modulate the expression of genes associated with cancer such as mutant p53, Bcl-2, bax, p21 and PCNA.

Probiotics have been shown to reduce cholesterol and triacylglycerol plasma concentrations, (Kiessling *et al.* 2002; Taranto *et al.* 1998). Cholesterol absorption is dependant on the solubilisation of cholesterol with bile acids. Probiotics are thought to influence cholesterol levels due to deconjugation of bile acids by bacterial enzymes (bile salt hydrolase) giving rise to deconjugated bile salts which are less soluble and less efficiently reabsorbed from the intestinal lumen than their conjugated counterparts, leading to larger amounts of free bile acids being excreted in the faeces. Also, free bile salts are less efficient in the solubilisation and absorption of lipids in the gut. Therefore, the deconjugation of bile acids by probiotic bacteria could lead to a reduction in serum cholesterol either by increasing the

demand of cholesterol for de novo synthesis of bile acids to replace that lost in faeces or by reducing cholesterol solubility and, thereby, absorption of cholesterol throughout the intestinal lumen. Gilliland *et al.* (1985) reported a significant relationship between cholesterol assimilation by lactobacilli and their degree of bile deconjugation.

A meta-analysis performed by Agerholm-Larsen *et al.* (2000) found that probiotic intervention significantly decreased total and LDL cholesterol. du Toit *et al.* (1998) performed a feeding trial with a mix of three lactobacilli strains in minipigs and found that serum cholesterol levels were decreased three weeks after beginning intervention. The effects of consumption of probiotics and prebiotics on serum lipid levels in humans was reviewed by Pereira & Gibson (2002). They concluded that in hyperlipidemic subjects probiotic consumption reduces total cholesterol levels, whereas in normal subjects, effects on serum triglycerides are the dominant feature.

The aims of this study were:

- 1) To investigate if synbiotic consumption can alter the composition of the gastrointestinal flora.
- 2) To determine if synbiotic consumption alters the physiochemical environment of the gut lumen e.g. short-chain fatty acids.
- 3) To determine if synbiotic consumption protects against damage and inflammation of the colonic mucosa e.g. cytotoxic potential of FW and calprotectin expression.
- 4) To investigate if synbiotic consumption can influence cholesterol levels.

Table 1: Selective media employed for the culture of different bacteria

Bacteria	Agar	Supplement
Lactobacilli	Rogosa Agar	1.32ml/L of glacial acetic acid
Bifidobacteria	MRS Agar	0.2% lithium chloride 0.3% sodium propionate 0.05% cysteine hydrochloride 5% sheeps blood
Enterococci	KF Streptococcus Agar	10ml/L 1% TTC, (2,3,5-Triphenyltetrazolium chloride)
Clostridium perfringens	OPSP Agar	Perfringens A Selective Supplement, SR076E (Oxoid) Perfringens B Selective Supplement, SR077E (Oxoid)
Coliforms	MacConkey agar No.3	
Bacteroides	Wilkins & Chalgren Agar	Anaerobic supplement SR108 (Oxoid) 5% horse blood

Methods

Work described in this chapter was conducted in collaboration with Pernilla Karlsson and Prof. Joseph Rafter of the Department of Medical Nutrition, Karolinska Institutet, Huddinge, Sweden, Dr Jan Van Loo and Dr Nadine Jonkers of Orafit, Tienen, Belgium and Dr Tony Fitzgerald of the Biochemistry Department, The Mercy University Hospital, Cork.

Faecal Flora Analysis

Subjects provided faecal samples at the beginning (week 0), mid way through (week 6) and at the end of intervention (week 12). Total stool from one passage was collected in a plastic container and mixed using a sterile wooden spatula. An aliquot was used to make a faecal slurry with sterile 60% PBS / 40% Glycerol / 0.05% cysteine. Faecal slurries were stored at -80°C until required.

Faecal slurries were thawed at room temperature, serially diluted 10-fold in sterile PBS / 0.05% cysteine and either spread-plated or pour-plated in appropriate dilutions on suitable media (*Table 1*).

Plates were incubated in gas pak jars (BBL) with CO_2 generating kits (Anaerocult A; Merck), for 48 to 72 hrs at 37°C . Mac Conkey agar No. 3 and OPSP plates were incubated for 18-24 hours at 37°C . Colony identities were confirmed by Gram stain. Microbial numbers were calculated as colony forming units per g of wet weight of faeces. The identity of colonies on MRS blood plates however was further investigated as this medium is not fully selective for Bifidobacteria.

Bifidobacteria are characterized by a unique hexose metabolism that occurs via a phosphoketolase pathway often termed the 'bifid shunt'. Fructose-6-phosphate phosphoketolase (F6PPK) is a key enzyme of the 'bifid shunt' and its presence is the most common diagnostic test for this genus, as it is not present in other Gram-positive intestinal bacteria.

Representative colonies were selected from MRS blood plates and inoculated into MRS broth supplemented with 0.05% cysteine and grown anaerobically for 48hrs at 37°C. 1ml of turbid culture was centrifuged at 1700 x g for 5 mins. The pellet was washed twice in 1ml of 0.05M phosphate buffer with 0.05% cysteine HCL, pH6.5 (0.5% PB/HCL). The washed pellet was resuspended in 200µl of 0.5% PB/HCL, and 0.25% Triton X-100, mixed vigorously for two minutes to induce cell lysis, and incubated at room temperature for 10mins. Following incubation 50µl of a sodium fluoride (6mg/ml) / sodium iodoacetate (10mg/ml) solution and 50µl of a fructose-6-phosphate solution (80mg/ml) were added. Samples were mixed on a vortex and incubated for 1hour at 37°C. 300µl of 13.9% (w/v) hydroxylamine HCL (freshly neutralized with NaOH to pH 6.5) was added, followed by incubation for 10mins at room temperature. Finally 200µl of 15% trichloroacetic acid, 200µl of 4M HCL, and 200µl of 5% FeCl₃.6H₂O in 0.1M HCL was added. Immediate development of a purple colour indicated a positive result for the presence of F6PPK. A yellow colour indicates a negative result. Only colonies with the same colony morphology as the F6PPK positive colonies were counted as Bifidobacteria.

Calprotectin

Frozen faecal samples were thawed at room temperature. Approximately 100 mg (40-120 mg) faecal sample was collected with a disposable inoculation loop and placed into a 15 ml screw cap tube. The weight of the faeces was recorded and the loop handle was broken off, leaving the loop with faeces and 4-6 cm of the handle inside the tube (to act as agitator). Pre-diluted extraction buffer (Nycomed, Norway) containing urea and citrate was added in a weight/volume ratio of 1:50. The tubes were mixed on a vortex for 30 sec followed by homogenisation for 30 min at 1400 rpm on a shaker (Ika-Vibrax-VXR, IKA). 1 ml of the homogenate was transferred to a microcentrifuge tube (Eppendorf) and centrifuged for 20 min at 10000 x g. After centrifugation 0.5 ml of the supernatant was transferred to a new microcentrifuge tube and analysed immediately or stored at -20°C until analysis.

The calprotectin concentrations of faecal supernatants were assessed by a calprotectin enzyme linked immunosorbent assay (Oslo, Norge). Standards and positive controls supplied in the kit were included in each run of the assay according to manufacturer's instructions. The faecal supernatants were diluted 1:50 in assay buffer (0.15M NaCl, 0.5mM MgCl₂, 50mM TRIS, 2.5mM KCl, 0.25 mM Merthiolate, 0.05% Tween 20, pH 8, supplemented with 10g/L bovine serum albumin) and 100µl of each sample was added in duplicate to cells of a 96 well microtiter plate that had previously been coated with polyclonal antibody to calprotectin.

The plate was then incubated at room temperature for 45mins followed by washing four times with washing solution (0.15M NaCl, 0.5mM MgCl₂, 50mM Tris, 2.5mM

KCl, 0.25 mM merthiolate, 0.05% Tween 20, pH 8). 100µl conjugate (purified rabbit anti-human calprotectin IgG antibodies conjugated with alkaline phosphatase) were added to each well and the plate was incubated for 45mins at room temperature. At the end of the incubation period the plate was washed four times with washing solution as above and 100µl of substrate solution (p-nitrophenylphosphate dissolved in 1% diethanol amine, MgCl₂ 0.01g/L, merthiolate 0.01 g/L, pH 9.5) were added to each well.

After 15 min incubation in the dark at room temperature the optical density was read at 405 nm in a spectrophotometer. The calprotectin concentrations in the faecal samples were calculated from the standard curve obtained with the standards of known concentration. The concentrations of extracted calprotectin were expressed as µg/g of stool.

Cytotoxicity

The Alamar Blue cytotoxicity kit (Alamar, USA) was used to examine cytotoxic effects of faecal water in cells. HT-29 cells (2.5×10^4 cells/well) were seeded in wells of 96-well plates and cultured for 36 hours at 37°C in a humidified atmosphere containing 5% CO₂ in air. Medium was removed and deoxycholic acid (DCA) and faecal water diluted (1:10, 1:20, and 1:30) in 0.1% Dulbecco's Modified Eagle Medium (DMEM) and each sample was added to eight wells of the 96 well plate. After 24 hours, the test agents were removed and a 10% solution of Alamar Blue dye was added. The cells were incubated for 3 hours with Alamar Blue, thereafter the colour change was measured spectrophotometrically at 600 and 570 nm

(SPECTRAmax 250, Molecular Devices Corporation). The mean absorbance of each well was calculated and % cell death was expressed as:

$$100\% - \left(\frac{\text{absorbance in sample well}}{\text{maximal absorbance in DMEM well (negative control)}} \times 100 \right) ,$$

Faecal Short-Chain Fatty Acids (SCFAs)

Faecal samples were obtained at week 0, week 6 and week 12 and stored at -80°C until analysed. Faecal SCFAs were determined using gas chromatography and flame ionisation detection (GC VEGA 6000, Carlo Elba, Milano, Italy).

Faecal samples were weighed and diluted with an internal standard solution (2-methyl valeric acid, 0.01%). The solution was acidified with 0.5 ml H_2SO_4 (pH 2), and SCFAs were extracted by shaking with 2.0 ml diethylether and subsequent centrifugation (10 min at 600 x g). The ether phase was injected directly onto a pre-heated (150°C) column (BT21-FFAPP 25 m x 0.53 mm x 0.5 mm, Achrom NV/SA, Belgium), using N_2 as the carrier gas and the detection temperature was set at 230°C . Peaks were integrated using the Atlas software (ThermoLab Systems, The Netherlands).

Cholesterol

Blood was collected in serum-gel blood collection tubes (Sarstedt). The tubes were centrifuged at 300 x g for 5mins and the upper serum layer was removed, aliquoted and stored at -80°C until analysed. Serum total cholesterol, HDL cholesterol, and triglycerides were determined enzymatically on an Olympus Chemistry- Immuno Analyzer in the Biochemistry Department, Mercy University Hospital. LDL cholesterol was calculated from the Friedewald formula, (Friedewald *et al.* 1972).

Results

Faecal Flora Analysis

Subjects provided faecal samples at the beginning (week 0), mid way through (week 6) and at the end of intervention (week 12). The composition of the faecal flora was assessed using standard plate count methods.

Synbiotic consumption modified the composition of the faecal flora. In cancer subjects consuming synbiotic (n=19) there was a trend towards increase in the numbers of lactobacilli (p=0.0674) and enterococci (p=0.0558) although this increase did not reach significance. Bifidobacteria were significantly increased at weeks 6 (p=0.0045) and 12 (p=0.0009) compared to week 0. The numbers of coliforms were significantly lower (p=0.0262) at week 12 compared to week 0 while there was no change in the numbers of *Clostridium perfringens* and bacteroides (Figure 1).

In contrast the numbers of lactobacilli, enterococci, coliforms, *Clostridium perfringens* and bacteroides in cancer subjects consuming placebo (n=14) were not altered during the twelve-week intervention period. There was however a significant reduction in the numbers of bifidobacteria at weeks 6 (p=0.0093) and 12 (p=0.0046) compared to week 0 (Figure 2).

Similarly synbiotic consumption in polyp subjects (n=19) altered the composition of the gut flora. The numbers of lactobacilli at week 12 (p=0.0204) and bifidobacteria at weeks 6 (p=0.0143) and 12 (p<0.0001) were significantly increased compared to week 0. *Clostridium perfringens* were significantly decreased at week 12 (p=0.0303)

and coliforms at week 6 ($p=0.0410$) compared to week 0. The numbers of enterococci and bacteroides were unaltered (*Figure 3*).

The faecal flora of polyp subjects consuming placebo ($n=18$) was not significantly altered during the twelve-week intervention period. There was however a trend towards higher numbers of bacteroides at week 12 compared to week 0 (*Figure 4*).

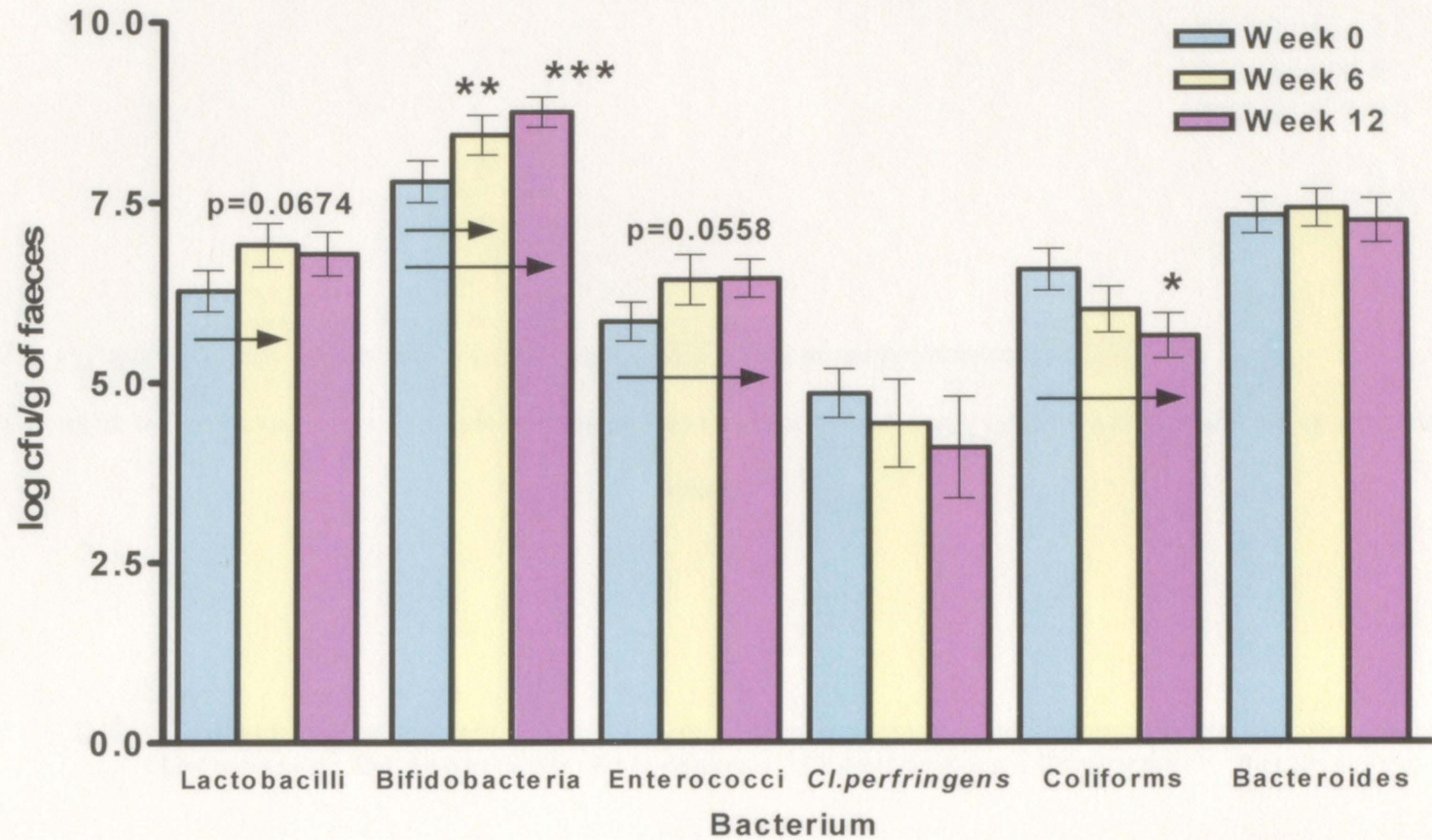
Initial bacterial numbers in cancer (synbiotic & placebo) subjects and polyp (synbiotic & placebo) subjects at week 0 were analysed (*Table 2*). Lactobacilli ($p=0.0401$) and enterococci ($p=0.0029$) levels were significantly lower in cancer subjects compared to polyp subjects. Bifidobacteria, *Clostridium perfringens*, coliforms and bacteroides levels were not significantly different.

Figure 1

Faecal flora composition of cancer subjects consuming synbiotic (n=19) at the beginning (week 0), mid-way through (week 6) and the end of intervention (week 12). Bifidobacteria levels at weeks 6 and 12, lactobacilli levels at week 6 and enterococci levels at week 12 were increased while coliforms decreased compared to week 0.

Figure 1

Synbiotic Cancer Subjects



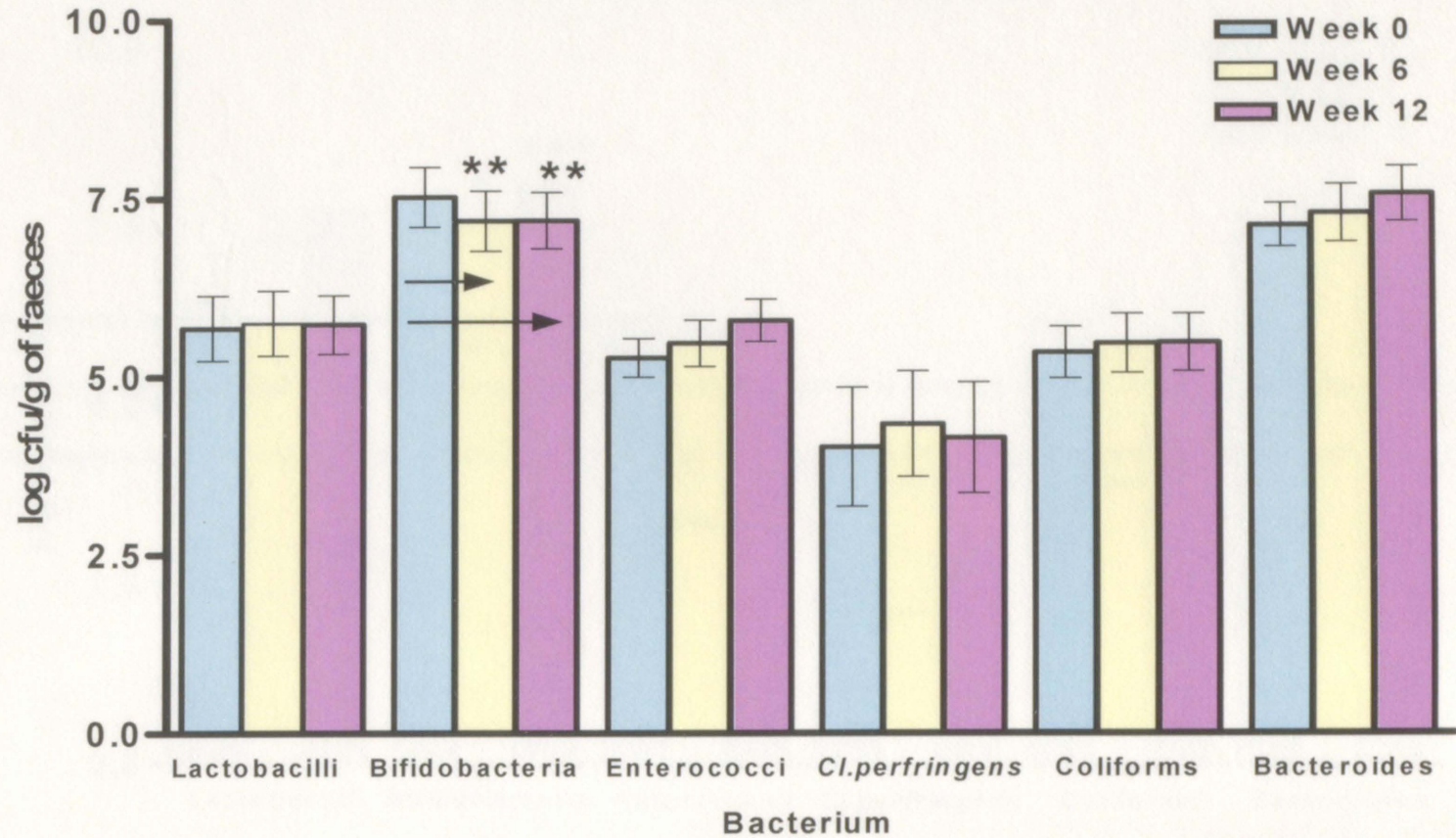
* $P=0.01$ to 0.05 ** $P=0.001$ to 0.01 *** $P<0.0001$

Figure 2

Faecal flora composition of cancer subjects consuming placebo (n=14) at the beginning (week 0), mid-way through (week 6) and the end of intervention (week 12). Bifidobacteria levels at weeks 6 and 12 were significantly decreased compared to week 0.

Figure 2

Placebo Cancer Subjects



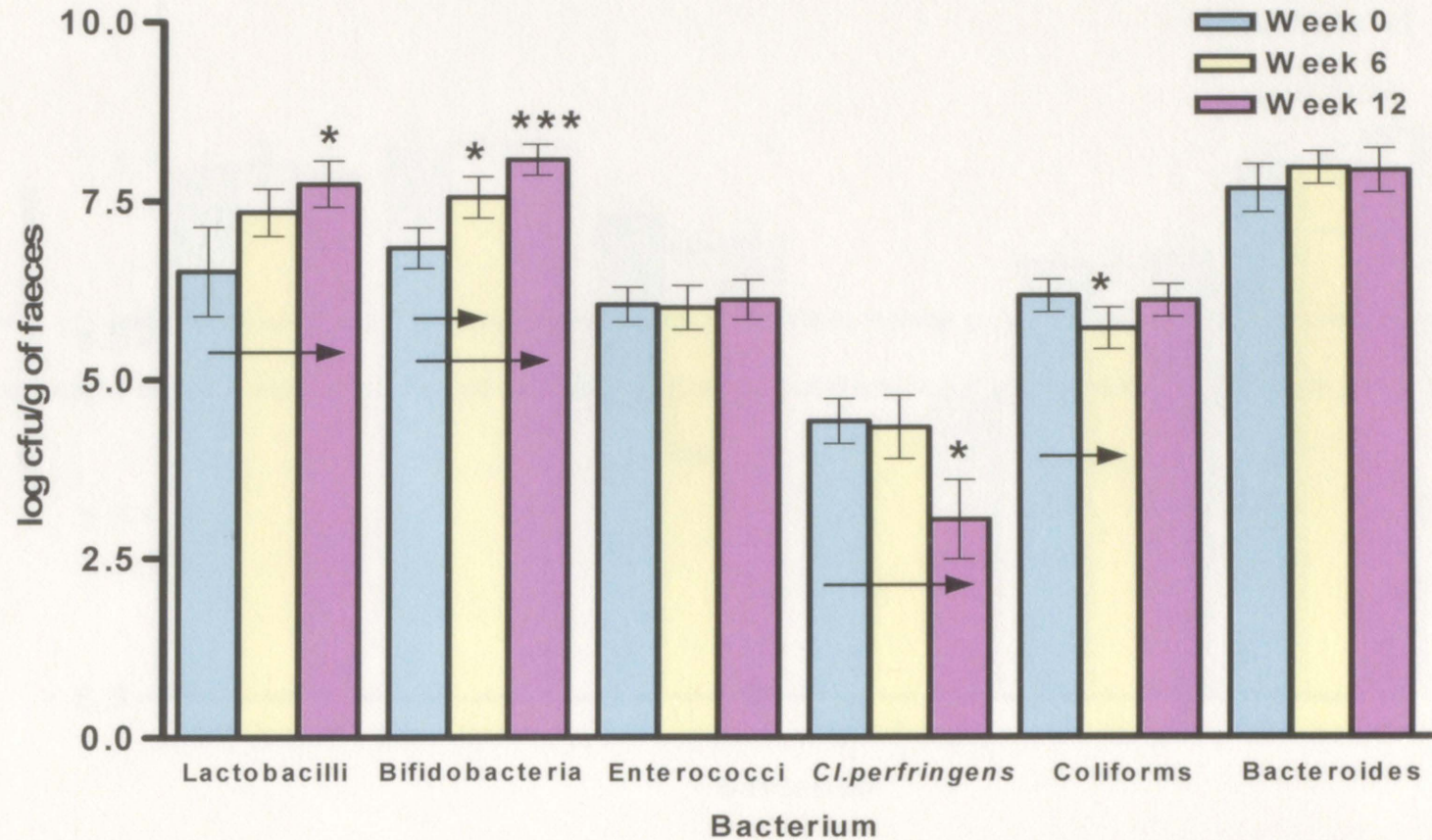
* P=0.01 to 0.05 ** P=0.001 to 0.01 *** P<0.0001

Figure 3

Faecal flora composition of polyp subjects consuming synbiotic (n=19) at the beginning (week 0), mid-way through (week 6) and the end of intervention (week 12). Bifidobacteria levels at weeks 6 and 12 and lactobacilli levels at week 12 were increased while *Clostridium perfringens* at week 12 and coliforms at week 6 were decreased compared to week 0.

Figure 3

Synbiotic Polyp Subjects



* P=0.01 to 0.05 ** P=0.001 to 0.01 *** P<0.0001

Figure 4

Faecal flora composition of polyp subjects consuming placebo (n=18) at the beginning (week 0), mid-way through (week 6) and the end of intervention (week 12). Bacteroides were significantly increased at week 12 compared to week 0. Other members of the flora were unaltered.

Figure 4

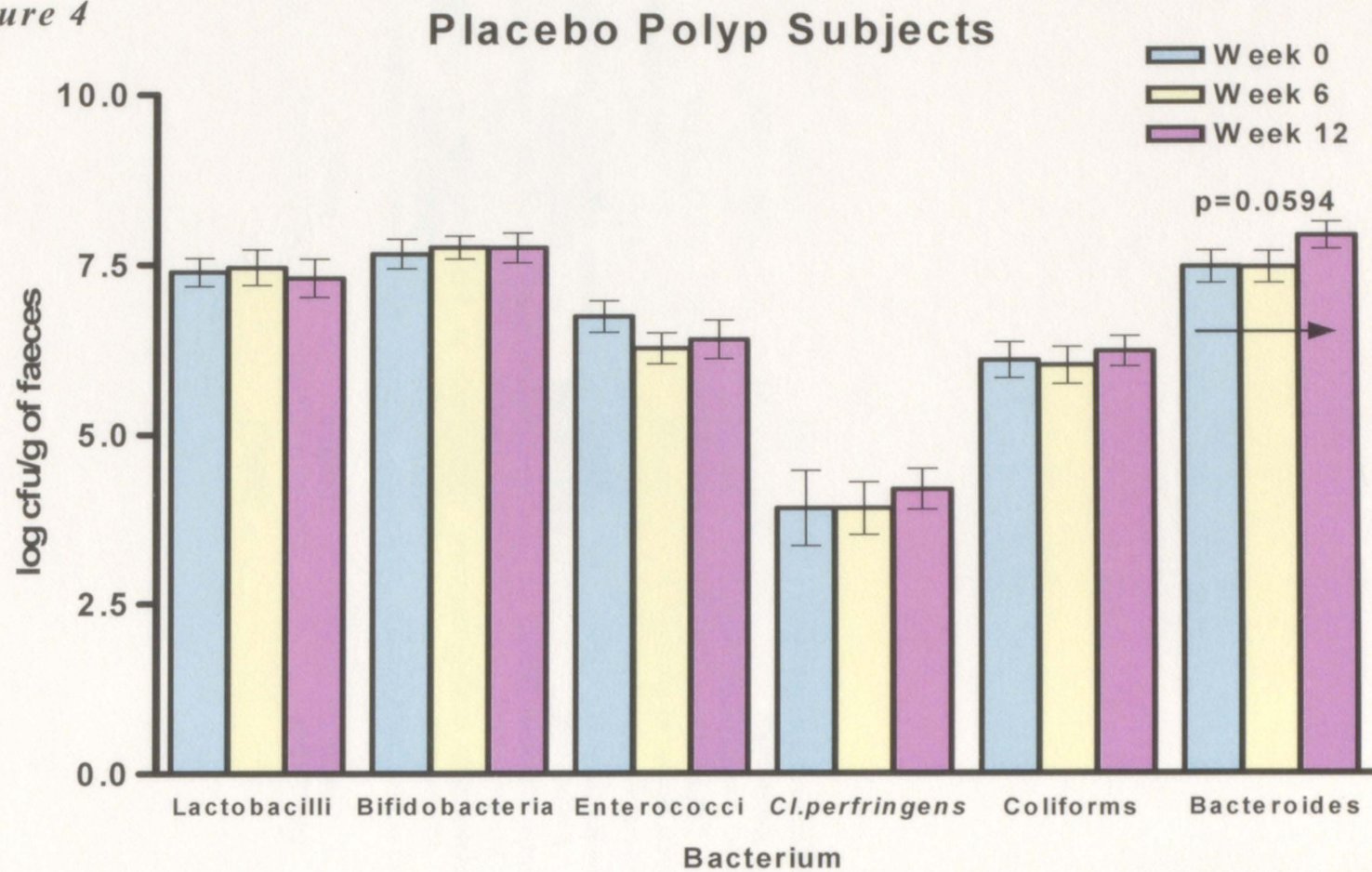


Table 2

Comparison of faecal flora composition in all cancer subjects compared to all polyp subjects at the beginning of intervention. Lactobacilli and enterococci were significantly higher in polyp subjects compared to cancer subjects. Results are expressed as mean $\pm$ standard error. Statistical significance was assessed using an un-paired t-test.

Table 2			
Biomarker (units)	Cancer Subjects Week 0	Polyp Subjects Week 0	P value
Faecal Flora			
Lactobacilli (cfu/g wet weight of faeces)	6.059 ± 0.248 (n=30)	5.956 ± 0.331 (n=36)	0.0401
Bifidobacteria (cfu/g wet weight of faeces)	7.676 ± 0.244 (n=32)	7.243 ± 0.193 (n=37)	n.s.
Enterococci (cfu/g wet weight of faeces)	5.588 ± 0.190 (n=32)	6.407 ± 0.171 (n=40)	0.0029
<i>Clostridium perfringens</i> (cfu/g wet weight of faeces)	4.561 ± 0.360 (n=21)	4.133 ± 0.337 (n=37)	n.s.
Coliforms (cfu/g wet weight of faeces)	6.024 ± 0.251 (n=32)	6.123 ± 0.176 (n= 39)	n.s.
Bacteroides (cfu/g wet weight of faeces)	7.232 ± 0.193 (n=32)	7.558 ± 0.200 (n=39)	n.s.

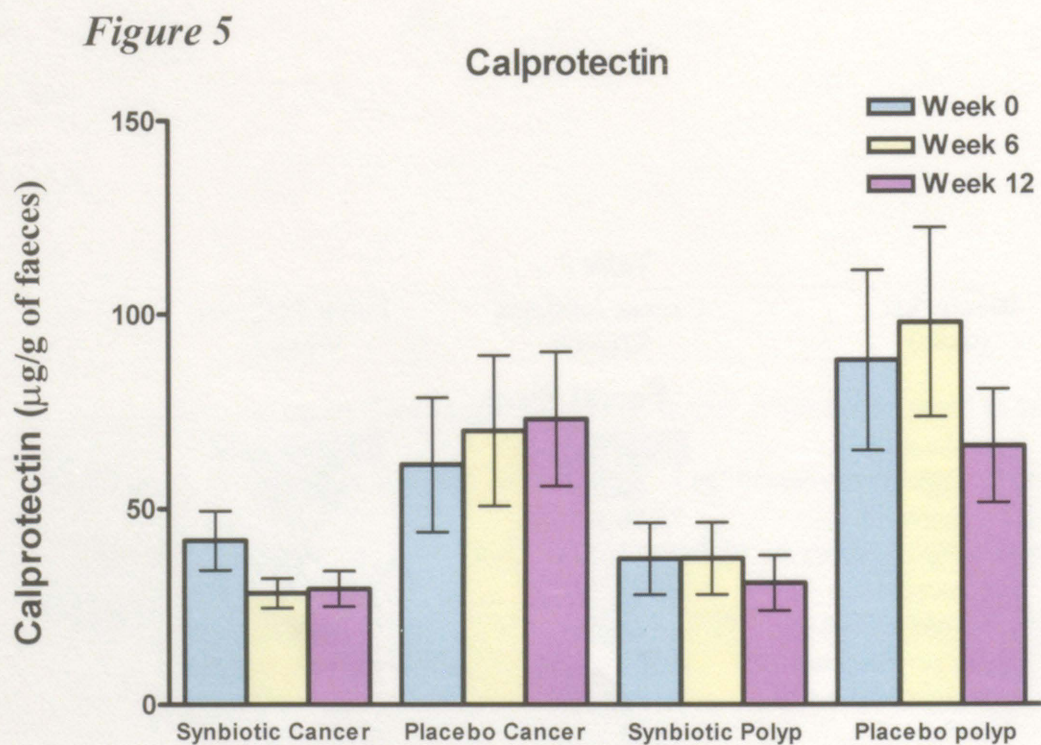


Figure 5: Faecal calprotectin levels at the beginning of intervention (week 0), mid- way through intervention (week 6) and the end of intervention (week 12) in cancer synbiotic (n=18) and placebo subjects (n=14) and polyp synbiotic (n=19) and placebo (n=21) subjects. Results are expressed as mean $\pm$ standard error.

Calprotectin

Faecal calprotectin levels were assessed by ELISA at weeks 0, 6 and 12. There was no significant change in faecal calprotectin levels in cancer or polyp subjects , subjects during the twelve week intervention period (*Figure 5*).

Initial calprotectin levels in the cancer (synbiotic & placebo) subjects and polyp (synbiotic & placebo) subjects at week 0 were not significantly different (*Table 4*). Results are expressed as mean $\pm$ standard error. Statistical significance was assessed using an un-paired t-test.

Table 4

	Cancer Subjects	Polyp Subjects	P value
	Week 0	Week 0	
Calprotectin	48.40 $\pm$ 7.76	65.29 $\pm$ 12.96	n.s.
(μ g/g of faeces)	(n=36)	(n=43)	

Figure 6

Cytotoxic Potential of Faecal Water

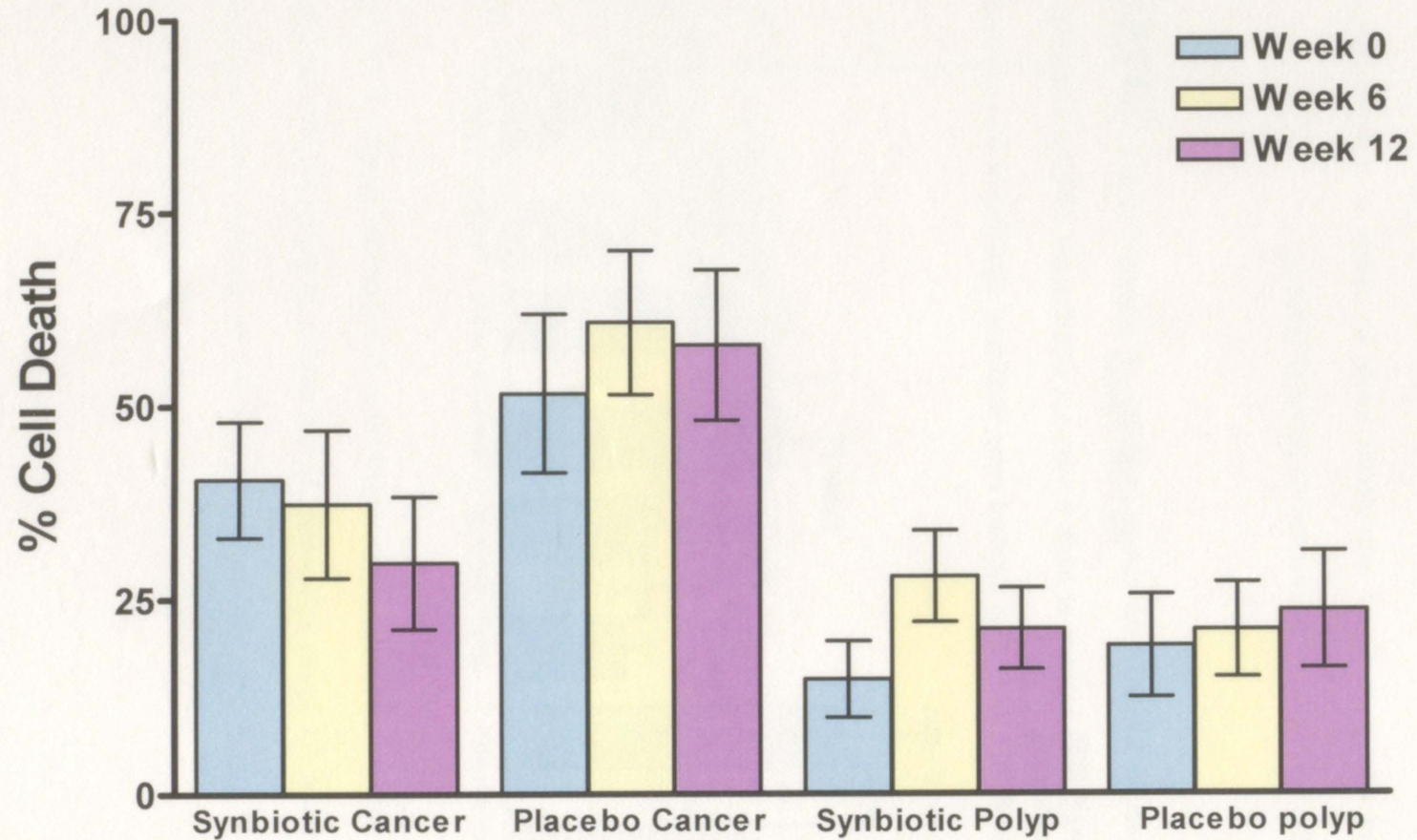


Figure 6: Percentage cell death following incubation with FW derived from cancer and polyp synbiotic and placebo subjects at the beginning (week 0), mid-way through (week 6) and the end of intervention (week 12). Results are expressed as mean $\pm$ standard error.

Cytotoxicity

FW was isolated from faecal samples at the beginning (week 0), mid-way (week 6) and the end of intervention (week 12). The cytotoxic effect of FW in HT-29 cells was assessed using the Alamar Blue cytotoxicity kit. The cytotoxic potential of FW (% cell death) was unaltered in the cancer synbiotic (n=16) and placebo subjects (n=12) and in the polyp synbiotic (n=16) and placebo (n=18) subjects during the twelve week intervention period (*Figure 6*).

The cytotoxicity of FW derived from cancer (synbiotic & placebo) and polyp (synbiotic & placebo) subjects at the beginning of intervention (week 0) was analysed (*Table 5*). The percentage cell death was significantly higher following incubation with FW derived from cancer subjects compared to FW derived from polyp subjects indicating the FW from cancer subjects was more cytotoxic than FW from polyp subjects. Results are expressed as mean ± standard error. Statistical significance was assessed using an un-paired t-test.

Table 5

	Cancer Subjects Week 0	Polyp Subjects Week 0	P value
Cytotoxicity (% cell death)	22.78 ± 5.66 (n=33)	18.86 ± 3.91 (n=37)	0.0004

Short-Chain Fatty Acids (SCFAs)

Faecal SCFAs were determined using gas chromatography and flame ionisation detection. In polyp subjects consuming both synbiotic (n=8) and placebo (n=11) , there was no significant change in the total SCFA levels or in the individual levels of acetic, propionic, isobutyric, butyric, isovaleric, valeric or hexonic acids during the twelve week period (*Tables 6(a) & (b)*).

SCFA levels were also unaltered in cancer subjects during the twelve-week period, however the numbers of subjects in this analysis were small (synbiotic n=3, placebo n=2) due to small sample size (*Tables 6(c) & (d)*).

Initial SCFA concentration in the cancer (synbiotic & placebo) subjects and polyp (synbiotic & placebo) subjects at week 0 were analysed (*Table 7*). The concentration of proprionic (p=0.0358), isobutyric (p=0.0113) and isovaleric (p=0.0161) acids in faeces was significantly higher in cancer subjects compared to polyp subjects. In polyp subjects the concentration of acetic acid and total acids tended to be lower and hexonic acid tended to be higher compared to cancer subjects but the difference did not reach significance. However the statistical power of this analysis was limited due to the small number of subjects in the cancer group (n=5 cancer, n=19 polyp).

Table 6 (a)

Short chain fatty acid (SCFA) concentrations in faeces from synbiotic polyp subjects at the beginning (week 0), mid-way through (week 6) and the end of intervention (week 12). There was no effect of synbiotic consumption on SCFA concentrations. Results are expressed as mean $\pm$ standard error.

Table 6 (b)

Short chain fatty acid (SCFA) concentrations in faeces from placebo polyp subjects at the beginning (week 0), mid-way through (week 6) and the end of intervention (week 12). SCFA concentrations were unaltered during the twelve-week intervention period. Results are expressed as mean $\pm$ standard error.

Table 6(a): SCFA

SCFA (mg / g)	Synbiotic Polyp Subjects (n=8)			
	Week 0	Week 6	Week 12	P value
Acetic Acid	347.0 ± 70.52	374.0 ± 78.00	342.1 ± 75.32	n.s.
Propionic Acid	109.9 ± 23.54	137.5 ± 37.31	108.6 ± 23.23	n.s.
Isobutyric Acid	16.81 ± 2.37	19.91 ± 3.82	14.10 ± 2.40	n.s.
Butyric Acid	195.3 ± 65.73	192.3 ± 61.78	190.9 ± 59.40	n.s.
Isovaleric Acid	26.75 ± 3.56	31.75 ± 6.69	23.98 ± 4.06	n.s.
Valeric Acid	22.69 ± 4.85	18.95 ± 6.46	19.50 ± 6.14	n.s.
Hexonic Acid	3.62 ± 1.66	3.81 ± 1.86	6.40 ± 4.38	n.s.
Total Acids	722.0 ± 158.6	778.2 ± 181.2	705.6 ± 161.4	n.s.

Table 6(b): SCFA

SCFA (mg / g)	Placebo Polyp Subjects (n=11)			
	Week 0	Week 6	Week 12	P value
Acetic Acid	380.5 ± 55.87	396.1 ± 41.92	367.7 ± 48.36	n.s.
Propionic Acid	132.5 ± 22.47	136.9 ± 17.92	134.6 ± 19.89	n.s.
Isobutyric Acid	14.04 ± 0.99	16.22 ± 1.09	16.82 ± 2.00	n.s.
Butyric Acid	171.1 ± 45.70	167.4 ± 35.62	171.8 ± 33.13	n.s.
Isovaleric Acid	22.55 ± 1.85	25.64 ± 2.15	27.91 ± 3.48	n.s.
Valeric Acid	30.00 ± 4.48	31.36 ± 4.38	30.23 ± 5.12	n.s.
Hexonic Acid	6.46 ± 3.16	11.41 ± 8.02	5.55 ± 3.67	n.s.
Total Acids	757.0 ± 122.7	785.0 ± 95.48	754.7 ± 104.1	n.s.

Table 6 (c)

Short chain fatty acid (SCFA) concentrations in faeces from synbiotic cancer subjects at the beginning (week 0), mid-way through (week 6) and the end of intervention (week 12). There was no effect of synbiotic consumption on SCFA concentrations. Results are expressed as mean $\pm$ standard error.

Table 6 (d)

Short chain fatty acid (SCFA) concentrations in faeces from placebo cancer subjects at the beginning (week 0), mid-way through (week 6) and the end of intervention (week 12). SCFA concentrations were unaltered during the twelve-week intervention period. Results are expressed as mean $\pm$ standard error.

Table 6 (c): SCFA

SCFA (mg / g)	Synbiotic Cancer Subjects (n=3)			
	Week 0	Week 6	Week 12	P value
Acetic Acid	466.0 ± 74.10	323.7 ± 131.1	316.0 ± 92.65	n.s.
Propionic Acid	181.0 ± 79.00	109.3 ± 36.92	120.0 ± 44.31	n.s.
Isobutyric Acid	20.07 ± 5.80	12.10 ± 2.90	11.00 ± 2.89	n.s.
Butyric Acid	126.0 ± 71.53	127.6 ± 103.1	115.7 ± 86.8	n.s.
Isovaleric Acid	34.33 ± 11.41	18.90 ± 5.23	17.47 ± 6.00	n.s.
Valeric Acid	17.90 ± 7.90	11.90 ± 8.13	11.17 ± 7.54	n.s.
Hexonic Acid	1.230 ± 0.573	0.407 ± 0.139	0.548 ± 0.217	n.s.
Total Acids	846.5 ± 156.4	603.9 ± 274.6	591.8 ± 205.7	n.s.

Table 6 (d): SCFA

SCFA (mg / g)	Placebo Cancer Subjects (n=2)			
	Week 0	Week 6	Week 12	P value
Acetic Acid	577.5 ± 30.5	554.0 ± 123.0	507.0 ± 132.0	n.s.
Propionic Acid	260.5 ± 57.5	267.0 ± 82.0	246.0 ± 102.0	n.s.
Isobutyric Acid	28.00 ± 0.09	24.50 ± 9.50	28.50 ± 16.50	n.s.
Butyric Acid	240.5 ± 44.5	215.5 ± 9.5	193.5 ± 44.5	n.s.
Isovaleric Acid	40.50 ± 1.50	41.50 ± 12.50	48.00 ± 27.00	n.s.
Valeric Acid	58.50 ± 1.50	62.50 ± 5.50	43.00 ± 4.00	n.s.
Hexonic Acid	0.925 ± 0.575	1.060 ± 0.740	1.050 ± 0.050	n.s.
Total Acids	1206 ± 128	1166 ± 222	1067 ± 326	n.s.

Table 7

SCFA concentration in the cancer (synbiotic & placebo) subjects and polyp (synbiotic & placebo) subjects at the beginning of intervention (week 0). Propionic, isobutyric and isovaleric acids in faeces were significantly higher in cancer subjects compared to polyp subjects. Results are expressed as mean $\pm$ standard error. Statistical significance was assessed using an un-paired t-test.

Table 7

Short Chain Fatty Acids (units)	Cancer Subjects Week 0	Polyp Subjects Week 0	P value
Acetic Acid (mg/g of faeces)	510.6 ± 49.9 (n=5)	366.4 ± 42.8 (n=19)	n.s.
Propionic Acid (mg/g of faeces)	212.8 ± 50.2 (n=5)	122.9 ± 16.1 (n=19)	0.0358
Isobutyric Acid (mg/g of faeces)	13.24 ± 3.71 (n=5)	15.21 ± 1.16 (n=19)	0.0113
Butyric Acid (mg/g of faeces)	171.8 ± 50.2 (n=5)	181.3 ± 37.2 (n=19)	n.s.
Isovaleric Acid (mg/g of faeces)	26.80 ± 6.44 (n=5)	24.32 ± 1.83 (n=19)	0.0161
Valeric Acid (mg/g of faeces)	34.14 ± 10.86 (n=5)	26.92 ± 3.32 (n=19)	n.s.
Hexonic Acid (mg/g of faeces)	1.108 ± 0.370 (n=5)	5.265 ± 1.943 (n=19)	n.s.
Total Acids (mg/g of faeces)	990.5 ± 129.5 (n=5)	742.3 ± 94.8 (n=19)	n.s.

Cholesterol

There was no effect of synbiotic intervention on serum levels of total, HDL and LDL cholesterol and triglycerides. Total cholesterol levels were slightly, but not significantly above the normal level of <5.5 mmol/L in all subjects (*Table 8(a)*). HDL (*Table 8(b)*) and LDL (*Table 8(c)*) cholesterol levels were within the recommended normal range of <1.5 mmol/L and <4.0 mmol/L respectively for all subjects. Triglyceride levels were slightly elevated above the recommended normal level of <1.7 mmol/L (*Table 8(d)*).

Table 8(a)

Total cholesterol in synbiotic and placebo cancer and polyp subjects at the beginning (week 0), mid-way through (week 6) and the end of intervention (week 12). Total cholesterol levels were unaltered during the twelve-week intervention period. Normal levels are <5.5 mmol/L. Results are expressed as mean $\pm$ standard error.

Table 8(b)

HDL cholesterol in synbiotic and placebo cancer and polyp subjects at the beginning (week 0), mid-way through (week 6) and the end of intervention (week 12). HDL cholesterol levels were unaltered during the twelve-week intervention period. Normal levels are >1.5 mmol/L. Results are expressed as mean $\pm$ standard error.

Table 8(c)

LDL cholesterol in synbiotic and placebo cancer and polyp subjects at the beginning (week 0), mid-way through (week 6) and the end of intervention (week 12). LDL cholesterol levels were unaltered during the twelve-week intervention period. Normal levels are <4.0 mmol/L. Results are expressed as mean $\pm$ standard error.

Table 8(d)

Triglyceride levels in synbiotic and placebo cancer and polyp subjects at the beginning (week 0), mid-way through (week 6) and the end of intervention (week 12). Triglyceride levels were unaltered during the twelve-week intervention period. Normal levels are <1.7 mmol/L. Results are expressed as mean $\pm$ standard error.

Table 8(a): Total cholesterol (mmol/L)

Total Cholesterol	Week 0	Week 6	Week 12	P value
Synbiotic Cancer (n=14)	5.750 ± 0.378	5.829 ± 0.234	5.736 ± 0.245	n.s.
Placebo Cancer (n=12)	6.008 ± 0.405	5.817 ± 0.410	5.733 ± 0.340	n.s.
Synbiotic Polyp (n=18)	5.828 ± 0.201	5.900 ± 0.187	5.694 ± 0.171	n.s.
Placebo Polyp (n=21)	5.614 ± 0.247	5.729 ± 0.220	5.757 ± 0.211	n.s.

Table 8(b): HDL cholesterol (mmol/L)

HDL Cholesterol	Week 0	Week 6	Week 12	P value
Synbiotic Cancer (n=14)	1.279 ± 0.101	1.314 ± 0.103	1.321 ± 0.098	n.s.
Placebo Cancer (n=12)	1.458 ± 0.120	1.400 ± 0.097	1.408 ± 0.104	n.s.
Synbiotic Polyp (n=18)	1.350 ± 0.068	1.333 ± 0.076	1.356 ± 0.066	n.s.
Placebo Polyp (n=20)	1.315 ± 0.070	1.355 ± 0.068	1.360 ± 0.069	n.s.

Table 8(c): LDL cholesterol (mmol/L)

LDL Cholesterol	Week 0	Week 6	Week 12	P value
Synbiotic Cancer (n=13)	3.462 ± 0.39	3.523 ± 0.213	3.377 ± 0.273	n.s.
Placebo Cancer (n=9)	3.467 ± 0.480	3.511 ± 1.336	3.367 ± 0.340	n.s.
Synbiotic Polyp (n=17)	3.600 ± 0.177	3.665 ± 0.184	3.559 ± 0.152	n.s.
Placebo Polyp (n=19)	3.489 ± 0.201	3.384 ± 0.217	3.474 ± 0.221	n.s.

Table 8(d): Triglycerides (mmol/L)

Triglycerides	Week 0	Week 6	Week 12	P value
Synbiotic Cancer (n=14)	2.149 ± 0.354	2.244 ± 0.320	2.211 ± 0.277	n.s.
Placebo Cancer (n=12)	2.752 ± 0.458	2.372 ± 0.338	2.707 ± 0.485	n.s.
Synbiotic Polyp (n=17)	1.832 ± 0.253	1.954 ± 0.272	1.743 ± 0.236	n.s.
Placebo Polyp (n=20)	1.872 ± 0.265	2.376 ± 0.287	2.298 ± 0.501	n.s.

Discussion

The human gastrointestinal tract contains more than 500 different bacterial species, the greatest concentration being found in the colon. Imbalance in the gastrointestinal flora has been associated with increased risk of colon cancer (Gorbach & Goldin 1990). Many studies have shown that pre-, pro- and synbiotic consumption can modulate the gut flora, in general increasing the levels of beneficial bacteria such as bifidobacteria and lactobacilli and decreasing the levels of detrimental bacteria such as *C. perfringens* and coliforms. A protective role of pre-, pro-, and synbiotics against colon carcinogenesis has been demonstrated in animal studies (Femia *et al.* 2002; Gallaher & Khil 1999; Goldin *et al.* 1996) however studies in humans are limited.

In the current study we report that a synbiotic combination of *Lactobacillus rhamnosus* GG, *Bifidobacterium lactis* Bb12 and Synergy[®]1 modulated the flora of cancer and polyp subjects. Lactobacilli and bifidobacteria in cancer and polyp subjects and enterococci in cancer subjects were increased by synbiotic consumption with concomitant decreases in coliforms in cancer subjects and *C. perfringens* in polyp subjects. In the placebo group of cancer subjects there was a reduction in bifidobacteria the cause of which is unknown. These results correlate with other studies reporting increases in lactobacilli and bifidobacteria (Kruse *et al.* 1999; Tuohy *et al.* 2001) and decreases in clostridia and coliforms (Spanhaak *et al.* 1998), following probiotic consumption. We also compared the initial levels of faecal bacteria in cancer and polyp subjects and found that polyp subjects have significantly higher levels of lactobacilli and enterococci than cancer subjects.

Elevated levels of faecal calprotectin have been found in patients with colonic polyps and colon cancer (Kristinsson *et al.* 1998; Roseth *et al.* 1993; Tibble *et al.* 2001) and active inflammatory bowel disease (Roseth *et al.* 1993; Tibble *et al.* 2000). In this study there was no influence of synbiotic consumption on faecal calprotectin levels. This lack of effect may be due to the fact that calprotectin levels in our study group were not significantly elevated compared to the normal level of $<60 \mu\text{g/g}$. Calprotectin levels at the beginning of intervention were $48.40 \pm 7.76 \mu\text{g/g}$ for all cancer (synbiotic and placebo) and $65.29 \pm 12.96 \mu\text{g/g}$ for all polyp (synbiotic and placebo) and were not elevated compared to normal levels. This indicates that in this study subjects did not have active intestinal inflammation and therefore calprotectin levels were not elevated.

Studies have shown that subjects at high risk of colon cancer have a high mucosal proliferative activity as compared to controls. This increase in proliferative activity is to replace cells killed either by apoptosis or by cytotoxic damage. *In vitro* faecal water cytotoxicity towards colon cells is often employed as a biomarker in intervention studies. Studies have shown that dietary interventions such as consumption of prebiotics or shifting from dairy product rich to a dairy product free diet influences FW cytotoxicity (Glinghammar *et al.* 1997; van Muster *et al.* 1994). However in this study there was no significant effect of synbiotic intervention on the cytotoxic potential of FW derived from cancer or polyp subjects. Interestingly FW derived from cancer subjects was more cytotoxic than FW derived from polyp subjects suggesting that the colonic mucosa of cancer subjects is exposed to a higher level of cytotoxicity than the mucosa of polyp subjects which may lead to increased proliferative activity and increased cancer risk.

Short chain fatty acids (SCFAs) are produced from carbohydrate fermentation by colonic bacteria and are a major source of energy for liver cells, colonocytes and peripheral tissues. Low faecal SCFA concentrations have been associated with increased cancer risk (Segal *et al.* 1995). We have previously shown that consumption of the synbiotic combination employed in this study increase caecal butyrate in azoxymethane treated rats (Femia *et al.* 2002). In this study however synbiotic consumption did not influence the faecal levels of acetic, propionic, isobutyric, butyric, isovaleric, valeric, hexonic or total short chain fatty acids.

The concentration of propionic, isobutyric and isovaleric acids were significantly higher in cancer subjects compared to polyp subjects at the beginning of intervention. However the number of cancer subjects for which data is available is small and a larger study group would be required to confirm this result. Also >95% of SCFA are produced and absorbed in the colon and the use of faecal measurements may not be a true indication of SCFAs produced in the colon (Dunne 2001; Topping & Clifton 2001). Measurement of SCFAs in colonic contents sampled at endoscopy would provide a truer representation of the colonic SCFA profile.

One beneficial effect that has been suggested to result from human consumption of pre- and probiotics is a reduction in serum cholesterol levels, as suggested by the results of several human and animal studies. However results from human studies are not always consistent (Taylor & Williams 1998). In this study there was no effect of synbiotic intervention on serum levels of total, HDL and LDL cholesterol and triglycerides and although cholesterol levels were not optimal in our study group

levels were not significantly outside the normal range to warrant medical intervention.

In conclusion consumption of a synbiotic combination of *Lactobacillus rhamnosus* GG, *Bifidobacterium lactis* Bb12 and Synergy[®]1 modulated the flora of cancer and polyp subjects increasing the numbers of beneficial bacteria such as lactobacilli and bifidobacteria and reducing putrefactive bacteria such as coliforms and *C. perfringens*. This change in the composition of the gut flora did not alter the expression of faecal calprotectin, the concentration of faecal SCFAs, the cytotoxic potential of FW or serum cholesterol levels suggesting that these parameters may not be suitable biomarkers in an intervention trial of this type. However comparison of cancer vs. polyp subjects at the beginning of intervention showed that the number of lactobacilli and enterococci, were lower and the cytotoxic potential of FW and the concentration of propionic, isobutyric and isovaleric acids are significantly higher in cancer subjects than in polyp subjects. Further investigation of these parameters may help identify subjects at risk of cancer.

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Chapter 7

**Gene expression profiles of the colonic mucosa
in normal, cancer and polyp subjects and the
influence of synbiotic consumption on gene
expression**

Abstract

The progression from normal epithelium to adenoma, invasive carcinoma and metastasis is a multi-step process involving several genetic changes. Genes implicated in this process include those associated with proliferation, apoptosis and DNA repair. Studies of gene expression profiles of normal, adenoma and tumour tissues have identified genes which are differentially expressed in diseased states compared to normal. The aim of this study was to determine the gene expression profile in rectal biopsies from normal, cancer and polyp subjects and to identify genes which are highly expressed in the colonic mucosa. We also investigated the subsequent effect of synbiotic consumption on gene expression in cancer and polyp subjects. Using cDNA array analysis of 588 cancer associated genes we have identified 14 genes which are highly expressed in the colonic mucosa of normal, cancer and polyp subjects as well as genes which are highly expressed in subsets of subjects. We have also identified 30 genes, which are expressed at significantly different levels in cancer, polyp and normal subjects. Synbiotic consumption modulated the expression of genes associated with differentiation and tight junction integrity such as TEK, MT3, PDGFRA and protocadherin 1 and may indicate potential mechanisms by which synbiotics reduce cancer risk by modulating intestinal barrier integrity and cytoprotective gene expression. Further analysis of expression profiles of these genes in a larger number of subjects over a number of time-points are needed to confirm the highly and differentially expressed genes identified in this study as well as the influence of synbiotic consumption on gene expression and will determine if analysis of these genes are useful as markers of cancer risk or as intervention markers.

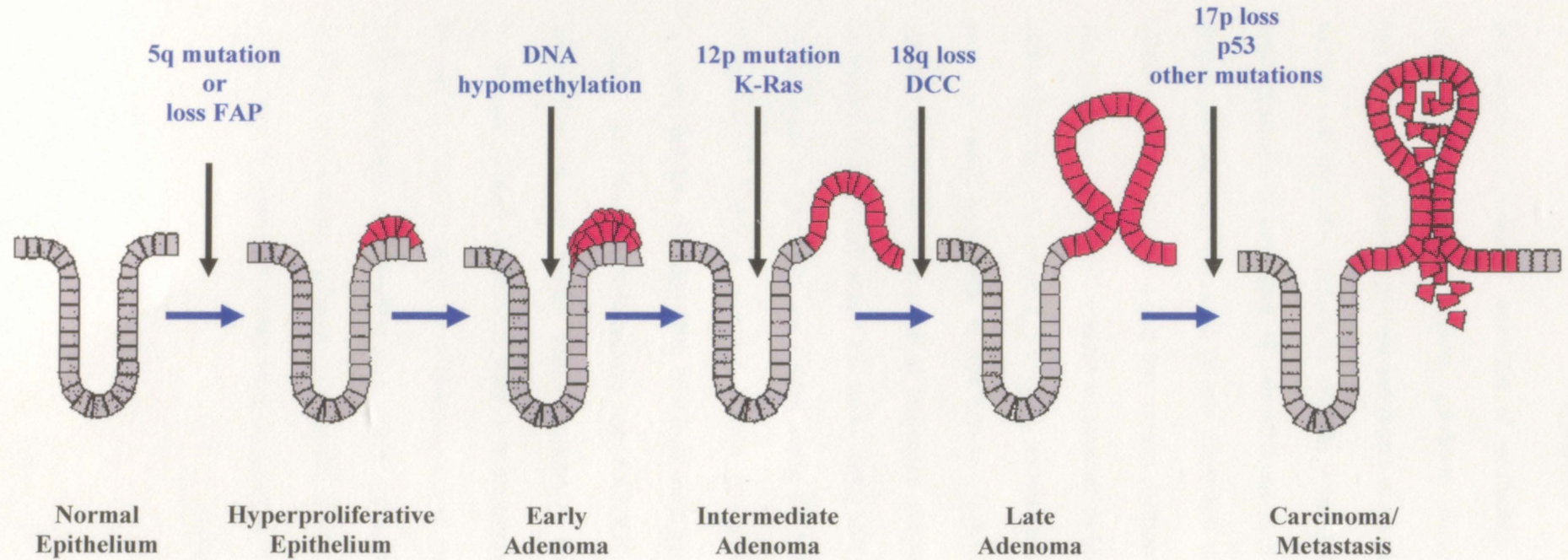


Figure 1: 'A Genetic Model for Colorectal Carcinogenesis' adapted from (Fearon & Vogelstein 1990). Tumourigenesis proceeds through a number of genetic alterations including mutation of oncogenes and tumour suppressors. However, not all mutations are necessary for tumour formation and although genetic alterations often occur according to a certain sequence it is the total accumulation of these changes, rather than their order with respect to one other, which determines the biological properties of the tumour.

Introduction

The adenoma-carcinoma sequence, initially postulated by Fearon and Vogelstein accounts for approximately 90% of cases of colorectal cancers (Vogelstein *et al.* 1988). This sequence refers to the multi-step progression from normal epithelium to hyperproliferative epithelium, to focally dysplastic crypts, to tubular adenoma, to dysplastic or villous adenoma and finally onto invasive carcinoma and metastasis. Several gene changes have been identified to be present at different stages of this cascade. Two major molecular patterns of change exist, namely chromosomal instability (85%) and microsatellite instability (15%) (Kinzler & Vogelstein 1998; Lengauer & Issa 1998; Lindblom 2001). Changes that occur due to chromosomal instability include alteration in chromosome number (aneuploidy) and detectable loss of portions of chromosomes 5q, 18q, 17p, and mutations in the k-ras oncogene. Loss of 5q involves the APC gene, which is an example of one of the earliest changes in tumour evolution. Microsatellite instability cancers are tumours with a mostly intact chromosome complement, but due to defects in DNA repair mechanisms, important cancer-associated genes remain unchecked. In 1990 Fearon and Vogelstein published 'A Genetic Model for Colorectal Carcinogenesis', describing some of the molecular events in the carcinogenic process and suggesting the possible sequence in which these events occur (*Figure 1*).

The knowledge gained from the study of cancer genetics has lead to the identification of markers that can aid early diagnosis and that are relatively specific to malignant and premalignant disease. These changes can be detected in colorectal biopsies but also non-invasively, by detection in the stools, as cells containing these

abnormal DNA sequences are shed into the gastrointestinal lumen by exfoliation (Duffy 1995).

These genetic changes can be detected using several different traditional techniques, such as subtractive hybridization and PCR for individual genes, but these methods are often time-consuming and limited in the number of genes which can be investigated at any one time. With the advent of microarray technology, the investigation of the expression of thousands of genes can be performed within a single patient sample.

Various studies have reported the expression profiles of colorectal normal tissue, adenomas and adenocarcinoma. Notterman *et al.* (2001) used an oligonucleotide array (GeneChip, Affymetrix) and found that only 1.8% of 4000 genes were differentially expressed in the tumours compared to matched normal tissue. This low level of difference may be due to the close relationship of the tissues studied. They were also able to successfully distinguish adenoma from adenocarcinoma and normal tissue by means of a hierarchical clustering algorithm. Kitahara *et al.* (2001) studied the different patterns of expression of 9216 genes in normal and neoplastic colorectal tissue. Overall both of these studies identified one to two hundred genes involved in tumour progression, but only three of these genes were held in common. These genes, pyroline 5-carboxylate reductase, BENE and carbonic anhydrase II, were differentially expressed in adenocarcinoma and normal tissue in both studies. There were however many differences in materials and methodology in these studies, which highlights the importance of study design and interpretation of data sets.

A study conducted using gene array analysis of left-sided colorectal tumours showed that most genetic changes occur at an early stage of cancer development and far fewer genes change their expression at the stage of spread to lymph nodes or distant metastasis (Birkenkamp-Demtroder *et al.* 2002).

Using laser mediated microdissection and RNA amplification, another group studied gene changes in areas of low grade dysplasia within colonic adenomas and found up-regulation of proliferation associated genes such as ras-oncogene related p21-rac1 and PAK p38 α and also found down-regulation in apoptosis related genes; FAST kinase and p53 which reveals an extra insight into the early steps towards colorectal cancer (Lechner *et al.* 2003). Bertucci *et al.* (2004) classified tumours by identifying sets of genes that discriminated between normal and tumour tissues, tumours associated or not with lymph node invasion or genetic instability, and tumours from the right or left colon. This group also identified a gene set that divided patients by prognosis; with significantly different 5-year survival (100% in one group and 40% in the other group; $p=0.005$). Protein expression was also studied which confirmed the up-regulation of the protein NM23 in colorectal cancer and a down-regulation in poor prognosis tumours.

In terms of pharmacogenetic studies, two publications in *Nature Genetics* observed differential expression of genes among the 60 cell lines used in the National Cancer Institute's screen for anticancer drugs (Ross *et al.* 2000; Scherf *et al.* 2000). However the clinical relevance of these genes needs to be confirmed in large groups of patients.

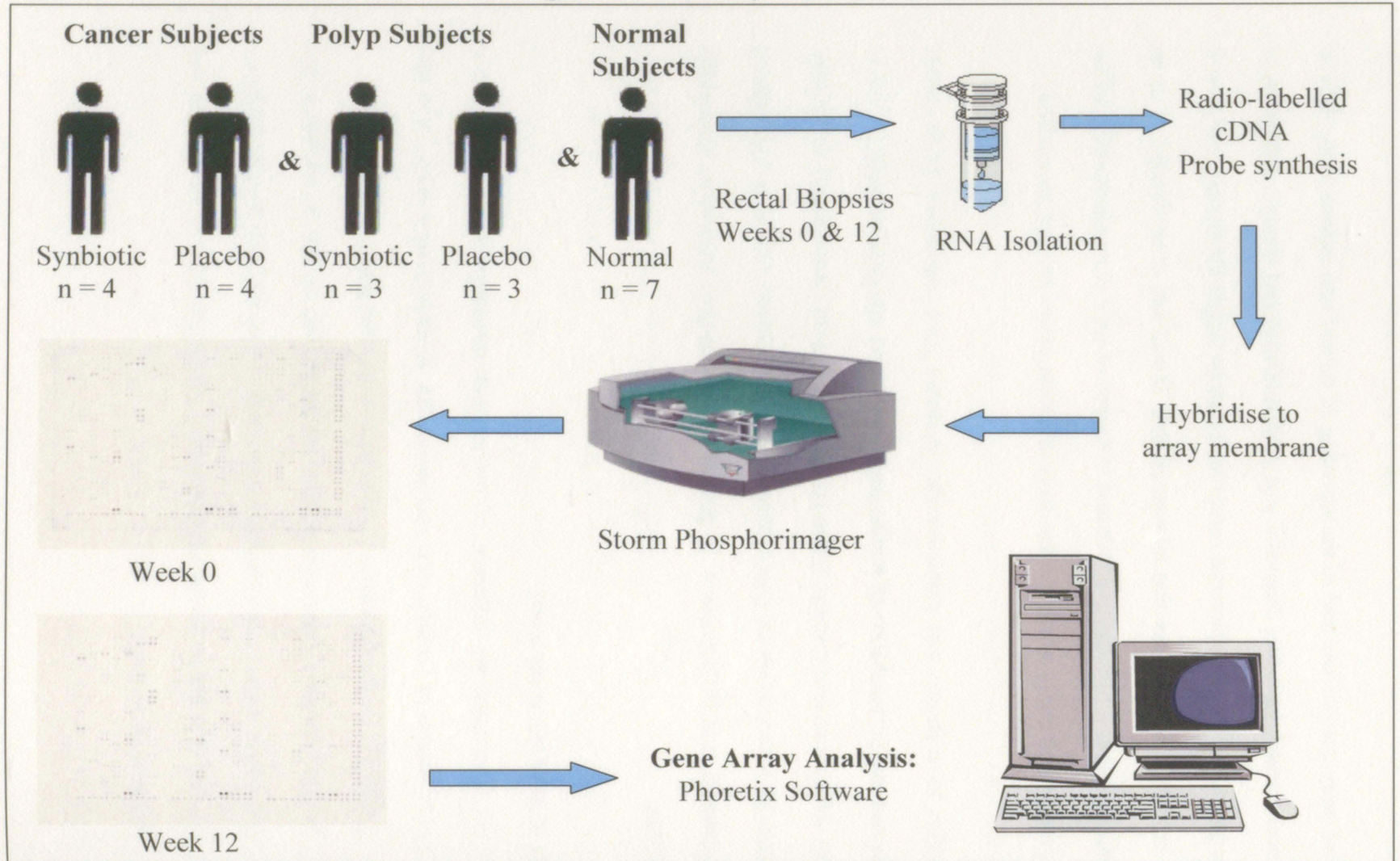
Gene array analysis has lead to the unfolding of several new aspects in the field of cancer research including diagnosis, patient stratification and therapy. All factors of the genetic pathway in colorectal cancer are potential targets for therapy. However it is important to remember that microarray data should not to be thought of as an endpoint but as a hypothesis-driven tool to further advance our understanding of the cancer process and to enable the discovery of new therapies and new biomarkers.

Studies have shown that probiotics can modulate gene expression. Sheil (2003) reported that co incubation of human peripheral blood mononuclear cells (PBMCs) with different probiotic strains led to profound changes in cytokine gene expression. Although a proportion of genes were consistently altered following exposure to Lactobacilli and Bifidobacteria, in general changes in gene expression were strain specific.

The aims of this study were:

- 1) To investigate the influence of twelve-week consumption of this synbiotic combination of *Lactobacillus rhamnosus* GG, *Bifidobacterium lactis* Bb12 and Synergy[®]1 on the expression of 588 cancer associated genes.
- 2) To generate gene expression profiles for the colonic mucosa of normal, polyp and cancer subjects, and identify genes highly expressed in the colonic mucosa.
- 3) To identify genes useful as biomarkers of cancer risk or as markers for intervention studies.

Figure 2: Gene array method



Methods

Work described in this chapter was performed in collaboration with Dr Mary Bennett and Dr John MacSharry of the Department of Medicine, University College Cork, Cork.

cDNA gene expression was performed on colorectal biopsies from normal, cancer and polyp subjects (*Figure 2*).

Total RNA Extraction

2 rectal biopsies from cancer and polyps subjects were obtained by sigmoidoscopy at the beginning (week 0) and the end of intervention (week 12), placed in a collection tube containing 'RNA later' (Ambion) and transported on ice to the laboratory. Biopsies were also obtained from normal subjects at one time-point. Biopsies were stabilised overnight in 'RNA later' at 4°C and subsequently transferred to -80°C. Total RNA was extracted using a commercially available kit, (Stratagene, Absolutely RNA, 400800) as per manufacturers instructions. Briefly biopsies were disrupted in lysis buffer using a homogeniser (Powergen 125, Fisher Scientific) and the lysate was applied to an RNA binding spin column. Following washing, RNase free DNase was applied to the column to remove contaminating DNA and incubated at 37°C for 15mins. Additional washes removed the DNase and impurities, and total RNA was eluted in a low ionic strength buffer and stored at -80°C.

RNA was quantified by spectrophotometry and RNA integrity was examined by running RNA in an ethidium bromide stained 2% agarose gel in TBE. Absence of

contaminating DNA was confirmed by performing a Polymerase Chain Reaction (PCR) for G3PDH. If this reaction yields a product contaminating DNA is present.

Gene Expression Analysis

Gene expression in biopsies was investigated using an Atlas™ human cancer cDNA expression array (BD Biosciences, Clontech) which contains 588 double spotted cDNAs.

- **³³P cDNA synthesis**

Radio-labelled cDNA was synthesized from total RNA by RT-PCR using [α -³³P] dATP (Amersham). Briefly 2.5µg of total RNA in 2µl DEPC (Diethyl pyrocarbonate) treated H₂O was incubated with 1µl of CDS primer mix (Clontech) at 70°C for two minutes followed by 2 minutes at 48°C. Following incubation the reverse transcription reagents (*Table 1*) were added to the RNA/CDS primer mix which was then incubated at 48°C for an hour to allow the cDNA synthesis reaction to occur. This reaction was terminated by the addition of 1µl 10X termination buffer (0.1M EDTA [pH8.0], 1mg/ml glycogen).

Table 1: Volume of reverse transcription reagents added to RNA/CDS primer mix

Reverse Transcription Reagents	Stock Solution	Volume
5X Reaction Buffer (Clontech)	250mM Tris-Hcl [pH8.3], 375mM KCL, 15mM MgCl ₂	2µl
10X dNTP Mix (Clontech)	5mM each dCTP, dGTP, dTTP	1µl
[α ³³ P] dATP (Amersham Life Science)	(>2,5000 Ci/mmol, 10µCi/µl)	3.5µl
DTT (Clontech)	100mM	0.5µl
MMLV reverse transcriptase (Clontech)	100 U/µl	1µl

- **Column Chromatography**

The ^{33}P -labeled cDNA probe was purified from unincorporated ^{33}P -labeled nucleotide by column chromatography using the NucleoSpin Extraction Kit supplied, which involves a number of wash steps and the purified ^{33}P -labeled cDNA probe was collected. ^{33}P incorporation was determined by scintillation count. Counts within the range of $2\text{--}10 \times 10^6$ cpm indicate successful incorporation.

- **Hybridisation**

0.5mg of sheared salmon testes DNA (Sigma) was denatured at $95\text{--}100^\circ\text{C}$ for 5 mins and the chilled immediately on ice. The Atlas nylon membranes were then prehybridised with 5ml warm ExpressHyb and the salmon testes DNA for 30 mins at 68°C in roller bottles rotating at 5-7rpm. The purified ^{33}P -labeled cDNA was mixed with 11 μl of 10X denaturing solution (1M NaOH, 10mM EDTA) and incubated at 68°C for 20 mins. Following this 5 μl of $\text{C}_0\text{t-1}$ DNA and 115 μl of 2X neutralising solution (1M NaH_2PO_4 [pH7]) were added and a final 10 minute incubation at 68°C performed. The denatured probe was then added to the prehybridisation solution in the roller bottles and hybridisation was performed overnight at 68°C . The next day the membranes were washed 4 times (30 mins each) with prewarmed wash solution 1 (2X SSC, 1% SDS), 68°C followed by 1 wash (30 mins) with wash solution 2 (0.1X SSC, 0.5% SDS) at 68°C rotating at 12-15 rpm and a final 5 min wash with 200ml of 2X SSC. The membranes were then wrapped in cling film and exposed to a phosphorimaging screen (Molecular Dymnamics) for 5 days. Phosphor screens were scanned using a Storm 860 scanner (Molecular Dynamics, Amersham Biosciences). The screens were then scanned at 50 μm pixel size using a storm phosphorimager (Molecular Dynamics, Amersham Biosciences).

- **Array Analysis**

Array images were analysed using Phoretix™ Array Software (Nonlinear dynamics). The background was subtracted and the intensity was normalised to negative control spots and to the housekeeping gene β -actin and expression was expressed as a percentage of β -actin expression. The normalised data was imported to Microsoft Excel (Microsoft Corporation) and genes which were expressed at greater than 40% of β -actin in 8 cancer and 6 polyp subjects at the beginning (week 0) and end (week 12) of intervention and in the 6 normal subjects were considered to be highly expressed in the colonic mucosa.

To identify genes differentially expressed in cancer, polyp and normal subjects the ratios of difference of each gene for cancer versus normal, polyp versus normal and cancer versus polyp subjects was determined and significantly different genes were identified by unpaired t- test. For this analysis one cancer subject was excluded (Syn-39) as the level of gene expression in this subject was much higher than other cancer subjects and was considered an outlier (*Appendix I*).

In cancer and polyp subjects consuming synbiotic and placebo the ratios of difference in gene expression at the beginning compared to the end of intervention was also calculated.

Finally genes were classified and grouped according to function which was determined using Genecards (<http://bioinformatics.weizmann.ac.il/cards>), Entrez Gene (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene), and the PubMed database (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed).

Results

cDNA array analysis of 588 cancer associated genes was performed on biopsies from cancer, polyp and normal subjects.

Constitutive Genes

Genes expressed at a level above 40% of beta actin expression in all subjects at all times were considered to be highly expressed. 14 genes were identified as highly expressed in the colonic mucosa of all subjects (6 polyp and 8 cancer subjects at weeks 0 and 12 and 6 normal subjects) (*Table 2*).

Table 2

Gene Symbol	Gene Name
Differentiation	
<i>Cell cycle</i>	
CDK6	cyclin-dependent kinase 6
NME2	protein expressed in non-metastatic cells 2 (NM23B)
UBC	ubiquitin C
RPL13A	ribosomal protein L13a
RPS9	ribosomal protein S9
<i>apoptosis</i>	
GAPD	glyceraldehyde-3-phosphate dehydrogenase
Cell Cell Interaction	
RHOA	ras homolog gene family, member A
ARHGDIB	Rho GDP dissociation inhibitor (GDI) alpha
CD9	CD9 antigen (p24)
CDH5	cadherin 5, type 2, VE-cadherin (vascular epithelium)
CD59	CD59 antigen p18-20
Cell Structure	
KRT19	keratin 19
VIM	vimentin
ACTB	actin, beta

Further analysis revealed some genes although not highly expressed in all subjects were highly expressed in subsets of subjects (*Table 3*). Interestingly there were no highly expressed genes unique to cancer subjects.

Table 3

Gene Symbol	Gene Name
Highly Expressed in Cancer & Polyp Subjects	
HLA-C	major histocompatibility complex, class I, C
KRT8	keratin 8
Highly Expressed in Cancer & Normal Subjects	
JUP	junction plakoglobin
K-Alpha-1	tubulin, alpha, ubiquitous
Highly Expressed in Normal Subjects	
GRB2	growth factor receptor-bound protein 2
LITAF	LPS-induced TNF-alpha factor
FN1	fibronectin 1
Highly Expressed in Polyp Subjects	
RHOC	ras homolog gene family, member C

Accession	Gene	Normal Subjects							Cancer Subjects								Polyp Subjects								Canc vs Norm	p Value	Polyp vs Norm	p Value	Canc vs Polyp	p Value
Number		1	3	4	5	6	7	Ave	Syn05	Syn13	Syn15	Syn30	Syn38	Syn58	Syn77	Ave	Syn40	Syn65	Syn70	Syn42	Syn49	Syn74	Ave							
Cell Cycle																														
U47413	cyclin G1	18.05	6.80	15.51	8.94	6.59	6.60	10.41	4.19	4.91	7.71	7.11	4.82	6.83	3.08	5.52	3.91	4.79	5.32	9.85	10.79	8.92	7.23	1.89	0.034	1.44	n.s.			
U39657	MAP2K6	18.38	11.87	12.16	11.39	4.30	7.30	10.86	4.30	5.90	11.39	7.34	4.09	6.66	3.67	6.19	3.46	4.30	3.23	8.94	6.16	13.45	6.59	1.76	0.048	1.65	n.s.			
M59911	Integrin, alpha 3	17.70	33.23	25.01	31.43	21.83	25.58	25.79	15.79	16.64	46.68	19.27	28.98	17.26	29.38	24.85	18.73	17.01	15.40	24.81	8.05	20.59	17.43			1.48	0.030			
Cell Adhesion Receptors/Proteins																														
Z26317	desmoglein 2	19.13	10.07	9.93	11.48	13.28	19.24	13.85	11.84	5.91	15.22	9.60	15.46	11.30	8.91	11.18	16.13	12.07	14.74	23.91	34.87	17.74	19.91			1.44	n.s.	1.78	0.027	
Apoptosis Associated Proteins																														
U56390	caspase 9	9.96	4.62	3.36	10.37	2.18	5.06	5.92	6.16	1.77	19.53	15.72	7.21	7.19	8.34	9.41	8.09	11.52	6.38	11.05	11.54	15.82	10.73	1.59	n.s.	1.81	0.032			
Y10256	MAP3K14	1.99	6.06	3.11	5.39	0.28	2.95	3.29	3.79	1.69	10.77	10.20	8.01	7.32	6.20	6.85	6.01	4.73	4.06	7.90	5.28	14.38	7.06	2.08	0.045	2.14	n.s.			
U45880	BIRC4	3.19	4.10	2.88	4.30	0.23	1.66	2.72	3.79	1.64	11.33	2.28	2.81	5.07	1.97	4.13	4.97	2.42	4.28	5.29	5.72	9.24	5.32	1.51	n.s.	1.95	0.042			
Cell Receptors																														
M21574	PDGFRA	15.66	25.94	30.51	13.96	13.42	33.86	22.22	4.64	11.68	9.08	5.12	10.29	28.63	12.64	11.72	10.36	5.64	5.22	26.51	18.04	32.26	16.34	1.90	0.048					
X06374	PDGFA	5.79	6.62	4.06	11.59	5.96	7.66	6.95	6.36	6.25	8.52	8.45	10.77	10.20	9.59	8.59	11.70	10.14	5.95	10.93	14.66	13.73	11.18			1.61	0.027			
M57399	pleiotrophin	14.56	15.25	20.77	17.91	11.86	18.50	16.47	3.99	6.90	43.63	14.56	34.98	57.33	34.53	27.99	18.31	22.84	18.26	47.07	30.69	38.38	28.92	1.70	n.s.	1.76	0.028			
Cell Signaling, Extracellular Communication Proteins																														
D30751	BMP4	6.13	11.49	4.54	9.94	2.15	5.65	6.65	5.63	3.68	16.68	8.52	5.48	12.58	9.69	8.89	10.84	12.87	9.42	11.22	14.88	12.50	11.95			1.80	0.008			
L38734	ephrin-B2	6.61	8.47	3.37	6.24	1.49	2.70	4.81	3.54	3.31	14.10	6.99	5.41	10.71	8.82	7.52	9.03	7.45	6.34	10.13	13.43	13.24	9.94	1.56	n.s.	2.07	0.010			
X02851	interleukin 1, alpha	1.48	2.91	3.73	1.57	0.43	1.13	1.87	2.88	2.24	10.17	4.59	3.59	2.94	1.57	3.99	2.42	3.39	3.73	2.30	4.47	3.73	3.34	2.13	n.s.	1.78	0.035			
M62403	IGFBP4	6.71	6.11	3.00	7.27	6.82	3.33	5.54	6.79	3.15	9.33	9.44	4.50	5.42	2.89	5.93	9.49	11.02	6.64	9.54	12.14	8.11	9.49			1.71	0.005	1.60	0.022	
Intracellular Transducers/Effectors/Modulators																														
Y00815	PTPRF	3.28	5.82	7.32	4.27	3.53	3.87	4.68	5.72	3.41	10.95	5.89	3.78	9.12	7.16	6.57	6.63	5.82	6.67	6.05	11.25	8.26	7.44	1.41	n.s.	1.59	0.026			
U24153	PAK2	10.40	7.57	6.02	11.95	1.20	6.07	7.03	4.63	3.38	13.45	7.80	14.37	14.39	14.72	10.39	9.41	10.67	11.10	11.76	26.84	18.26	15.00	1.48	n.s.	2.13	0.042	1.44	n.s.	
M31470	RHOQ	3.35	6.64	3.89	3.50	1.76	4.72	3.97	3.80	2.97	9.79	6.51	5.92	8.05	3.69	5.82	3.07	5.76	5.54	9.80	10.80	12.76	7.95	1.46	n.s.	2.00	0.037			
X06820	RHOB	37.50	16.15	12.09	42.85	38.92	53.16	33.44	43.38	17.63	53.86	47.52	49.70	57.22	45.37	44.98	47.99	39.72	36.97	71.10	91.41	72.68	59.98			1.79	0.037			
U02570	ARHGAP1	2.21	4.30	2.58	1.64	0.83	0.85	2.07	1.92	1.88	8.08	4.75	2.45	6.06	2.53	3.66	1.49	3.69	4.90	3.53	5.16	6.75	4.25	1.77	n.s.	2.06	0.036			
Z18951	caveolin 1	3.87	5.42	4.29	3.60	4.81	2.90	4.14	4.56	4.00	10.57	6.85	8.17	7.30	5.88	6.76	3.08	6.28	4.00	13.65	10.28	10.52	7.97	1.63	0.022	1.92	n.s.			
Metalloproteinases & Protease Inhibitors																														
X05232	MMP3	3.14	2.67	3.52	3.33	0.89	1.37	2.49	3.05	2.78	5.33	4.31	5.30	6.87	3.35	4.43	2.37	2.83	4.52	2.67	7.87	9.37	4.94	1.76	0.023	1.99	n.s.			
M11313	A2M	11.28	7.84	7.02	12.14	3.32	3.88	7.58	5.85	2.07	7.10	10.70	12.57	11.70	14.80	9.22	9.72	13.33	12.67	11.58	25.69	15.60	14.76			1.95	0.026	1.60	n.s.	
Z30183	TIMP3	15.14	16.31	18.41	18.89	11.91	16.50	16.19	16.77	13.30	30.92	16.08	25.22	39.72	40.16	26.02	22.66	14.04	20.37	37.86	43.24	41.64	29.95	1.61	n.s.	1.85	0.024			
Cytoskeleton/Motility Proteins																														
X51521	villin 2	35.79	30.70	12.78	32.18	24.97	31.39	27.97	28.56	24.81	55.52	40.11	46.70	65.92	43.62	43.63	38.59	28.56	28.81	39.41	76.91	34.28	41.09	1.56	0.038	1.47	n.s.			
DNA Synthesis, Recombination & Repair																														
X06745	POLA	4.53	5.35	3.27	3.08	1.79	4.65	3.78	3.75	6.35	9.91	6.29	2.53	6.51	2.08	5.63	4.79	3.37	5.29	7.65	8.88	11.67	6.94	1.49	n.s.	1.84	0.042			
U07418	MLH1	4.98	4.39	3.89	1.76	0.91	1.21	2.86	1.90	1.58	6.50	5.63	1.86	3.35	1.13	3.13	4.91	3.95	3.65	4.17	5.35	7.58	4.93			1.73	0.050	1.57	n.s.	
U35835	PRKDC	9.83	11.42	7.96	10.64	7.72	23.20	11.79	5.60	6.46	12.64	9.39	5.81	9.39	3.95	7.58	16.04	5.20	5.81	19.18	20.40	15.62	13.71	1.56	n.s.			1.81	0.049	
U17075	CDKN2B	6.81	4.92	4.13	6.04	5.40	1.46	4.79	4.14	2.59	8.46	6.29	2.69	4.84	4.07	4.72	6.32	7.48	6.64	9.21	11.19	7.26	8.01			1.67	0.013	1.70	0.012	
X90392	DNASE1L1	4.92	4.37	3.20	2.60	1.44	1.80	3.05	2.46	2.38	6.80	5.40	1.96	2.42	2.60	3.43	2.83	3.70	4.10	7.23	9.46	7.53	5.81			1.90	0.047	1.69	n.s.	
Angiogenic factors																														
U48801	VEGFB	4.63	5.25	6.45	6.46	2.76	3.36	4.82	7.63	3.21	8.30	6.04	7.86	8.94	8.25	7.17	7.26	8.08	10.61	8.09	13.55	10.80	9.73	1.49	0.037	2.02	0.002			

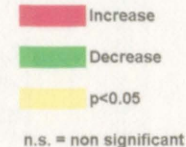
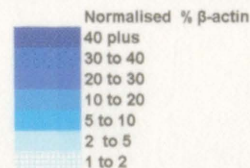


Table 4: The ratios of difference of each gene for cancer versus normal, polyp versus normal and cancer versus polyp subjects. All genes are normalised to the housekeeping gene β -actin and expressed as % of β -actin. Significantly different genes were identified by unpaired t- test. For a full list of gene names see [Appendix III](#)

Differentially Expressed Genes

Ratios of difference of gene expression for all 588 genes in cancer versus normal, polyp versus normal and cancer versus polyp subjects were calculated. For this analysis one cancer subjects was excluded (Syn-39) as an outlier because the level of gene expression in this subject was much higher than other cancer subjects (**Appendix I**). Genes with a ratio of difference of 1.4 or above were identified (**Appendix II**) and statistical analysis (unpaired t-test) revealed 30 genes, which are expressed at significantly different levels in cancer, polyp and normal subjects (*Table 4*).

Accession number		Synbiotic Cancer				Placebo Cancer				Synbiotic Polyp			Placebo Polyp		
		Syn 5	Syn 13	Syn 39	Syn 58	Syn 15	Syn 30	Syn 38	Syn 77	Syn 40	Syn 65	Syn 70	Syn42	Syn49	Syn74
	Transcription Activators & Repressors														
L23959	TFDP1									1.6565	1.5081				
	Cell cycle														
L22005	cell division cycle 34	1.584			1.555								1.7104	1.8497	
D13639	cyclin D2	2.455	1.7522	1.6122										1.929	2.085
U00001	CDC27					2.0131		1.5026	4.3849						
M80629	CDC2L5					1.784		1.655							
X59798	cyclin D1					1.829			1.743						
X85134	RBBP5						1.941	1.502							
Y11416	tumor protein p73	1.5738			1.7457										
D13365	MT3	2.239	1.978												
	Apoptosis														
L41690	TRADD			1.597	1.5179		1.629		1.645						
U13699	caspase 1						1.525	1.834							
U28014	caspase 4													1.7983	1.5811
L08246	MCL1												1.864	2.336	
U23765	BAK1												2.0396	3.4283	1.9397
	Cell Receptors														
M21574	PDGFRA	1.6886		1.6113											
M35410	IGFBP2													3.047	2.289
AAA36418	protocadherin 1		1.7523		1.878										
U43901	LAMR1					1.652	1.704								
M73780	integrin, beta 8					1.806			1.791						
X53586	integrin, alpha 6						1.783		1.504						
L34774	OPCML			1.7209	1.8441										
Z26317	desmoglein 2												2.6376	2.7356	
	Cell Signaling, Extracellular Communication Proteins														
L09753	TNFSF8	1.5092		1.6807	1.5462										
L06801	interleukin 13	1.926	1.5	2.022	2.001										
M74178	MST1	1.5088		1.7081											
U46010	HGF			1.7553	1.8801										
M61176	BDNF					1.838			1.526						
U24163	FRZB										1.745	1.7939			
	Intracellular Transducers/Effectors/Modulators														
U46461	DVL1L1												1.648	1.5447	
M64595	RAC2												1.956	4.622	4.193
U07707	EPS15						1.837	1.501							
L06139	TEK			1.7539	1.7633										
L20688	ARHGDI8													4.324	4.0733
X78817	ARHGAP4													2.0328	2.2589
	Oncogenes & Tumor Suppressors														
X03663	CSF1R													3.0478	4.365
	Ribosomal Proteins														
X65923	FAU												1.6412	1.792	1.5156
	Protein Turnover														
X57766	MMP11	1.644			1.5374										
J05070	MMP9													4.5301	5.9587
	Cytoskeleton/Motility Proteins														
M77349	TGF- β induced													4.7	2.321
	Angiogenic factors														
M32977	VEGF	1.962		1.985											

Increased expression

Decreased expression

No change

Table 5: Genes in which expression was changed at the end of intervention (Wk 12) compared to the beginning of intervention (Wk 0) in two or more subjects. All genes are normalised to the housekeeping gene β -actin and expressed as % of β -actin. For a full list of gene names see *Appendix III*

Influence of Synbiotic Intervention on Gene Expression

cDNA array analysis was performed on biopsies from cancer (synbiotic n=4, placebo n=4) and polyp (synbiotic n=3, placebo n=3) subjects at the beginning and after twelve weeks intervention. For each subject the ratios of expression at the beginning of intervention (week 0) compared to the end of intervention (week 12) were calculated. Genes with ratios of difference of 1.5 or greater were considered significantly different. *Table 5* shows 40 genes in which major changes in expression occurred in two or more subjects at the end of the twelve-week intervention period compared to the beginning.

Discussion

The aetiology of colorectal cancer is heterogeneous, with environment or genetics playing varying key roles in different patients (Potter 1999). The influence of genetic factors is well documented and there are several rare genetic syndromes (HNPCC, FAP) that carry a markedly increased risk for CRC. However about 80% of colorectal cancer cases are sporadic with no evidence of a heritable component. Although the genetic process of sporadic cancer is not understood, genes described in the Fearon and Vogelstein cascade (APC, genes on 18q, ras, and p53), have been confirmed as affected in sporadic cancers.

Tumour development and progression involves the accumulation of numerous genetic alterations and occurs over a long time period. Techniques commonly used to measure gene expression such as Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Northern blot analysis etc are time consuming and often require large amounts of RNA. These techniques are further limited in the number of genes which can be investigated at any one time. However in recent years the advent of cDNA microarray analysis technology has provided the means by which the expression of the entire human genome can be determined.

By employing gene array analysis of 588 cancer related genes we have generated an expression profile for the colorectal mucosa. We have identified 14 genes which are highly expressed in the colorectal mucosa of normal, previously re-sected colon cancer and polypectomised subjects. A number of these genes are involved in the cell cycle and apoptosis (CDK6, NME2, UBC, RPL13A, RPS9 and GAPD). Genes

involved in cell-cell interactions were also highly expressed (RHOA, ARHGDIB, CD9, CDH5 and CD59), as were structural genes (KRT19, VIM and ACTB).

Interestingly, we found two genes (HLA-C and KRT8), which were highly expressed in cancer and polyp subjects but not in normal subjects. The genes JUP and K-Alpha-1 were highly expressed in cancer and normal subjects but were not found at these levels in polyp subjects. We also identified highly expressed genes, which were unique to normal (GRB2, LITAF, FN1) and polyp (RHOC) subjects. There were no highly expressed genes unique to cancer subjects. Further analysis of these genes and others in a larger number of subjects over a number of time-points are needed to confirm the expression of highly genes identified in this study and will determine if analysis of these genes are useful as markers of cancer risk.

Analysis of the expression of 588 genes in cancer, polyp and normal subjects revealed 30 genes, which were significantly differentially expressed in these subjects. VEGF which is a growth factor involved in angiogenesis was more highly expressed in both cancer and polyp subjects than in normal subjects. Cell cycle genes such as cyclin G1, MAP2K6 and integrin alpha 3 were more highly expressed in normal subjects than in cancer and polyp subjects. Cyclin G1 is a member of the cyclin family, which play a role in the cell cycle by regulating the activities of cyclin-dependent protein kinases (CDKs) and CDK inhibitors. Transcriptional activation of cyclin G1 can be induced by tumour protein p53, transforming growth factor β (TGF- β), BMP-4, p63, and p73. MAP2K6 encodes a protein, which phosphorylates and activates p38 mitogen-activated protein kinase (p38 MAPK) in response to inflammatory cytokines or environmental stress. Qi *et al.* (2004) reported

that p38 MAPK activation selectively induced cell death in K-ras-mutated human colon cancer cells. Integrin alpha 3 is a receptor for fibronectin, laminin, collagen, epiligrin and thrombospondin. Integrin alpha 3 has been shown to be upregulated in oesophageal adenocarcinoma tissue compared to normal oesophageal tissue (Hourihan *et al.* 2003).

In contrast to cell cycle genes, genes involved in apoptosis (caspase 9, MAP3K14 and BIRC4) were more highly expressed in cancer and polyp subjects than in normal subjects. Caspase 9 is involved in the activation cascade of caspases responsible for apoptosis. The caspase 9 protein is processed by caspase APAF1 and this step is thought to be one of the earliest in the caspase activation cascade. MAP3K14 is involved in the activation of the transcription factor Nuclear factor of kappaB (NF- κ B) which plays a role in the regulation of cell proliferation, apoptosis and cell migration. NF- κ B activation also leads to production of enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (Cox-2), which enhance the production of reactive oxygen species, leading to additional DNA damage (Karin *et al.* 2002). BIRC4 is known to inhibit apoptosis by binding to tumour necrosis factor receptor-associated factors TRAF1 and TRAF2. BIRC4 also inhibits caspase 9. MAP3K14 also plays a role in apoptosis as well as inducing nitric oxide synthase (iNOS) and cyclooxygenase-2 (Cox-2) production through NF- κ B activation, leading to the production of reactive oxygen species and additional DNA damage.

Interestingly genes, which code for cell signalling and communication proteins (BMP4, ephrin-B2, interleukin 1, alpha, IGFBP4) are more highly expressed in polyp and in some cases cancer subjects compared to normal subjects. These genes

are known to be involved in the regulation of apoptosis and proliferation. BMP4 is a member of the bone morphogenetic protein family, which is part of the transforming growth factor-beta superfamily. Nishanian *et al.* (2004) reported that BMP4 is involved in signalling in apoptosis and as a negative regulator of proliferation through interaction with the Wnt signalling pathway. Ephrin-B2 encodes a member of the ephrin and EPH-related receptor family, which is the largest subfamily of receptor protein-tyrosine kinases. Recently, it has been demonstrated that over-expression of specific Ephs and ephrins is associated with a poor prognosis in human tumours. EphB and the ephrin-B subfamilies are coexpressed in human colorectal cancer, and ephrin-B2 is expressed at higher levels in human colorectal cancer than in adjacent normal mucosa (Liu *et al.* 2002). IL-1 alpha is involved in the inflammatory response and induction of apoptosis. Abdul & Hoosein (2002) reported that IL-1 alpha expression was observed in three of the seven colon cancer cell lines and was associated with high CEA secretion and aggressive growth behavior. IFGBP4 is a member of the insulin-like growth factor binding protein (IGFBP) family, which bind insulin-like growth factors (IGFs) I and II. IGF-II is expressed commonly in colorectal tumours. IGFBP-4 counteracts the tumour promoting activities of IGF-II by binding this growth factor (Diehl *et al.* 2005).

Genes coding for proteins classed as intracellular transducers, effectors and modulators such as PTPRF, PAK2, RHOQ, RHOB and ARHGAP1 were also more highly expressed in polyp subjects than normal subjects and tended to be increased in cancer subjects compared to normal subjects although this increase did not reach significance. PTPFR is a member of the protein tyrosine phosphatase (PTP) family, which are signalling molecules that regulate a variety of cellular processes including

cell growth, differentiation, mitotic cycle, and oncogenic transformation. Recently PTPRF has been identified as a putative molecular marker for assessing the metastatic potential of prostate cancer (Trojan *et al.* 2005). PAK2 is a member of the p21 activated kinases (PAK) which are critical effectors that link Rho GTPases to cytoskeleton reorganisation and nuclear signalling. The protein encoded by this gene is activated by proteolytic cleavage during caspase-mediated apoptosis, and may play a role in regulating the apoptotic events in the dying cell. Huang *et al.* (2004) reported that Pak2 negatively regulates the oncogenic activities of Myc and suggest that this negative regulation of Myc is due to the tumour suppressive activity of Pak2. RHOQ and RHOB are plasma membrane bound small GTPases involved in mitogen-activated protein kinase (MAPK) cascades, cell cycle progression and transformation. RhoB mediates apoptosis in neoplastically transformed cells after DNA damage and has been reported to modulate COX-2 transcription and COX-2 mRNA stability (Shao *et al.* 2000). ARHGAP1 is a GTPase activator for the Rho, Rac and Cdc42 proteins. Another gene in the intracellular transducers, effectors and modulators group is caveolin 1 which is more highly expressed in cancer subjects than in normal subjects. Caveolin 1 encodes one of the principal proteins of caveolae, the vesicular invaginations of the plasma membrane. Several reports have suggested that caveolin-1 may have a role in cellular transformation and tumourigenesis and is over-expressed in colon cancers (Fine *et al.* 2001; Patlolla *et al.* 2004). However some investigators have reported that caveolin 1 is down regulated in human colon cancer (Bender *et al.* 2000).

Genes coding for matrix metalloproteinases and their inhibitors (MMP3, A2M and

TIMP3) were identified as being differentially expressed in cancer and polyp subjects compared to normal subjects. MMP3 is a member of the matrix metalloproteinase (MMP) family involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodelling, as well as in disease processes, such as arthritis and metastasis. MMP3 encodes an enzyme which degrades fibronectin, laminin, collagens III, IV, IX, and X, and cartilage proteoglycans. The enzyme is thought to be involved in wound repair, progression of atherosclerosis, and tumour initiation. (Roeb *et al.* 2004) reported that MMP-3, MMP-7 and MMP-13 were more highly expressed in tumour tissue than in distant normal tissue. A2M is a protease inhibitor and cytokine transporter, which inhibits proteases such as trypsin, thrombin and collagenase. This gene has been found to be differentially expressed in normal and tumour tissue in hepatocellular carcinoma (Kurokawa *et al.* 2003). TIMP3 is a member of the tissue inhibitor of metalloproteinase (TIMP), gene family, which are natural inhibitors of MMPs. TIMP3 is known to act on MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-13, MMP-14 and MMP-15.

Villin 2 is more highly expressed in cancer subjects than in normal subjects and is involved in connecting major cytoskeletal structures to the plasma membrane and plays a role in cell surface structure adhesion, migration, and organization. Nishizuka *et al.* (2003) suggested that villin 2 may be suitable as a diagnostic marker for colon cancer.

Genes involved in DNA synthesis, recombination and repair (POLA, MLH1, PRKDC, CDKN2B and DNASE1L1) were more highly expressed in polyp subjects

compared to normal and cancer subjects. POLA is a key replication enzyme, primarily responsible for the synthesis of DNA. MLH1 was identified as a locus frequently mutated in hereditary nonpolyposis colon cancer (HNPCC). It is a human homolog of the *E. coli* DNA mismatch repair gene mutL, consistent with the characteristic alterations in microsatellite sequences found in HNPCC. PRKDC encodes a Serine/Threonine kinase involved in DNA double-strand break repair. The protein must be bound to DNA to express its catalytic properties. CDKN2B encodes a cyclin-dependent kinase inhibitor, which forms a complex with CDK4 or CDK6, and prevents the activation of the CDK kinases, therefore functioning as a cell growth regulator that controls cell cycle G1 progression. DNASE1L1 gene encodes a member of the deoxyribonuclease family and is thought to be a lysosomal type of endonuclease.

Comparison of gene expression in cancer and polyp subjects at the end of the twelve-week intervention period revealed a number of genes, which were altered by synbiotic consumption. In cancer subjects, synbiotic consumption altered the expression of genes involved in the cell cycle such as cell division cycle 34, cyclin D2, tumor protein p73 and MT3. CDC34 encodes a gene, which is required for ubiquitin-mediated degradation of cell cycle G1 regulators, and for the initiation of DNA replication. Cyclin D2 regulates the activities of CDKs and CDK inhibitors, forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. Cyclin D2 has also been shown to interact with and be involved in the phosphorylation of tumour suppressor protein Rb. Tumour protein p73 is involved in the apoptotic response to DNA damage and when overproduced, activates transcription from p53-responsive

promoters and induces apoptosis. It has been suggested that p73 has tumour suppressor properties however over-expression of p73 causes progression in some tumour types. MT3 expression deficiency has been implicated in carcinogenesis, however there is also a possible involvement of MT3 over expression and resistance of tumours to anti-cancer therapy.

Interestingly in the placebo group of cancer subjects there was an increase in four cell cycle genes, which were not altered in the synbiotic group (CDC27, CDC2L5, cyclin D1 and RBBP5). CDC27 has been shown to interact with mitotic checkpoint proteins including Mad2, p55CDC and BUBR1, and thus may be involved in controlling the timing of mitosis. CDC2L5 is a member of the cyclin-dependent serine/threonine protein kinase family, which play an essential role as master switches in cell cycle control however the exact function of CDC2L5 has not yet been determined. Cyclin D1 has a similar function to cyclin D2 as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition and cyclin D1 also interacts with the tumour suppressor protein Rb. RBBP5 encodes a protein which is among several proteins that bind directly to retinoblastoma protein, a cell proliferation regulator. RBBP5 binds preferentially to underphosphorylated retinoblastoma protein and has been reported to be coamplified with MDM4 in malignant gliomas (Riemenschneider *et al.* 1999).

In the cancer group of synbiotic subjects the expression of a number of genes encoding cell receptors was altered (PDGFRA, protocadherin 1 and OPCML). The PDGFRA gene encodes a cell surface tyrosine kinase receptor for members of the platelet-derived growth factor (PDGF) family. PDGFRA binds both PDGFA and

PDGFB. The protocadherin 1 encoded protein is a membrane protein found at cell-cell boundaries and involved in cell adhesion. OPCML is a member of the IgLON family of immunoglobulin and encodes a plasma membrane protein, which binds opioids in the presence of acidic lipids and is thought to play a role in cell contact. The expression of these genes were unaltered in the placebo group of cancer subjects, however there was an increase in the expression of LAMR1, integrin beta 8 and integrin alpha 6. LAMR1 encodes a receptor for the laminin family of extracellular matrix glycoproteins which are the major noncollagenous constituent of basement membranes and are involved in cell adhesion, differentiation, migration, signalling and metastasis. LAMR1 has been reported to be more highly expressed in colon carcinoma tissue than in normal colon tissue. Integrin beta 8 is a member of the integrin family of transmembrane proteins and is a receptor for fibronectin. Integrin alpha 6 combines with integrin beta-1 to form a receptor for laminin on platelets and also combines with integrin beta-4 as a receptor for laminin in epithelial cells. Increased expression is associated with colon cancer metastasis to the liver.

In cancer subjects synbiotic consumption also altered the expression of TNFSF8, interleukin 13, MST1, HGF, TEK, MMP11 and VEGF. TNFSF8 encodes a cytokine that belongs to the TNF ligand family, is a ligand for TNFRSF8/CD30 and induces proliferation of T cells. Interleukin 13 encodes an immunoregulatory cytokine produced primarily by activated Th2 cells. IL-13 inhibits inflammatory cytokine production and synergises with IL-2 in regulating IFN- γ synthesis. IL-13 was upregulated in two of the four synbiotic subjects and down-regulated in the other two subjects. Interestingly PBMCs derived from cancer synbiotic subjects produced more IFN- γ at the end of intervention compared to the beginning. MST1 is a

pleiotropic growth factor that signals via the Ron receptor tyrosine kinase. HGF regulates cell growth, cell motility, and morphogenesis by activating a tyrosine kinase signaling cascade after binding to the proto-oncogenic c-Met receptor. Its ability to stimulate mitogenesis, cell motility, and matrix invasion gives it a central role in angiogenesis, tumorigenesis, and tissue regeneration. TEK encodes a protein tyrosine-kinase transmembrane receptor for angiopoietin 1 and possibly regulates endothelial cell proliferation, differentiation and the proper patterning of endothelial cells during blood vessel formation. MMP11 encodes an enzyme, which is activated intracellularly by furin within the constitutive secretory pathway in contrast to most MMP's, which are secreted as inactive pro-proteins, and are activated when cleaved by extracellular proteinases. Also in contrast to other MMP's, this enzyme cleaves alpha 1-proteinase inhibitor but weakly degrades structural proteins of the extracellular matrix. VEGF encodes a growth factor, which is active in angiogenesis, vasculogenesis and endothelial cell growth and induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis, and induces permeabilisation of blood vessels.

In contrast to cancer subjects synbiotic consumption in polyp subjects altered the expression of only two genes. TFDP1 can stimulate E2F transcription. The E2F transcription factor family regulates the expression of various cellular promoters, particularly those involved in the cell cycle and seems to play a role in both proliferation and apoptosis. FRZB acts as a soluble Wnt-binding protein that antagonizes Wnt signalling. FRZB has been reported to be expressed in normal colonic tissue from subjects with colon cancer but not in the tumour tissue. FRZB is not expressed in the normal colonic mucosa (Byun *et al.* 2005).

Interestingly we have identified a number of genes unique to the placebo polyp group in which expression levels are changed after 12 weeks. These included genes involved in cell cycle and apoptosis such as cell division cycle 34, cyclin D2, caspase 4, MCL1 and BAK1, cell receptors IGFBP2 and desmoglein 2, intracellular transducers / effectors or modulators such as DVL1L1, RAC2, ARHGDIB, ARHGAP4, CSF1R and other genes such as FAU, MMP9 and TGF- β induced. Reduced expression of these genes is interesting in light of the proliferation results where synbiotic consumption reduced the proliferative activity of the the colonic mucosa in polyp subjects. It may be that these genes are involved in proliferation and that synbiotic consumption prevents this reduction in gene expression therefore reducing the proliferative activity of the colonic mucosa.

In conclusion by using cDNA array analysis of 588 cancer associated genes we have identified 14 genes which are highly expressed in the colonic mucosa of normal, cancer and polyp subjects as well as genes which are highly expressed in subsets of subjects. We have also identified 30 genes, which are expressed at significantly different levels in cancer, polyp and normal subjects. Further analysis of these genes and others in a larger number of subjects over a number of time-points are needed to confirm the expression of constitutive and differentially expressed genes identified in this study and will determine if analysis of these genes are useful as markers of cancer risk.

Analysis of gene expression in cancer and polyp subjects before and after intervention with either a synbiotic or placebo revealed a number of genes, the expression of which were altered by synbiotic consumption. Changes in genes

associated with differentiation and tight junction integrity such as TEK, MT3, PDGFRA and protocadherin 1 in subjects who consumed synbiotic may indicate potential mechanisms by which synbiotics reduce cancer risk by modulating intestinal barrier integrity and cytoprotective gene expression. The reduced expression 15 genes in the placebo polyp group and the possible association with proliferation warrants further investigation.

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Chapter 8

Conclusion

Conclusion

Colorectal cancer is the fourth most common cause of death from cancer in the world and the second most common (behind lung cancer) in developed countries. The lifetime colorectal cancer risk in the general population is 5%, but this figure rises dramatically with age: by the age of 70, approximately half the Western population will have developed an adenoma (Fodde *et al.* 2001).

Cancer develops over a long period of time and symptoms of colorectal cancer generally present at a late stage in the disease process. This long time period poses a difficult problem in identifying subjects at risk of CRC and also when studying the relationship between suspected aetiological agents of colon cancer and the end point of tumour formation. The currently available biochemical markers for CRC such as CEA and CA19-9 are not markers of cancer risk and therefore are not particularly useful in cancer risk lowering studies or in evaluating the effectiveness of prevention strategies.

Traditionally colorectal cancer risk has been assessed by monitoring the frequency of polyp relapse, by measuring proliferation or apoptosis in the colonic mucosa or the presence of aberrant crypt foci. Although assessment of these parameters has proven useful in the detection of early stage and recurrent disease, the invasive endoscopic examination and sampling required for their analysis means that measurement of these markers is time consuming and expensive and can not be extended to the general population.

In order to identify at risk subjects as well as early stage cancers, there is a real need for the development of new, non-invasive, reliable markers, predictive of tumour formation, which are altered by changing risk. The ideal biomarker of cancer risk identifies an early alteration during the premalignant phase of neoplasia and predicts with a degree of sensitivity and specificity the future progression and likely prognostic outcome of the disease.

The aim of this study was to investigate a range of potential CRC biomarkers, and to assess their suitability as biomarkers of CRC risk. The influence of synbiotic consumption on these parameters was used to determine their usefulness as biomarkers in intervention studies. It was decided to investigate these potential biomarkers in two study populations from different disease backgrounds; cancer subjects who had defined surgically resected frank cancer and polyp subjects who had previously had polyps removed. Both the cancer and polyp subjects had undergone pre-neoplastic changes, however the cancer subjects had progressed to frank cancer while polyp subjects were considered to be at an intermediate stage.

Before beginning the intervention study it was confirmed that the probiotics employed in this study had the ability to transit the gastrointestinal tract in the presence of the prebiotic so that any observed effect could be associated with synbiotic consumption. In the intervention study synbiotic consumption had a beneficial effect on the gut flora increasing the numbers of lactobacilli and bifidobacteria while decreasing the numbers of putrefactive bacteria such as coliforms and *Cl. perfringens* recovered from the faeces. The altered composition of the faecal flora did not however alter the expression of faecal calprotectin, the

concentration of faecal SCFAs, the cytotoxic potential of FW or serum cholesterol levels during the twelve week intervention period suggesting that these parameters may not be suitable biomarkers in an intervention trial of this type. However significant differences in the number of lactobacilli and enterococci, the cytotoxic potential of FW and the concentration of propionic, isobutyric and isovaleric acids in cancer subjects compared to polyp subjects were observed suggesting that these parameters may be useful as biomarkers of cancer risk.

Natural killer cell activity, IL-12 production by PBMCs and the level of PGE₂ in FW was identified as being significantly different in cancer subjects as compared to polyp subjects. NK cell activity was found to be significantly higher in polyp subjects compared to cancer subjects in this study. Previous studies have reported that high NK cell activity of PBMCs is associated with reduced cancer risk (Imai *et al.* 2000). IL-12 production by PBMCs from cancer subjects was significantly higher than production by PBMCs from polyp subjects in this study. Two studies by O'Hara *et al.* (1998a,b) showed that CRC patients have lower preoperative levels of IL-12 compared to healthy controls and lower levels of IL-12 production was associated with advanced disease. In the current study the levels of PGE₂ in FW of cancer subjects was significantly higher than in FW from polyp subjects. Similarly Lauritsen *et al.* (1984) reported that the levels of PGE₂ in FW from patients with active ulcerative colitis is significantly higher than in FW from healthy volunteers. NK cell activity, IL-12 production and the levels of PGE₂ in FW may have potential as biomarkers for cancer risk, however further analysis in a larger study population is needed.

Prebiotics and probiotics have been reported to have numerous influences on the immune system (Blum & Schiffrin 2003; Cross 2002; Erickson & Hubbard 2000). In contrast in the current study synbiotic consumption did not significantly modulate the systemic immune system. It is however possible that modulation of the immune system by synbiotics occurred in a site specific manner and future studies should investigate the immune response in tissues such as the Peyer's patch and the mesenteric lymph node.

One of the most commonly used cancer risk biomarkers is the proliferative activity of the colonic mucosa. In the current study synbiotic intervention significantly reduced proliferation in the colonic mucosa of polyp subjects suggesting a protective effect of synbiotics on cancer risk. Interestingly synbiotic intervention did not influence the tumour promoting activities (tight junction integrity and invasive potential) of FW of either cancer or polyp subjects. However FW derived from polyp subjects significantly increased tight junction integrity (TER) compared to FW derived from cancer subjects indicating that the composition and / or activity of FW from polyp subjects is significantly different from that of cancer subjects. Also of note was the finding that the activity of the detoxifying enzyme glutathione-S-transferase (GST) was increased in cancer subjects indicating the induction of a putative cancer protective state due to synbiotic consumption. The levels of CEA and CA19-9 were unaltered by synbiotic consumption showing that synbiotic intervention does not adversely affect these commonly used diagnostic tools, thus allowing these markers to be used to monitor possible recurrence of disease when the synbiotic is included in the diet.

The level of genotoxic damage in the colon and in PBMCs and the genotoxic potential of FW are other putative cancer risk biomarkers which have received much attention in recent years. A high level of genotoxic damage in the colon is associated with increased risk of colon cancer (Hambly *et al.* 1997; Pool-Zobel & Leucht 1997) as is highly genotoxic FW (Oßwald *et al.* 2000; Rieger *et al.* 1999). Dietary intervention studies as well as studies with pre-, pro- and synbiotics have been shown to inhibit DNA damage in the colon as well as reduce the genotoxic potential of FW.

In the current study synbiotic consumption was found to protect against *in vivo* genotoxic damage of the colonic mucosa in cancer and polyp subjects, reduce the genotoxic potential of FW derived from polyp subjects, as well as influence the level of DNA damage in PBMCs derived from polyp subjects. Cancer subjects have significantly higher DNA damage in the colonic mucosa and significantly lower damage in PBMCs than polyp subjects. Therefore it seems reasonable to further investigate the usefulness of *in vivo* genotoxic damage as well as the genotoxic potential of FW as both biomarkers of cancer risk and intervention markers. However large-scale studies comparing the levels of the markers in healthy and diseased states are needed, and, as gender, smoking status and genetic polymorphisms seem to play a role in susceptibility to genotoxic damage future studies should be designed, and sufficient participants enrolled, to control for such factors.

Current knowledge points to the fact that cancer develops from the accumulation of a number of mutations in a single cell. In recent years, the advent of cDNA microarray

technology has greatly improved the potential for investigating the molecular mechanisms of cancer and the identification of molecular markers of cancer risk. In the current study we used cDNA array analysis to identify genes which are highly expressed in the colonic mucosa of normal, cancer and polyp subjects. We also identified genes which were differentially expressed in cancer, polyp and normal subjects.

Synbiotic consumption in cancer subjects modulated the expression of genes involved in differentiation and tight junction integrity such as TEK, MT3, PDGFRA and protocadherin 1 as well as cell cycle and apoptosis genes. Interestingly the expression of 15 other genes was reduced in the placebo polyp group but the expression of these same genes were unaltered in the synbiotic group. The possible association of this altered gene expression and the reduction in proliferative activity in the synbiotic polyp group warrants further investigation.

In summary, several putative biomarkers of cancer risk have been identified including immune parameters (NK cell activity, PGE₂ in FW, IL-12 production by PBMCs), the tumour promoting (TER) and genotoxic potential of FW, the level of genotoxic damage in the colonic mucosa and the composition and metabolic activity (SCFA) of the microflora. Synbiotic consumption altered the composition of the microflora to a more beneficial type and modulated gene expression in both cancer and polyp subjects. Synbiotic consumption also significantly reduced the proliferative activity and genotoxic damage in the colonic mucosa, the genotoxic potential of FW and the level of DNA damage in PBMCs in polyp subjects. In cancer subjects synbiotic consumption protected against further DNA damage in the

colonic mucosa and induced the activity of the detoxifying enzyme GST. These results suggest that polyp subjects are more amenable to the protective effects of synbiotics than cancer subjects although beneficial effects are also seen in this group.

Synbiotics offer great potential as a preventative strategy. Future studies in humans should further elucidate the mechanisms involved in their preventative action and their possible role as an early intervention strategy. Studies should be randomised, double-blinded and placebo controlled, and performed in a well-defined population. Careful synbiotic selection from well characterised pre- and probiotics, based on the ability to ameliorate disease progression is essential.

Most cancer risk studies are performed in subjects who have either a personal or family history of cancer and are generally older than 40. Recently researchers have suggested the need for long-term trials (20 to 30 years) in the general population beginning at a much younger age. They hypothesise that many of the changes which predispose to cancer occur at an early age and that traditional trials do not detect these alterations (Dickersin 2005). Such trials, however will be time consuming and expensive and the availability of reliable non-invasive, reproducible biomarkers will be essential, especially with regard to subject recruitment and compliance.

The identification and use of proven CRC biomarkers will be essential in assessing synbiotic efficacy in cancer risk lowering studies. Progress in the area of detection of cancer and the development of early preventive measures, such as synbiotics, will lead to a dramatic change in the morbidity and mortality patterns of colorectal cancer in the developed world.

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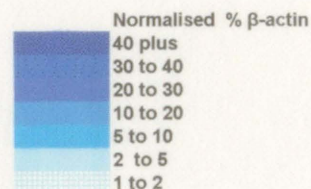
Last but not least, Mam and Dad, who gave me the confidence, and belief that I could be and I could do anything I wanted. Your support, encouragement and belief in me have gotten me this far and I hope that I have made you proud. Grandad, thanks for giving me a bed and putting up with me for the last few months, I promise to go out and get a real job now! Many thanks also to Mary and Pat Barry for giving me a place to stay and for feeding me (too well at times). Finally to Liam, my chauffeur, banker and therapist, who met my mad friends and mad family and was brave enough to stay around. Thank you for your kindness, encouragement and support and for never asking if I was nearly finished. This is it, I'm finally finished!!

Yvonne Clune

Yvonne Clune.

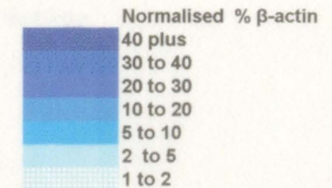
Appendix I

Accession	Gene Name	Cancer Subjects								
Number		Syn05	Syn13	Syn15	Syn30	Syn38	Syn39	Syn58	Syn77	Ave
	Transcription Activators & Repressors									
U76638	BARD1	1.73	1.80	8.09	3.75	1.74	11.62	5.51	2.52	4.59
M96577	E2F1	2.76	3.66	4.06	4.46	4.37	12.12	4.56	2.18	4.77
Y10479	E2F3	7.06	4.35	13.15	13.97	9.51	26.69	15.37	13.41	12.94
U15642	E2F5	2.81	1.37	10.44	4.47	6.20	16.05	7.70	3.97	6.62
M62829	EGR1	12.88	7.79	42.73	21.78	22.20	35.64	27.01	16.30	23.29
M91196	homeo box D3	4.00	1.94	15.19	5.07	4.15	19.59	10.80	2.96	7.96
D11117	ICSBP1	4.54	2.78	8.12	3.95	3.27	20.96	2.30	1.06	5.87
X14454	IRF1	18.42	7.13	49.50	23.79	24.75	28.34	36.27	20.49	26.08
X17620	NME1	135.46	120.85	243.35	137.11	154.67	415.25	229.89	144.76	197.67
L16785	NME2	6.18	2.65	9.23	9.39	9.58	18.41	10.81	9.06	9.41
M96684	PURA	6.53	32.25	15.42	15.05	6.81	12.59	11.72	12.89	14.16
X06538	RARA	14.83	5.88	20.80	12.29	16.84	22.04	21.41	18.78	16.62
X07282	RARB	2.08	2.26	8.22	2.42	2.99	17.05	3.56	3.33	5.24
M24857	RARG	3.37	4.71	6.66	4.33	3.23	15.08	6.56	2.54	5.81
M97935	STAT1	2.77	1.53	5.10	3.86	3.09	6.34	2.73	1.50	3.36
M97934	STAT2	19.27	28.12	9.70	27.70	15.32	26.77	30.27	23.82	22.62
U47686	STAT 5B	4.54	8.30	17.84	7.16	9.34	29.78	13.14	16.62	13.31
U59863	TANK	3.28	2.48	9.86	4.85	4.42	10.41	9.95	2.14	5.92
L23959	TFDP1	11.28	5.56	21.38	10.13	17.29	25.52	12.63	15.54	14.91
U18422	TFDP2	3.48	3.31	7.70	2.27	5.67	16.47	5.96	5.17	6.25
	Cell Cycle									
U33286	CSE1L	3.23	1.83	2.75	5.01	1.90	16.84	3.60	0.80	4.49
L34075	FRAP1	3.84	2.78	8.56	6.16	3.74	8.84	3.32	2.26	4.94
AF007111	Mdm2	2.87	3.34	7.87	3.78	2.12	24.87	6.00	2.63	6.68
Z12020	Mdm4	2.76	1.37	4.90	3.30	2.88	19.40	2.34	2.63	4.95
U63295	SIAH1	1.81	1.62	7.93	2.09	1.54	12.27	4.27	2.31	4.23
Y11416	tumor protein p73	3.73	8.03	4.83	3.28	4.02	17.59	7.62	6.90	7.00
	Cyclins									
X51688	cyclin A2	3.38	2.37	5.82	4.65	2.01	9.21	6.32	3.07	4.60
M25753	cyclin B1	4.33	5.13	11.44	7.45	7.27	26.48	11.69	5.82	9.95
M74091	cyclin C	3.82	1.53	6.10	3.69	3.04	10.56	3.70	1.48	4.24
X59798	cyclin D1	2.70	5.19	14.35	6.53	6.93	15.71	12.73	7.31	8.93
D13639	cyclin D2	8.17	6.78	56.84	8.28	37.88	36.30	70.62	46.36	33.90
M92287	cyclin D3	5.86	3.15	9.11	7.91	5.47	13.34	7.73	2.70	6.91
M73812	cyclin E1	4.01	3.93	9.10	7.90	4.04	9.82	4.57	1.49	5.61
U47413	cyclin G1	4.19	4.91	7.71	7.11	4.82	18.39	6.83	3.08	7.13
U47414	cyclin G2	2.13	1.75	13.08	5.73	3.54	18.95	5.41	1.35	6.49
U11791	cyclin H	6.23	3.34	17.88	10.16	7.27	25.58	17.90	4.77	11.64
	Cell cycle-regulating kinases									
L29222	CDC-like kinase 1	2.83	3.19	10.52	10.58	4.57	20.68	6.64	3.87	7.86
L29216	CDC-like kinase 2	2.72	3.61	16.83	9.41	4.83	12.97	7.83	4.87	7.86
L29220	CDC-like kinase 3	4.97	3.06	11.26	17.33	7.78	17.09	11.59	2.23	9.41
M68520	CDK 2	2.95	3.44	11.09	8.95	4.72	15.56	5.67	2.38	6.84
X66357	CDK 3	3.73	4.10	9.85	8.96	4.04	18.52	4.30	1.58	6.88
M14505	CDK 4	5.94	2.94	16.70	7.49	8.41	19.42	10.72	5.33	9.62
X66364	CDK 5	6.84	5.23	14.79	11.11	12.85	16.28	11.47	7.19	10.72



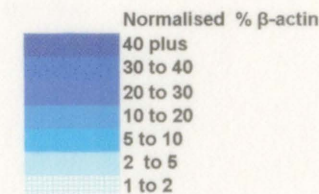
Appendix 1 : Gene expression in 8 cancer subjects. One subject (SYN-39) was excluded from all further analysis as the level of gene expression in this subject was much higher than other cancer subjects and was considered an outlier. For a full list of gene names see **Appendix III**

Accession Number	Gene Name	Cancer Subjects								
		Syn05	Syn13	Syn15	Syn30	Syn38	Syn39	Syn58	Syn77	Ave
	<i>Cell cycle-regulating kinases</i>									
X66365	CDK 6	74.73	73.57	156.78	100.69	111.67	202.01	193.92	126.05	129.93
L20320	CDK 7	2.76	2.23	5.65	7.85	3.21	15.77	5.79	1.70	5.62
L25676	CDK 9	8.43	4.58	12.95	15.32	12.42	24.16	15.46	10.70	13.00
L33264	CDK 10	6.21	5.79	15.61	10.15	12.34	26.87	10.28	9.73	12.12
X66358	CDKL1	4.20	3.77	11.95	6.02	2.76	18.38	5.27	1.98	6.79
X05360	CDC2	5.60	3.37	10.49	11.10	4.98	22.02	5.90	1.75	8.15
M80629	CDC2L5	2.83	5.03	16.41	6.64	4.62	20.58	18.24	5.78	10.01
X60188	MAPK3	31.60	20.72	79.20	30.90	43.92	44.64	45.01	34.93	41.36
U02687	FLT3	2.98	4.98	6.93	4.98	6.07	14.07	4.94	3.72	6.08
X59727	MAPK4	2.68	2.39	7.14	2.86	1.55	20.79	2.62	1.93	5.24
X80692	MAPK6	4.60	2.92	11.55	5.90	3.62	15.89	7.99	3.05	6.94
U25278	MAPK7	3.80	3.09	8.71	8.54	3.60	11.79	6.84	4.32	6.33
X79483	MAPK12	3.59	3.72	10.83	8.30	5.19	12.52	5.04	4.79	6.75
L35253	MAPK14	6.73	9.76	15.06	11.54	13.06	21.26	18.95	14.40	13.84
L05624	MAP2K1	4.75	3.17	9.34	7.47	6.90	12.52	7.74	2.97	6.86
U25265	MAP2K5	7.15	8.06	23.35	14.52	10.87	24.10	22.09	2.37	14.06
U39657	MAP2K6	4.30	5.90	11.39	7.34	4.09	12.22	6.66	3.67	6.94
U78876	MAP3K3	3.94	1.93	12.24	4.06	4.78	17.94	5.12	3.05	6.63
U01038	PLK1	3.39	3.39	9.19	5.04	3.39	15.71	5.58	2.81	6.06
	<i>CDK inhibitors</i>									
U09579	CDKN1A	51.31	53.17	117.82	71.72	60.47	89.09	103.34	67.90	76.85
U22398	CDKN1C	3.29	1.44	6.54	4.43	5.95	17.65	8.94	4.44	6.58
L27211	CDKN2A	5.28	34.72	5.33	9.37	8.25	27.16	8.54	12.63	13.91
U17075	CDKN2B	4.14	2.59	8.46	6.29	2.69	20.73	4.84	4.07	6.73
U40343	CDKND	11.67	5.21	14.57	23.62	16.31	24.76	11.35	12.47	14.99
U10564	WEE1	5.56	3.77	21.92	8.14	11.40	25.75	48.41	14.35	17.41
	<i>Other Cell Cycle Controls</i>									
U77949	CDC6	2.65	2.40	5.52	6.25	3.37	13.84	5.01	1.91	5.12
S72008	CDC10	8.35	19.98	52.43	14.57	17.83	51.13	37.65	21.83	27.97
U18291	CDC16	4.54	2.31	11.81	9.63	5.73	25.63	7.70	4.08	8.93
U63131	CDC37	18.82	10.04	38.28	24.97	31.00	25.85	31.45	22.35	25.34
S57153	ARID4A	3.90	4.21	16.41	3.27	4.20	24.70	9.55	1.44	8.46
M81933	CDC25A	3.59	2.24	9.48	6.15	2.39	27.31	5.18	2.65	7.37
M81934	CDC25B	4.88	2.35	12.59	11.43	7.19	13.30	13.09	7.75	9.07
M34065	CDC25C	2.85	9.33	7.31	7.27	3.08	17.24	8.07	3.12	7.28
U00001	CDC27	8.31	11.17	30.48	20.06	15.24	50.23	29.57	4.65	21.21
X80343	CDK5R1	1.84	2.19	6.50	8.77	3.06	8.03	5.30	3.16	4.85
U34051	CDK5R2	2.23	1.59	4.21	5.92	3.93	13.96	9.60	1.12	5.32
U66469	CGRRF1	2.27	1.98	7.35	2.79	2.40	9.61	5.58	1.37	4.17
L13698	GAS1	2.04	1.47	7.30	5.49	3.32	19.18	3.31	2.95	5.63
S66431	JARID1A	4.61	3.35	7.91	7.84	4.14	14.81	6.96	3.19	6.60
D13365	MT3	13.47	9.37	16.48	30.21	31.84	19.35	31.35	45.94	24.75
D63878	NEDD5	6.93	7.06	20.04	17.66	10.45	37.36	21.11	10.15	16.34
U49070	PIN1	5.21	6.40	14.85	13.67	12.29	9.16	17.12	10.31	11.12
X85134	RBBP5	6.73	8.43	15.01	5.32	7.40	17.56	13.06	9.77	10.41



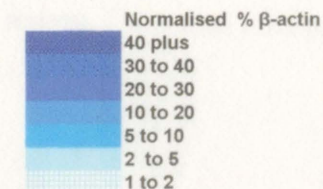
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Accession	Gene Name	Cancer Subjects								
Number		Syn05	Syn13	Syn15	Syn30	Syn38	Syn39	Syn58	Syn77	Ave
	<i>Other Cell Cycle Controls</i>									
X85133	RBBP6	3.09	3.51	10.92	8.77	6.63	20.98	6.65	8.42	8.62
U58334	TP53BP2	2.41	1.41	9.56	7.47	3.57	12.65	5.18	1.22	5.43
	<i>Cell Adhesion Receptors/Proteins</i>									
M81104	CD34	3.34	2.10	10.31	8.38	3.93	20.26	9.54	2.85	7.59
Z24680	GARP	2.35	0.91	5.10	3.33	2.12	13.67	7.89	1.28	4.58
	<i>Matrix Adhesion Receptors</i>									
M24795	CD36	2.46	4.59	6.42	2.20	3.27	18.56	5.86	2.86	5.78
M14219	decorin	5.77	5.02	12.32	12.73	10.71	29.97	10.56	11.52	12.32
X56654	desmoglein 1	2.10	1.62	10.12	6.15	3.28	26.01	3.80	0.87	6.74
Z26317	desmoglein 2	11.84	5.91	15.22	9.60	15.46	19.87	11.30	8.91	12.26
U43901	LAMR1	25.21	6.41	28.73	12.89	31.33	34.31	36.38	22.24	24.68
U14588	paxillin	31.56	36.97	58.76	36.13	45.02	35.13	49.45	40.68	41.71
L12350	thrombospondin 2	3.57	2.32	8.62	6.99	2.65	19.52	6.19	2.80	6.58
	<i>Cell-Cell Adhesion Receptors</i>									
M34064	cadherin 2	2.15	1.02	4.77	3.66	4.56	14.45	4.59	1.91	4.64
X63629	cadherin 3	2.56	2.09	3.53	3.83	3.38	11.84	9.67	2.02	4.86
L34059	cadherin 4	3.61	3.07	8.94	3.14	3.09	13.68	8.53	2.86	5.86
X79981	cadherin 5	87.11	79.42	198.09	108.22	129.79	213.14	174.64	136.80	140.90
D31784	cadherin 6	2.56	3.70	10.03	3.66	4.64	14.36	5.27	4.96	6.14
L34060	cadherin 8	2.76	4.82	6.50	2.84	3.23	20.18	5.48	1.72	5.94
L34056	cadherin 11	1.77	3.60	7.12	6.02	4.28	12.38	6.69	1.73	5.45
L34057	cadherin 12	2.47	2.62	8.39	4.79	1.56	17.15	7.35	1.80	5.77
L34058	cadherin 13	2.96	3.01	9.20	5.78	2.95	19.43	5.62	3.35	6.53
D83542	cadherin 15	2.03	0.89	9.27	3.36	3.29	8.79	3.32	2.20	4.14
D13866	catenin alpha 1	9.70	12.01	9.96	18.13	13.24	22.68	15.45	13.35	14.31
M94151	catenin alpha 2	3.91	11.35	8.53	3.67	4.84	23.02	4.74	4.70	8.09
X87838	catenin beta 1	18.86	14.20	42.19	20.32	26.15	35.52	33.92	26.95	27.26
X72925	desmocollin 1	1.81	1.14	5.14	2.53	2.57	13.15	2.51	1.76	3.82
X56807	desmocollin 2	2.47	1.74	9.05	3.44	1.25	17.05	3.95	1.42	5.05
X83929	desmocollin 3	2.46	5.57	8.10	3.24	1.59	13.60	4.28	3.13	5.24
M77830	desmoplakin	17.02	19.04	52.89	21.53	31.61	45.50	44.33	32.02	32.99
U53786	envoplakin	4.38	3.39	7.73	6.00	7.49	15.68	4.96	6.04	6.96
M74387	L1CAM	3.20	2.01	9.27	5.99	4.91	20.58	6.82	3.00	6.97
X16841	NCAM1	2.54	0.82	8.49	3.09	2.90	7.09	6.11	1.04	4.01
U72661	ninjurin 1	3.98	3.03	7.40	3.89	5.25	19.05	8.88	3.19	6.83
L34774	OPCML	4.90	1.63	7.19	3.79	2.29	12.80	5.98	1.92	5.06
U52111	plexin B3	2.25	4.45	5.20	3.55	3.26	19.21	7.22	2.78	5.99
AAA36418	protocadherin 1	22.99	8.74	15.00	22.35	20.89	20.05	15.93	24.03	18.75
L11373	PCDHGC3	3.72	2.94	14.27	6.50	6.90	19.63	15.08	9.32	9.79
U60800	SEMAD4	3.82	1.92	9.34	3.33	2.86	13.70	3.95	3.13	5.25
X85978	TAX1	1.76	1.38	8.83	3.29	2.08	12.96	3.74	2.05	4.51
	<i>Integrins</i>									
X68742	integrin, alpha 1	2.56	2.63	11.10	7.38	3.75	19.34	7.99	2.47	7.15
M28249	integrin, alpha 2	3.86	3.20	4.75	3.54	3.32	18.66	5.31	2.50	5.64
M59911	integrin, alpha 3	15.79	16.64	46.68	19.27	25.98	17.26	17.26	29.38	23.90
L12002	integrin, alpha 4	3.20	7.56	9.27	6.64	2.95	21.67	6.23	5.66	7.90



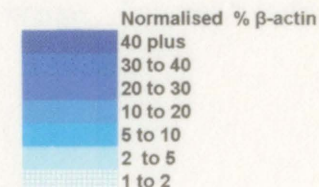
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Number		Syn05	Syn13	Syn15	Syn30	Syn38	Syn39	Syn58	Syn77	Ave
	<i>Integrins</i>									
X06256	integrin, alpha 5	3.84	2.30	6.47	4.60	2.84	29.28	11.14	2.44	7.86
X53586	integrin, alpha 6	23.80	24.48	78.59	29.99	40.68	30.28	67.01	35.90	41.34
X74295	integrin, alpha 7	2.56	2.72	6.68	4.87	3.44	11.54	6.82	3.60	5.28
L36531	integrin, alpha 8	2.87	0.90	11.48	8.12	4.76	15.69	7.15	1.50	6.56
D25303	integrin, alpha 9	2.59	1.70	7.27	4.37	3.90	10.89	6.09	4.23	5.13
L25851	integrin, alpha E	3.93	2.14	7.62	5.48	3.41	9.05	8.66	2.94	5.40
M34189	integrin, beta 1	2.31	0.73	10.70	6.60	1.34	15.40	8.92	1.90	5.99
J02703	integrin, beta 3	3.84	2.25	8.05	20.89	5.05	24.63	6.70	3.97	9.42
U37139	integrin beta 3 (BP)	2.00	2.57	7.03	3.82	2.51	14.49	4.90	1.37	4.83
X53587	integrin, beta 4	24.93	16.99	61.43	35.26	43.44	26.61	37.59	53.03	37.41
J05633	integrin, beta 5	4.92	4.13	11.14	18.79	3.37	7.17	7.67	3.37	7.57
M35198	integrin, beta 6	4.58	3.94	11.61	7.15	5.03	13.34	8.59	5.08	7.41
M62880	integrin, beta 7	8.44	8.60	50.57	21.59	16.78	42.09	19.87	19.60	23.44
M73780	integrin, beta 8	8.69	12.64	21.79	14.83	9.61	41.71	28.20	8.45	18.24
	<i>Immune System Proteins</i>									
M34671	CD59	102.83	104.61	221.03	97.81	98.46	216.75	197.48	101.19	142.52
M11886	HLA-C	89.99	45.04	80.99	98.63	64.12	53.09	72.15	97.87	75.23
M21626	TRAV14DV4	2.94	1.56	10.48	4.81	1.76	12.40	5.53	1.68	5.14
	<i>Oncogenes & Tumor Suppressors</i>									
U23435	abl-interactor 2	3.51	1.26	8.30	5.34	4.03	8.59	5.72	1.38	4.76
M74088	APC	8.17	8.56	27.89	5.48	11.51	36.07	18.26	16.53	16.56
U43746	BRCA2	3.14	24.32	7.01	3.81	2.05	23.74	4.15	2.06	8.78
X03663	CSF1R	8.20	4.42	10.09	11.32	12.15	18.90	10.57	17.02	11.58
X76132	DCC	4.03	2.79	16.87	6.97	4.27	18.50	7.10	5.06	8.20
U49089	DLG3	16.89	14.61	70.72	29.31	37.66	67.48	73.31	42.17	44.02
AF010314	ENC1	2.52	1.48	6.57	3.09	1.23	16.18	4.24	1.64	4.62
X00588	EGFR	9.31	11.31	33.36	18.74	19.26	19.48	12.25	20.66	18.04
M99487	FOLH1	3.66	2.39	9.61	2.81	1.81	18.86	5.72	1.24	5.76
M19722	FGR	5.79	2.21	4.75	7.53	1.79	8.30	5.79	2.46	4.83
L29511	GRB2	45.40	29.08	149.49	61.80	66.71	104.58	112.04	64.44	79.19
AF001954	ING1	6.12	2.90	12.66	13.30	7.30	17.13	6.82	2.58	8.60
M29039	JUNB	5.50	2.58	6.68	5.90	4.80	16.11	5.28	3.39	6.28
U43527	KISS1	2.93	1.85	4.67	3.01	2.12	10.20	4.96	1.14	3.86
X06182	KIT	3.02	1.43	5.97	7.88	2.81	21.30	3.14	1.59	5.89
X13967	LIF	3.80	4.06	12.90	3.93	4.06	12.93	7.68	3.42	6.60
AF010312	LITAF	36.74	29.52	91.03	41.01	62.59	80.74	90.05	56.22	60.99
J02958	MET	5.68	4.24	14.88	4.40	6.96	10.60	11.71	4.59	7.88
U35113	MTA1	5.81	3.61	11.86	5.43	6.12	19.25	9.13	6.60	8.47
U68162	MPL	2.86	2.58	8.07	2.40	2.69	14.35	2.54	2.28	4.72
AF010315	p53-induced protein	3.02	2.08	6.83	2.68	1.73	19.38	7.79	1.69	5.65
X02811	PDGFB	3.53	1.52	12.05	2.08	4.12	21.88	3.93	1.31	6.30
D89667	prefoldin 5	43.52	44.16	83.18	40.83	51.75	130.57	69.84	52.98	64.60
AF010310	PRODH	2.75	2.56	4.95	3.46	2.33	9.52	4.24	1.20	3.87
AF010316	PTGES	3.50	1.83	6.51	4.02	2.23	12.22	3.98	1.19	4.43
AF010309	TP5313	4.19	4.18	12.61	7.62	6.29	18.77	7.32	2.09	7.88
L25080	RHOA	122.79	128.04	256.75	149.57	150.35	441.82	255.09	185.46	211.23



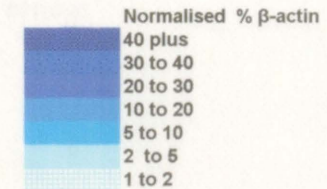
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	Oncogenes & Tumor Suppressors									
L25081	RHOC	58.05	39.56	105.63	37.14	66.10	66.82	77.67	61.00	64.00
D85815	RHOD	2.36	1.51	4.98	2.39	2.83	16.83	3.27	1.79	4.49
X95282	RHOE	7.81	7.52	27.92	10.03	21.53	51.47	39.89	24.70	23.86
X61587	RHOG	4.85	3.98	7.84	6.96	5.44	23.93	8.48	4.83	8.29
Z35227	RHOH	3.06	4.98	4.57	1.71	3.14	15.75	4.49	1.57	4.91
M31213	oncogene RET	2.27	1.27	6.64	5.35	2.67	17.61	6.00	0.94	5.34
M15400	RB1	1.45	4.17	11.92	4.85	5.48	12.23	6.94	0.90	5.99
X74594	RBL2	4.44	2.95	16.53	12.18	7.19	13.59	10.76	2.65	8.78
U16296	TIAM1	3.34	2.05	8.63	3.91	3.02	15.93	8.03	2.97	5.98
Z29083	TPBG	3.93	1.43	8.91	6.50	3.43	10.23	1.47	1.48	4.67
M14694	tumor protein p53	3.42	1.60	10.91	6.65	2.28	30.03	4.44	1.44	7.59
M35296	ABL2	3.60	3.20	5.30	7.31	4.41	26.68	4.32	3.11	7.24
M63167	AKT1	2.43	1.02	7.05	4.24	2.11	11.57	4.23	1.24	4.23
M77198	AKT2	3.00	2.08	8.58	5.59	1.59	5.52	4.19	1.08	3.95
M11730	ERBB2	8.28	1.34	13.69	7.35	8.82	12.66	9.85	9.07	8.88
M29366	ERBB3	29.73	51.95	34.19	16.77	27.15	25.21	43.68	32.87	32.69
L07868	ERBB4	3.84	2.07	8.50	3.08	2.27	23.12	3.76	1.73	6.05
K00650	FOS	2.63	1.96	7.97	5.21	3.36	7.94	3.40	2.68	4.39
M54968	KRAS2	3.74	1.40	6.24	4.33	1.83	16.36	6.21	1.04	5.14
M13228	MYCN	2.16	1.87	3.41	2.94	3.60	16.45	3.18	2.11	4.46
M95712	BRAF	8.39	9.00	30.71	7.92	13.94	50.00	32.99	19.42	21.54
X03484	RAF1	7.04	4.93	19.72	11.59	9.53	13.12	10.46	10.66	10.88
NM_005417	SRC	3.43	2.49	7.18	12.26	4.89	13.68	8.20	2.93	6.88
M15990	YES1	6.46	2.82	22.81	8.22	8.69	31.28	20.15	7.25	13.46
	Extracellular Matrix Proteins									
M55172	aggrecan 1	9.80	51.42	77.91	63.71	34.81	124.34	21.15	33.54	52.08
J04599	biglycan	3.35	2.30	11.41	8.30	4.49	12.87	5.48	2.60	6.35
U16306	CSPG2	1.78	3.00	7.21	3.36	2.17	11.26	3.14	1.46	4.17
X02761	fibronectin 1	25.96	51.11	57.98	29.82	45.91	67.11	73.38	67.72	52.37
M85289	HSPG2	3.91	2.34	12.17	8.35	8.18	14.35	10.23	6.90	8.30
X78565	tenascin C	3.94	1.89	13.34	5.86	3.57	19.91	4.94	3.09	7.07
U86759	netrin 2-like	4.10	3.45	11.99	4.11	3.03	18.23	4.98	2.38	6.53
M30269	nidogen	6.77	2.10	9.57	6.96	4.52	16.86	9.65	3.35	7.47
J03040	SPARC	15.08	10.45	14.31	25.31	22.41	19.03	40.74	22.59	21.24
X98085	tenascin R	4.66	3.53	33.03	17.38	5.25	26.50	4.52	5.24	12.51
X14787	thrombospondin 1	2.53	1.63	10.45	4.60	2.74	13.44	5.14	1.85	5.30
X03168	vitronectin	5.31	5.46	31.76	21.00	12.27	46.94	10.69	14.48	18.49
	Trafficking/Targeting Proteins									
X55525	COL1A2	3.13	2.42	6.35	9.32	2.30	18.26	5.43	3.06	6.28
X16468	COL2A1	4.91	10.49	15.95	11.45	7.78	25.59	17.06	6.02	12.40
X14420	COL3A1	2.79	2.78	10.49	6.98	1.48	12.33	4.72	2.58	5.52
X05610	COL4A2	3.58	1.90	9.99	5.23	2.34	16.42	5.87	2.45	5.97
M92993	COL4A3	3.42	3.47	12.23	12.77	4.23	25.19	8.19	3.50	9.12
D21337	COL4A6	5.11	4.12	12.00	11.39	2.44	20.76	8.99	4.24	8.62
X15879	COL6A1	13.41	4.25	19.32	18.71	13.22	30.29	17.97	15.81	16.62
M34570	COL6A2	4.59	6.31	6.92	16.15	6.51	37.39	8.64	5.08	11.45



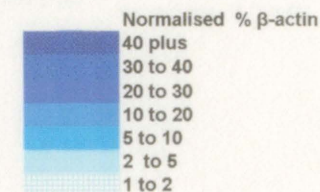
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	Trafficking/Targeting Proteins									
X52022	COL6A3	7.65	3.05	17.03	11.15	8.69	22.03	12.91	12.84	11.92
X57527	COL8A1	3.03	1.59	10.71	4.54	1.93	16.82	6.31	2.04	5.87
J04177	COL11A1	3.68	3.38	7.89	3.87	3.38	13.02	7.97	2.45	5.70
U32169	Col11A2	3.53	1.30	8.68	2.62	3.23	19.47	7.38	1.38	5.95
M92642	COL16A1	5.53	2.20	16.71	8.17	8.73	24.93	12.62	9.60	11.06
L22548	COL18A1	4.65	2.90	9.03	6.49	2.97	9.05	5.53	3.43	5.50
M61916	laminin, beta 1	7.58	5.18	10.63	12.56	8.29	25.61	12.38	10.28	11.56
S77512	laminin, beta 2	2.95	2.15	8.52	4.19	7.00	16.82	4.61	3.12	6.17
X70904	laminin, alpha 4	4.11	2.24	7.72	4.41	3.54	18.48	7.07	2.41	6.24
J03202	laminin, gamma 1	4.65	4.05	6.64	4.08	3.28	15.24	5.58	1.81	5.66
X97442	TMP21	7.66	13.84	37.32	22.72	20.01	23.32	35.77	20.61	22.65
	Metabolism									
X01677	GAPD	363.82	306.93	689.19	198.86	370.81	961.75	619.95	342.96	481.78
U90313	GSTO1	24.06	38.61	73.14	51.25	56.82	108.80	76.40	45.23	59.29
V00530	HPRT1	3.21	3.00	11.27	4.05	3.04	16.87	7.75	4.00	6.65
Y07604	NME4	11.51	6.67	20.09	17.69	19.20	23.58	13.60	17.89	16.28
M13267	SOD1	42.44	49.12	84.75	62.96	48.09	49.98	78.91	37.83	56.76
	Ribosomal Proteins									
X65923	FAU	39.01	20.17	45.33	46.18	45.76	49.56	33.44	46.21	40.71
X56932	ribosomal protein L13a	164.63	160.62	272.06	175.14	187.00	508.59	255.09	154.22	234.67
U14971	ribosomal protein S9	52.19	80.33	74.30	55.92	71.94	75.81	83.03	52.21	68.21
	Apoptosis Associated Proteins									
	<i>Death Receptor-Associated Proteins & Adaptors</i>									
U84388	CRADD	2.59	2.16	7.16	2.59	4.31	13.19	8.79	2.96	5.47
U82938	SIVA	4.24	1.08	7.04	3.17	2.23	14.47	6.29	1.74	5.03
AF015956	DAXX	5.37	2.12	3.37	8.13	3.76	11.11	6.88	6.30	5.88
L41690	TRADD	6.42	3.74	6.21	4.56	5.94	22.11	9.98	8.91	8.48
U78798	TRAF6	4.64	8.30	12.13	3.69	3.39	14.38	12.24	3.69	7.80
U90875	TNFRSF10A	2.52	0.65	7.85	1.64	2.67	13.73	2.45	1.36	4.11
	<i>Caspases</i>									
U13699	caspase 1	5.86	8.15	20.96	10.82	10.06	26.28	12.79	3.77	12.33
U13021	caspase 2	7.80	6.26	12.86	12.43	10.78	15.56	15.69	9.29	11.33
U13737	caspase 3	12.13	18.52	32.55	18.44	20.70	30.78	31.06	20.00	23.02
U28014	caspase 4	12.14	19.65	44.52	15.83	20.31	43.96	28.25	25.25	26.24
U20537	caspase 6	4.19	2.68	10.59	4.18	2.16	11.17	7.41	2.90	5.66
U37448	caspase 7	4.05	4.67	13.41	12.58	3.40	10.93	6.48	4.42	7.49
X98176	caspase 8	2.27	1.19	8.19	2.66	1.97	9.21	5.24	1.72	4.06
U56390	caspase 9	6.16	1.77	19.53	15.72	7.21	12.99	7.19	8.34	9.86
U60519	caspase 10	5.21	2.09	7.49	8.34	8.47	19.29	15.76	16.24	10.36
	<i>BCL2 Family Proteins</i>									
U23765	BAK	12.53	5.98	9.95	12.20	13.49	22.34	15.26	19.59	13.92
U66879	BAD	5.12	8.58	8.10	3.63	4.25	14.57	5.25	4.95	6.81
S83171	BAG1	15.70	19.80	26.37	19.61	22.20	40.09	41.25	26.31	26.41
L22474	BAX	9.04	4.55	15.11	11.92	13.81	12.22	7.74	9.37	10.47
M14745	BCL2	3.29	2.11	15.18	8.24	3.99	19.38	5.49	2.14	7.48
U29680	BCL2A1	1.83	1.21	4.62	6.51	3.80	11.75	4.52	1.32	4.44



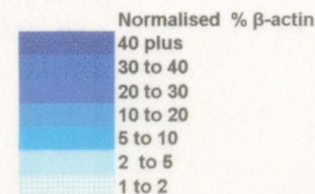
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	<i>BCL2 Family Proteins</i>									
U59747	BCL2L2	4.78	1.86	7.68	3.88	4.67	11.52	4.11	3.18	5.21
U34584	BIK	3.44	1.96	8.77	3.60	4.22	13.84	3.75	2.58	5.27
S82185	BRAG-1	3.22	2.27	8.17	2.50	2.16	16.70	3.18	1.43	4.95
L08246	MCL1	24.66	14.02	58.29	24.96	28.04	24.61	66.85	39.64	35.13
	<i>Death Kinases</i>									
X76104	DAPK1	8.41	7.07	17.68	10.07	15.22	16.73	14.19	17.57	13.36
Y10256	MAP3K14	3.79	1.69	10.77	10.20	8.01	15.85	7.32	6.20	7.98
X66363	PCTK1	6.48	3.64	11.63	15.28	9.05	12.04	9.33	11.40	9.85
X66360	PCTK2	2.48	1.53	11.14	4.80	2.97	18.17	5.23	1.20	5.94
X66362	PCTK3	3.20	3.54	11.03	8.64	13.67	13.43	10.78	7.55	8.98
	<i>Other Apoptosis-Associated Proteins</i>									
U45879	BIRC2	2.87	1.36	5.06	2.35	5.32	16.27	2.94	1.86	4.75
U45878	BIRC3	3.00	1.84	4.81	4.60	4.35	11.53	5.72	2.00	4.73
U45880	BIRC4	3.79	1.64	11.33	2.28	2.81	12.13	5.07	1.97	5.13
U75285	BIRC5	2.51	1.48	6.46	2.78	2.60	9.20	4.56	2.08	3.96
AF010127	CFLAR	2.50	2.02	7.21	2.43	6.33	18.16	8.46	4.31	6.43
U18321	DAP3	4.51	1.75	11.30	8.69	7.73	11.29	7.86	5.55	7.33
U91985	DFFA	2.82	1.74	3.66	4.47	2.64	14.49	4.07	1.88	4.47
S40706	DDIT3	10.89	45.55	30.84	23.00	26.72	65.91	37.28	29.19	33.67
S78085	PDCD2	9.00	13.40	21.49	8.58	9.96	28.57	20.25	10.66	15.24
AF022385	PDCD10	2.01	0.79	3.83	3.77	5.33	23.99	5.21	2.21	5.89
U25994	RIPK1	4.47	5.56	11.75	10.63	9.76	9.79	6.99	2.99	7.74
	<i>RNA processing, turnover, and transport</i>									
U12431	ELAVL2	3.64	32.73	6.03	10.04	3.22	16.38	4.56	3.11	9.96
	<i>Cell Receptors</i>									
	<i>Growth Factor & Chemokine Receptors</i>									
L17075	ACVRL1	5.30	2.42	8.06	6.96	10.20	21.54	9.43	5.29	8.65
M60459	EPOR	3.72	2.83	3.96	4.25	1.97	15.79	6.69	1.82	5.13
X68203	FLT4	4.38	3.32	13.51	4.26	3.86	30.10	4.52	4.01	8.51
L37882	FZD2	1.56	1.68	9.20	6.38	2.26	17.73	4.17	2.08	5.63
U28811	GLG1	8.75	6.94	23.08	19.52	17.11	19.54	16.45	16.32	15.96
X04434	IGF1R	3.38	3.26	8.15	4.09	3.79	18.60	4.28	3.04	6.07
Y00285	IGF2R	13.86	13.82	24.98	24.50	19.20	14.63	24.10	20.11	19.40
M35410	IGFBP2	25.38	4.38	9.72	19.76	16.25	11.88	11.87	4.57	12.97
U12140	NTRK2	2.93	12.16	30.94	6.97	9.61	15.93	9.97	6.02	11.81
U05012	NTRK3	3.60	0.81	10.77	1.84	2.40	18.54	2.14	2.54	5.33
M99437	Notc2 homolog 2	12.74	10.49	31.85	18.09	19.75	26.53	18.71	19.79	19.75
U77493	Notch homolog 2	3.14	5.69	4.14	5.99	5.37	32.11	3.53	3.05	7.87
U95299	Notch homolog 4	4.51	9.06	7.74	10.26	6.66	23.09	5.52	4.83	8.96
U43148	PTCH	2.65	2.59	4.19	3.00	2.49	14.21	5.24	2.55	4.61
M21574	PDGFRA	4.64	11.68	9.08	5.12	10.29	21.73	28.63	12.64	12.97
M21616	PDGFRB	2.70	1.46	9.45	4.67	2.89	13.10	3.40	2.87	5.06
L07594	TGFBR3	2.60	2.11	12.00	6.75	5.79	12.87	4.13	2.18	6.05
X85960	TRK-fused gene	13.28	19.57	62.05	21.47	18.42	31.91	66.51	20.08	31.66
AF016268	TNFRSF10B	3.74	12.17	9.85	3.44	3.27	10.55	5.11	3.48	6.45
Y09392	TNFRSF25	7.13	4.05	25.35	14.20	10.55	18.95	19.40	11.25	13.86



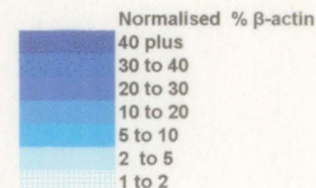
Appendix I : Gene expression in 8 cancer subjects. One subject (SYN-39) was excluded from all further analysis as the level of gene expression in this subject was much higher than other cancer subjects and was considered an outlier. For a full list of gene names see **Appendix III**

Accession Number	Gene Name	Cancer Subjects								
		Syn05	Syn13	Syn15	Syn30	Syn38	Syn39	Syn58	Syn77	Ave
	<i>Growth Factor & Chemokine Receptors</i>									
M33294	TNFRSF1A	9.87	5.68	15.44	14.19	13.57	17.66	14.94	10.98	12.79
M32315	TNFRSF1B	4.12	10.89	13.19	4.22	8.01	14.03	5.80	6.16	8.30
X60592	TNFRSF5	3.76	3.37	8.76	8.77	3.87	13.45	4.08	2.95	6.13
Z70519	TNFRSF6	3.82	6.39	11.86	8.33	4.21	9.04	4.57	2.23	6.30
	<i>FGF receptors</i>									
M37722	FGFR1	3.85	4.06	9.94	6.90	8.82	19.90	9.67	9.65	9.10
U11814	FGFR2	3.01	3.13	10.89	4.17	5.97	11.20	5.51	2.57	5.80
M58051	FGFR3	2.70	2.22	9.14	3.90	1.92	23.90	3.44	2.16	6.17
	<i>Ephrin receptors</i>									
M59371	EphA2	7.31	9.91	14.98	9.58	8.40	8.78	22.78	12.80	11.82
L40636	EphB1	1.62	1.17	9.63	2.48	1.84	14.31	3.90	2.09	4.63
L41939	EphB2	3.61	2.32	6.89	2.11	1.31	11.75	6.63	1.42	4.50
U07695	EphB4	2.57	0.92	8.44	2.09	2.23	7.13	3.80	1.37	3.57
	<i>Interleukin Receptors</i>									
M63099	interleukin 1 receptor	3.47	1.97	4.91	3.77	3.96	17.06	8.19	3.19	5.81
M20566	interleukin 6 receptor	6.02	5.77	13.44	7.11	5.33	6.82	8.76	3.95	7.15
	<i>Interferon receptors</i>									
J03171	IFNAR1	3.97	2.69	16.42	2.77	4.00	26.17	5.98	4.22	8.28
X77722	IFNAR2	2.70	1.58	9.13	2.80	1.98	16.00	12.45	2.05	6.08
A09781	IFNGR	2.18	1.64	10.04	4.31	2.33	13.59	3.58	1.44	4.89
J03143	IFNGR1	3.38	2.36	15.61	4.68	3.81	14.75	7.05	3.00	6.83
U05875	IFNGR2	7.98	6.08	36.01	7.86	17.69	40.17	31.41	16.57	20.47
	<i>Hormone Receptors</i>									
X52773	RXRA	8.41	3.71	7.82	7.76	7.90	10.65	5.94	4.27	7.05
M84820	RXRB	13.24	24.47	22.82	26.07	19.10	23.81	17.31	19.04	20.73
	<i>Protein Kinases</i>									
X61656	KDR	3.46	1.49	9.22	4.44	3.69	13.66	5.40	2.37	5.47
U33635	PTK7	3.69	1.41	5.79	2.83	2.25	15.64	7.16	2.76	5.19
Y09028	NTRK1	6.13	29.89	9.54	9.22	9.03	14.73	9.53	8.56	12.08
D17517	TYRO3	4.17	3.36	9.89	6.72	4.12	11.15	7.13	3.59	6.26
	<i>Other Receptors</i>									
X95425	EPHA5	2.47	1.35	3.68	3.16	1.70	13.00	3.89	1.53	3.85
U43318	FZD5	1.59	2.34	5.00	2.06	1.72	17.12	2.46	2.80	4.38
U82169	FZD9	1.79	2.49	5.15	2.54	2.19	20.45	8.45	1.67	5.59
X02492	GIP3	4.46	3.45	12.49	6.04	6.42	30.83	7.60	6.08	9.67
X13916	LRP1	6.15	11.91	15.57	6.26	12.69	15.69	15.27	13.24	12.09
U04441	LRP2	1.92	2.02	6.31	1.93	3.14	14.07	5.56	1.97	4.61
M14764	NGFR	3.66	3.07	11.30	3.21	2.96	9.97	5.19	1.64	5.12
U08839	PLAUR	3.14	2.22	9.51	3.04	4.32	16.30	5.88	3.64	6.00
X87852	SEX gene	5.43	11.18	12.59	7.92	9.74	25.27	20.51	14.61	13.40
X60957	TIE	3.20	2.10	7.42	3.27	4.26	13.89	4.63	4.02	5.35
	<i>Cell Signaling, Extracellular Communication Proteins</i>									
	<i>Neuropeptides</i>									
L19063	GDNF	2.52	1.56	6.33	5.01	2.23	11.04	6.88	2.84	4.80
X52599	NGFB	2.16	2.69	9.84	3.15	2.19	13.98	8.70	2.09	5.60
M73980	Notch homolog 1	5.33	8.10	5.95	7.10	6.03	15.00	9.00	6.97	7.93
S41541	NTF6G	4.03	1.06	9.08	9.59	5.16	21.03	5.27	2.99	7.27
X53655	neurotrophin 3	4.34	2.00	3.41	4.65	5.04	22.54	3.55	4.22	6.22



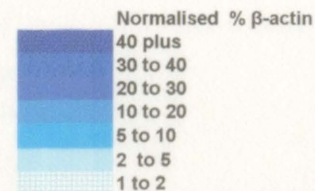
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Accession Number	Gene Name	Cancer Subjects								
		Syn05	Syn13	Syn15	Syn30	Syn38	Syn39	Syn58	Syn77	Ave
	<i>Growth Factors, Cytokines & Chemokines</i>									
M30704	amphiregulin	1.95	2.62	4.61	2.62	2.41	11.49	4.41	2.19	4.04
M15530	B-cell growth factor 1	2.63	1.29	5.42	5.03	2.16	16.50	5.88	2.43	5.17
M61176	BDNF	2.65	1.63	8.39	5.19	1.94	13.79	3.49	2.80	4.98
M73238	CNTFR	2.18	2.38	7.00	3.29	2.64	13.66	3.21	2.94	4.66
M92934	CTGF	2.24	2.27	8.49	3.79	2.16	20.87	5.10	2.31	5.88
U15979	DLK1	2.22	1.76	6.50	2.64	3.01	22.55	3.14	2.75	5.57
M60278	DTR	4.58	2.80	11.70	3.91	4.52	12.01	13.04	6.86	7.42
U49262	DVL3	2.28	1.98	3.90	3.71	2.89	15.37	2.73	1.61	4.31
X04571	EGF	2.77	1.35	9.72	3.45	1.65	13.37	5.65	2.49	5.05
U04806	FLT3LG	3.98	1.18	8.25	4.48	3.09	18.65	4.65	3.32	5.95
M86492	GMFB	7.86	37.28	23.04	8.70	7.48	46.31	41.45	19.56	23.96
D49493	GDF10	2.07	7.94	11.15	2.10	2.66	11.84	5.90	1.23	5.61
U46010	HGF	3.50	1.01	10.85	3.58	1.75	13.99	6.11	1.82	5.32
D16431	HDGF	16.29	6.52	32.82	14.20	22.39	19.04	19.05	16.88	18.35
D14012	HGF activator	2.16	1.47	6.99	3.19	2.25	13.78	7.68	2.55	5.01
A25270	IK cytokine	11.86	14.06	63.01	19.07	29.47	38.99	50.95	35.67	32.88
M59964	KITLG	4.18	4.74	11.54	6.36	3.56	21.20	15.48	4.69	8.97
M74178	MST1	3.98	3.15	25.91	8.42	11.79	13.44	21.91	8.89	12.18
X72755	CXCL9	2.27	2.64	7.03	2.81	2.92	20.01	3.55	1.32	5.32
L12260	neuregulin 1	4.01	1.98	8.97	4.66	3.89	12.97	9.52	3.08	6.13
X54936	PGF	3.69	31.18	5.95	10.31	4.70	28.07	8.14	6.89	12.36
X06374	PDGFA associated protein 1	6.36	6.25	8.52	8.45	10.77	12.51	10.20	9.59	9.08
M57399	pleiotrophin	3.99	6.90	43.63	14.56	34.98	62.86	57.33	34.53	32.34
M54995	PPBP	8.61	9.98	16.45	7.34	10.23	22.74	9.51	8.52	11.67
L42379	quiescin Q6	2.58	1.61	6.56	2.96	2.21	13.51	3.02	2.59	4.38
L26081	SEMA3A	2.68	5.93	7.88	4.28	3.73	17.10	5.45	2.07	6.14
AB000220	SEMA3C	3.14	1.21	5.51	3.34	2.66	11.89	5.90	1.71	4.42
U33920	SEMA3F	3.92	2.64	8.26	7.09	8.26	23.19	7.42	6.71	8.43
X02530	CXCL10	3.19	1.23	14.50	3.17	1.92	11.74	5.71	1.37	5.35
L38518	SHH	3.36	10.32	7.28	8.20	5.21	12.27	5.44	5.03	7.14
L36034	CXCL12	5.46	7.01	10.51	3.28	10.85	11.50	5.56	11.48	8.20
M96956	TDGF3	2.76	3.08	9.04	3.84	3.18	26.43	9.57	5.70	7.95
L36052	thrombopoietin	2.35	1.23	11.21	2.67	2.68	10.70	5.96	1.18	4.74
K03222	TGFA	5.09	2.78	8.59	5.25	5.22	26.43	6.47	4.40	8.03
X02812	TGFB1	3.57	1.64	11.60	4.11	4.78	23.26	5.22	4.14	7.29
M19154	TGFB2	3.60	22.87	10.02	4.74	3.02	15.87	6.40	3.23	8.72
J03241	TGFB3	2.48	2.49	5.09	3.27	1.65	15.04	3.32	1.74	4.38
L07414	TNFSF5	3.74	27.08	6.91	2.98	1.58	10.57	4.97	1.37	7.40
D38122	TNFSF6	4.68	1.24	4.73	7.52	2.58	11.10	6.61	2.65	5.14
L08096	TNFSF7	3.18	1.43	6.34	2.64	2.24	17.40	2.93	3.48	4.95
L09753	TNFSF8	3.69	28.20	7.20	2.67	2.56	11.79	5.78	1.06	7.87
U57059	TNFSF10	4.52	4.88	11.17	7.09	5.86	13.88	5.13	3.78	7.04
	<i>Fibroblast Growth Factor</i>									
X51943	FGF1	1.78	2.06	5.46	1.84	1.25	10.41	1.94	3.18	3.49
M27968	FGF2	2.76	3.22	6.91	4.09	2.28	9.06	10.37	1.91	5.07
X14445	FGF3	7.32	9.63	9.78	3.82	1.81	16.15	6.42	4.53	7.43



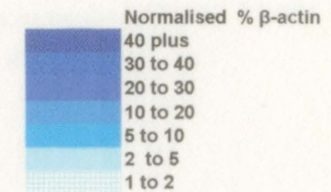
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Accession Number	Gene Name	Cancer Subjects								
		Syn05	Syn13	Syn15	Syn30	Syn38	Syn39	Syn58	Syn77	Ave
<i>Fibroblast Growth Factor</i>										
M37825	FGF5	2.96	2.19	8.40	3.74	2.45	15.41	5.21	1.92	5.28
X63454	FGF6	4.15	1.24	10.58	4.59	3.31	21.79	3.03	3.42	6.51
M60828	FGF7	4.50	3.42	20.80	5.59	6.42	20.09	14.20	5.63	10.08
U36223	FGF8	4.50	12.20	12.55	6.36	8.47	19.17	11.06	12.18	10.81
D14838	FGF9	2.44	1.50	6.54	2.62	3.84	15.60	4.31	2.21	4.88
U66197	FGF12	2.26	0.87	4.66	3.15	1.60	16.95	5.61	2.61	4.71
L03840	FGFR4	3.80	5.33	10.80	3.88	2.40	12.81	7.92	4.38	6.41
<i>Bone Morphogenetic Protein</i>										
M22488	BMP1	7.09	4.66	15.45	5.21	9.42	20.10	8.11	11.38	10.17
M22489	BMP2	4.03	2.97	10.21	7.67	6.41	16.37	9.00	7.19	8.04
M22491	BMP3	10.37	4.14	25.96	2.56	5.24	16.91	6.30	1.95	9.18
D30751	BMP4	5.63	3.68	16.68	8.52	5.48	18.67	12.58	9.69	10.11
M60314	BMP5	4.98	5.60	11.34	4.38	5.85	32.77	17.00	5.01	10.86
M60315	BMP6	5.51	2.03	11.00	1.87	2.58	12.03	5.11	3.34	5.43
M60316	BMP7	1.43	2.61	5.97	2.98	2.53	22.74	5.23	1.43	5.61
M97016	BMP8A	2.88	1.17	8.50	3.99	2.37	13.59	7.14	3.10	5.34
<i>Ephrins</i>										
M57730	ephrin-A1	2.33	1.32	6.19	3.68	2.45	19.47	3.23	1.85	5.06
U26403	ephrin-A5	2.13	2.44	3.65	2.74	1.91	11.24	4.98	2.24	3.91
U09304	ephrin-B1	2.15	1.83	7.53	2.56	2.45	15.71	2.44	1.42	4.51
L38734	ephrin-B2	3.54	3.31	14.10	6.99	5.41	20.33	10.71	8.62	9.12
U66406	ephrin-B3	2.15	1.89	8.09	2.53	2.48	13.36	5.61	1.59	4.71
<i>Interleukins</i>										
X02851	interleukin 1, alpha	2.88	2.24	10.17	4.59	3.59	13.40	2.94	1.57	5.17
K02770	interleukin 1, beta	32.48	51.99	34.28	25.91	33.20	49.16	60.90	44.73	41.58
A14844	interleukin 2	3.20	25.33	5.27	3.24	4.53	8.61	4.58	1.82	7.07
M14743	interleukin 3	4.14	4.78	6.73	2.36	2.12	12.85	3.67	2.38	4.88
M13982	interleukin 4	2.50	2.38	8.25	1.69	2.22	18.28	4.53	1.49	5.17
X04688	interleukin 5	2.87	1.66	9.00	5.17	2.18	16.10	4.22	1.23	5.30
X04602	interleukin 6	6.37	10.84	39.40	17.35	14.49	44.02	11.95	14.12	19.82
J04156	interleukin 7	3.11	3.74	5.40	2.75	2.39	19.76	4.48	1.38	5.37
X17543	interleukin 9	2.24	1.02	7.62	4.09	2.20	21.89	4.99	1.33	5.67
M57627	interleukin 10	3.76	3.67	5.40	6.17	5.36	18.01	5.40	7.82	6.95
M57765	interleukin 11	3.86	3.09	6.19	2.60	2.88	14.27	3.27	3.54	4.96
M65291	interleukin 12A	3.26	2.39	8.76	3.53	1.89	14.59	2.35	2.22	4.87
M65290	interleukin 12B	3.78	24.88	6.54	3.72	2.37	15.48	5.81	2.10	8.08
L06801	interleukin 13	12.65	3.60	23.34	26.44	17.65	39.53	6.80	13.41	17.92
L15344	interleukin 14	2.20	2.02	6.12	3.58	2.90	19.61	6.41	2.21	5.63
U14407	interleukin 15	4.35	1.25	7.52	2.31	4.88	9.39	3.63	1.85	4.40
U32659	interleukin 17	2.77	1.83	7.31	3.38	2.63	14.91	8.04	1.55	5.30
<i>Interferon</i>										
M28622	IFNB1	4.40	3.09	6.63	3.83	1.56	16.04	5.63	2.17	5.42
J00209	interferon, alpha 10	1.82	5.02	8.78	6.36	3.16	13.28	3.82	2.64	5.61
X01992	interferon gamma	2.77	1.68	8.09	5.07	2.50	26.71	11.43	2.55	7.60



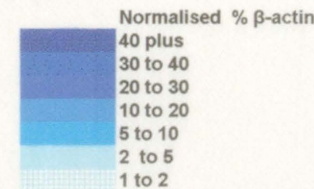
Appendix 1: Gene expression in 8 cancer subjects. One subject (SYN-39) was excluded from all further analysis as the level of gene expression in this subject was much higher than other cancer subjects and was considered an outlier. For a full list of gene names see *Appendix III*

Accession	Gene Name	Cancer Subjects								
		Syn05	Syn13	Syn15	Syn30	Syn38	Syn39	Syn58	Syn77	Ave
	<i>Insulin-like Growth Factor Binding Protein</i>									
M27544	IGF1	6.00	1.75	11.53	9.38	4.17	18.00	3.93	7.81	7.82
M31159	IGFBP3	6.01	3.88	10.45	7.00	16.64	13.44	12.27	14.56	10.53
M62403	IGFBP4	6.79	3.15	9.33	9.44	4.50	7.16	5.42	2.89	6.08
M65062	IGFBP5	3.05	1.76	7.25	6.45	3.59	14.08	2.93	3.90	5.37
M62402	IGFBP6	2.09	1.73	4.20	4.33	2.53	14.76	4.99	2.03	4.58
	<i>Other Extracellular Communication Proteins</i>									
U83508	angiopoietin 1	3.31	3.05	7.05	4.83	2.56	15.86	3.42	2.92	5.37
M38690	CD9 antigen (p24)	102.90	112.85	189.12	94.81	126.17	239.97	236.04	123.49	153.17
U24163	FRZB	4.21	5.63	14.28	11.49	7.05	17.42	7.41	6.26	9.22
AF028593	jagged 1	9.32	13.20	41.37	22.70	17.41	34.41	38.97	21.55	24.87
AF003521	jagged 2	4.01	5.94	6.16	6.40	3.70	16.44	4.89	3.69	6.40
U94354	LFNG	3.23	6.83	10.45	4.00	3.48	16.31	4.26	3.62	6.52
M25639	MIF	4.93	1.57	10.88	14.03	7.87	19.51	8.00	4.62	8.93
U94352	MFNG	3.70	1.83	7.19	6.16	3.94	15.70	8.63	4.35	6.43
U41745	PDAP1	7.19	3.86	14.65	6.79	12.30	23.41	14.88	12.71	11.97
AF017986	SFRP2	3.68	2.27	4.09	4.06	2.60	15.12	7.62	2.50	5.24
AF017988	SFRP5	3.19	1.81	4.98	2.55	3.32	9.72	6.84	2.30	4.34
X07876	WNT2	2.71	2.63	8.12	3.74	2.22	12.77	4.34	1.74	4.78
Z71621	WNT2B	3.57	4.42	6.92	8.24	4.67	11.31	6.93	4.48	6.32
L20861	WNT5A	4.36	7.68	11.59	10.38	10.71	21.85	4.81	5.76	9.62
X91940	WNT8B	5.72	16.50	10.16	12.72	4.76	18.98	7.50	9.80	10.77
X97057	WNT10B	2.23	1.93	5.58	3.39	1.13	7.70	4.37	0.53	3.36
	<i>Intracellular Transducers/Effectors/Modulators</i>									
M76125	AXL	5.82	3.55	15.02	9.22	12.40	9.90	8.30	11.59	9.47
U24166	MAPRE1	21.98	24.88	49.35	40.44	34.78	32.46	49.28	37.72	36.36
L11353	neurofibromin 2	4.70	3.98	12.71	3.96	4.13	9.37	4.94	3.84	5.95
Y00815	PTPRF	5.72	3.41	10.95	5.89	3.78	20.58	9.12	7.16	8.33
L06139	TEK	2.74	3.59	4.62	3.04	2.92	19.50	7.97	3.09	5.93
U77845	TRIP	1.81	2.66	4.53	3.18	2.96	17.16	3.10	0.94	4.54
	<i>Adaptor & Receptor-Associated Proteins</i>									
U07707	EPS15	6.41	6.08	8.75	7.31	6.92	16.26	18.03	11.22	10.12
U12535	EPS8	4.87	6.46	11.88	9.08	8.99	20.11	11.08	8.74	10.12
U69276	GRB10	2.69	1.70	7.50	4.56	4.91	14.50	6.76	2.48	5.63
AK026432	HCK	2.37	1.01	7.33	2.69	1.17	20.90	6.66	2.38	5.56
U40282	ILK	8.61	3.58	20.39	8.42	13.58	15.76	17.54	13.63	12.69
M63959	LRPAP1	6.54	4.35	8.38	13.49	9.56	13.64	5.23	7.88	8.63
U84401	SMO	3.93	3.46	6.06	5.23	4.96	15.57	6.90	4.76	6.36
U12597	TRAF2	2.51	1.42	6.83	4.04	2.83	13.42	4.49	1.80	4.67
U21092	TRAF3	4.44	1.41	11.82	6.92	6.53	16.84	10.73	6.40	8.13
U69108	TRAF5	3.11	1.45	3.91	4.69	2.60	9.46	3.47	1.81	3.81
L05148	ZAP70	2.67	2.14	4.81	2.05	2.02	14.75	2.40	2.54	4.17
AF000974	TRIP6	3.82	2.15	6.84	2.73	3.18	16.33	5.06	1.77	5.23



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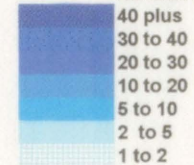
Accession Number	Gene Name	Cancer Subjects								
		Syn05	Syn13	Syn15	Syn30	Syn38	Syn39	Syn58	Syn77	Ave
	<i>Intracellular Kinase Network Members (non-receptor protein kinases)</i>									
X58957	BTK	3.12	1.38	7.58	2.07	2.82	14.73	4.01	3.01	4.84
X86779	FAST kinase	16.84	6.46	31.49	17.78	23.51	29.54	26.54	25.65	22.22
L26318	MAPK8	3.08	2.43	9.15	3.88	3.85	25.45	7.02	2.41	7.16
L31951	MAPK9	3.01	2.80	9.96	4.04	3.48	23.70	6.19	4.16	7.16
U34819	MAPK10	2.06	1.57	6.67	3.42	2.78	23.83	5.36	2.08	5.97
U24152	PAK1	7.02	8.47	33.05	10.37	18.70	29.99	23.50	16.66	18.47
U24153	PAK2	4.63	3.38	13.45	7.80	14.37	18.68	14.39	14.72	11.42
U43522	PAK2B	4.60	5.34	16.30	4.78	7.23	24.77	8.70	7.41	9.89
L13616	PTK2	5.87	4.30	13.77	5.05	9.64	18.51	11.83	6.16	9.39
U43195	ROCK1	1.86	0.76	9.28	3.29	2.08	11.53	5.61	2.51	4.61
	<i>G-Proteins</i>									
M35543	cell division cycle 42	9.47	4.85	26.57	16.99	15.50	27.73	19.78	19.62	17.56
Y07923	GTP-binding protein	3.29	2.07	10.81	3.30	5.22	15.39	4.08	2.33	5.81
X06820	RHOB	43.38	17.83	53.86	47.52	49.70	33.22	57.22	45.37	43.51
X95456	RHON	2.91	1.18	7.55	3.22	3.63	22.86	4.89	2.41	6.08
M31470	RHOQ	3.80	2.97	9.79	6.51	5.92	18.65	8.05	3.69	7.42
M29870	RAC1	27.31	31.75	78.78	26.37	30.68	51.06	88.12	33.80	45.98
M64595	RAC2	10.59	5.12	22.93	9.38	21.24	25.33	18.04	20.19	16.60
L24564	RRAD	3.06	0.61	4.76	5.43	2.32	9.66	4.63	1.90	4.04
	<i>GTP/GDP Exchangers & G-Protein GTPase Activity Modulators</i>									
Z18951	caveolin 1	4.56	4.00	10.57	6.85	8.17	17.99	7.30	5.88	8.16
U32114	caveolin 2	2.22	3.83	12.05	6.40	5.28	13.96	6.15	3.82	6.71
U11690	faciogenital dysplasia	3.99	2.57	11.58	2.27	2.70	14.30	4.73	2.00	5.51
U59752	PSCD2	20.07	26.21	53.63	24.65	33.73	51.50	47.06	33.10	36.24
U02570	ARHGAP1	1.92	1.88	6.08	4.75	2.45	12.05	6.06	2.53	4.71
X78817	ARHGAP4	12.52	6.56	30.03	14.38	14.20	37.95	14.41	15.49	18.20
X69550	ARHGDI A	86.45	76.54	208.01	108.25	122.54	252.76	181.19	148.03	147.97
L20688	ARHGDB	8.30	3.35	11.62	10.80	9.62	18.12	11.18	14.17	10.89
U82532	ARHGDIG	2.52	2.06	6.52	2.28	2.22	17.87	7.13	1.87	5.31
	<i>Phospholipases & Phosphoinositol Kinases</i>									
U38545	phospholipase D1	7.46	2.91	15.47	5.20	7.10	16.21	12.07	5.63	9.00
M86400	YWHAZ	5.08	22.02	14.17	9.00	5.89	21.14	8.19	8.70	11.77
	<i>Other Intracellular Transducers/Effectors/Modulators</i>									
L22005	cell division cycle 34	21.53	27.99	46.53	31.51	36.62	57.23	44.28	35.42	37.64
U46461	DVL1L1	13.24	34.81	66.31	38.25	29.99	61.31	22.74	24.74	36.42
U56976	PDE1B	3.19	6.09	6.18	4.97	2.52	22.25	3.78	1.60	6.32
	Protein Turnover									
	<i>Serine Proteases</i>									
D49742	HABP2	4.63	2.77	9.72	6.19	2.68	14.99	2.36	3.45	5.85
X05199	plasminogen	2.23	1.95	4.91	2.36	3.70	13.46	5.38	2.03	4.50
M15518	PLAT	17.92	9.49	38.82	27.84	25.36	34.90	29.95	24.60	26.12
M15476	PLAU	4.13	1.16	12.24	7.61	4.44	25.34	4.59	3.04	7.82
	<i>Metalloproteinases</i>									
X05231	MMP1	1.78	2.57	7.69	2.65	3.00	21.41	4.10	2.47	5.71
J03210	MMP2	2.78	1.15	9.76	2.53	3.43	22.78	5.61	3.65	6.46
X05232	MMP3	3.05	2.78	5.33	4.31	5.30	17.56	6.87	3.35	6.07



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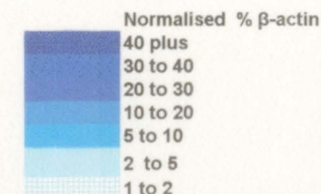
Accession Number	Gene Name	Cancer Subjects								
		Syn05	Syn13	Syn15	Syn30	Syn38	Syn39	Syn58	Syn77	Ave
	<i>Metalloproteinases</i>									
X07819	MMP7	3.10	1.91	4.49	2.71	2.51	10.01	3.49	1.39	3.70
J05556	MMP8	2.31	2.28	7.76	3.35	3.48	15.46	4.31	3.63	5.32
J05070	MMP9	5.81	5.91	7.75	6.60	4.53	10.15	6.72	5.04	6.56
X07820	MMP10	2.02	1.32	5.96	2.20	1.81	18.86	4.91	1.88	4.87
X57766	MMP11	6.83	15.81	12.69	22.31	18.75	25.60	18.92	22.91	17.97
L23808	MMP12	6.78	2.92	8.10	4.36	3.85	12.20	4.88	2.37	5.68
X75308	MMP13	4.30	2.20	8.31	3.02	2.70	15.07	7.59	2.11	5.66
D26512	MMP14	20.07	16.06	34.80	27.21	40.42	36.44	36.79	34.41	30.77
Z48482	MMP15	8.75	2.91	11.77	12.01	11.48	22.25	11.38	9.86	11.30
D85511	MMP16	3.02	5.72	8.88	4.95	2.93	16.70	7.18	3.57	6.62
X89576	MMP17	3.49	3.96	7.97	4.06	3.63	16.20	6.77	2.47	6.07
X92521	MMP19	1.87	2.71	5.61	2.01	3.33	16.54	3.52	1.79	4.67
U41766	ADAM9	4.34	6.72	13.12	7.26	6.95	16.55	9.88	6.53	8.92
U69611	ADAM17	6.62	9.22	15.22	10.81	11.17	13.58	18.29	12.05	12.12
	<i>Protease Inhibitors</i>									
M11313	A2M	5.85	2.07	7.10	10.70	12.57	17.26	11.70	14.60	10.23
M68516	SERPINA5	2.25	0.87	6.39	3.24	1.44	13.67	4.58	2.50	4.37
M18082	SERPINB2	3.67	2.69	7.76	7.72	4.44	17.59	4.08	3.42	6.42
X04429	SERPINE1	2.83	1.02	6.49	1.27	3.39	10.06	4.76	1.59	3.92
X03124	TIMP1	17.17	13.43	57.48	37.84	34.77	46.75	28.80	34.45	33.83
J05593	TIMP2	5.52	2.38	10.56	4.81	7.38	18.40	7.22	9.10	8.17
Z30183	TIMP3	16.77	13.30	30.92	16.08	25.22	22.75	39.72	40.16	25.61
U76456	TIMP4	3.41	1.13	6.68	5.98	2.66	16.52	4.89	2.60	5.48
	<i>Others Involved in Protein Turnover</i>									
L20471	BSG	56.64	44.78	38.41	34.93	42.75	31.90	61.82	38.39	43.70
M26880	ubiquitin C	159.41	169.08	364.18	154.85	201.18	437.66	261.18	193.00	242.56
	<i>Cytoskeleton/Motility Proteins</i>									
X00351	beta actin	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
U29343	RHAIM	2.33	1.15	6.86	4.04	2.44	13.42	4.78	1.33	4.54
M23410	junction plakoglobin	51.48	41.30	110.89	53.79	76.78	70.42	94.13	65.15	70.49
M77349	TGF- β 1	9.07	4.87	14.32	13.41	14.90	20.31	16.41	19.95	14.15
K00558	alpha tubulin	75.87	78.52	86.33	86.91	58.49	119.74	77.50	73.08	82.05
X51521	villin 2	28.56	24.81	55.52	40.11	46.70	43.46	65.92	43.82	43.61
X94991	zyxin	4.04	3.00	10.74	9.01	10.78	11.74	9.38	13.65	9.04
	<i>Intermediate Filament Proteins</i>									
M63618	DST	4.39	3.01	9.72	4.90	2.81	8.06	8.50	3.68	5.63
M99063	cytokeratin 2	3.22	1.25	7.71	3.51	5.10	10.21	2.97	0.60	4.32
U59167	desmin	19.08	12.53	24.42	29.38	52.44	22.47	30.12	68.46	32.36
M98776	keratin 1	5.43	4.34	9.36	7.98	8.24	15.17	4.65	3.42	7.32
M99061	keratin 2A	4.14	2.20	4.57	4.71	5.18	14.62	5.79	3.50	5.59
X67683	keratin 4	3.05	2.30	8.52	3.62	4.23	12.84	3.91	2.27	5.09
M21389	keratin 5	2.58	0.51	7.45	3.13	2.93	7.37	4.33	2.21	3.81
J00269	keratin 6A	2.88	0.98	6.73	3.04	2.49	10.92	5.29	1.85	4.27
L42592	keratin 6B	2.48	3.04	10.18	3.43	2.32	17.47	5.42	1.74	5.76
X03212	keratin 7	2.99	1.11	8.17	3.28	3.39	12.42	3.75	2.04	4.64
M34225	keratin 8	54.34	49.80	93.10	59.06	68.22	118.56	78.92	70.68	74.08
Z29074	keratin 9	4.44	1.48	4.47	3.53	2.84	17.43	3.65	1.86	4.96

Normalised % β -actin



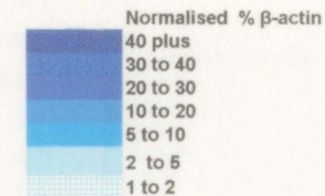
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Accession	Gene Name	Cancer Subjects								
Number		Syn05	Syn13	Syn15	Syn30	Syn38	Syn39	Syn58	Syn77	Ave
<i>Intermediate Filament Proteins</i>										
M19156	keratin 10	2.96	1.26	5.27	3.35	2.55	13.10	2.78	2.81	4.26
D78367	keratin 12	3.18	0.88	8.41	3.29	3.69	12.14	8.00	1.48	5.13
X52426	keratin 13	2.94	2.53	6.32	2.60	4.02	13.21	3.42	0.73	4.47
J00124	keratin 14	3.04	1.41	11.42	3.79	1.86	15.31	3.47	2.61	5.36
M26326	keratin 18	44.86	26.98	50.21	52.22	46.26	21.29	59.52	42.48	42.98
Y00503	keratin 19	67.71	61.28	88.23	140.54	91.49	40.78	98.22	64.72	81.62
M21772	K16:CK16	3.41	1.94	8.26	4.39	4.78	15.21	9.49	0.76	6.03
X56134	vimentin	62.19	58.32	207.40	44.73	57.66	278.57	171.03	84.66	120.57
<i>DNA Synthesis, Recombination & Repair</i>										
<i>DNA Replication: Polymerases, Replication Factors & Topoisomerases</i>										
M15796	PCNA	34.93	37.87	98.20	52.35	47.51	96.58	71.28	46.19	60.61
X06745	PLOA	3.75	8.35	9.91	6.29	2.53	19.13	6.51	2.08	7.32
M63488	RPA1	5.94	3.45	9.97	5.96	4.60	17.67	7.74	3.10	7.30
M87338	RFC2	3.92	31.96	12.17	4.76	4.19	16.33	4.46	3.81	10.20
L07541	RFC3	8.16	32.74	9.96	11.58	7.00	23.04	9.18	11.83	14.18
M87339	RFC4	4.32	10.90	12.26	5.90	5.22	20.72	6.32	5.02	8.83
L07540	RFC5	3.49	1.92	6.40	2.81	2.52	14.52	4.54	1.87	4.76
J03250	TOP1	4.88	3.10	11.28	6.20	2.94	12.33	7.32	4.26	6.54
J04088	TOP2A	3.74	3.05	7.26	6.89	3.78	24.09	7.23	5.15	7.65
<i>DNA Damage Signaling/Repair Proteins & DNA Ligases</i>										
U33841	ATM	5.47	35.32	6.48	10.26	3.63	20.73	13.28	10.19	13.17
M13194	ERCC1	7.52	4.83	20.83	12.58	18.85	23.99	20.23	15.96	15.60
X52221	ERCC2	2.78	4.56	7.43	6.33	2.44	13.00	7.51	2.54	5.82
M31899	ERCC3	3.93	6.54	10.72	7.56	5.24	9.79	7.30	2.88	6.74
L20046	ERCC5	8.02	6.44	25.25	21.09	17.66	21.04	21.98	17.65	17.39
X84740	LIG3	3.02	1.84	7.69	4.67	2.22	12.33	2.42	3.17	4.67
X83441	LIG4	1.20	1.11	10.48	4.53	2.67	14.58	3.26	2.61	5.05
U07418	MLH1	1.90	1.58	6.50	5.63	1.86	13.59	3.35	1.13	4.44
U04045	MSH2	3.36	2.60	10.38	8.73	3.47	16.47	9.00	4.77	7.35
U54777	MSH6	1.49	4.96	10.90	7.51	3.27	11.65	9.44	4.78	6.75
M29971	MGMT	3.46	4.96	8.62	4.56	3.81	5.29	7.72	2.99	5.17
U35835	PRKDC	5.60	6.46	12.64	9.39	5.61	11.54	9.39	3.95	8.07
D21235	RAD23A	19.73	21.89	25.01	33.10	33.25	33.56	28.34	33.38	28.53
U63139	RAD50	2.22	2.21	9.34	2.44	1.85	11.77	9.63	1.76	5.15
D13804	RAD51	2.95	2.48	12.53	4.85	2.52	10.08	5.29	3.18	5.48
U12134	RAD52	1.80	1.13	9.04	2.97	2.80	15.34	4.01	0.81	4.73
M32865	G22P1	7.53	4.02	13.35	11.66	11.56	15.95	11.35	7.92	10.41
<i>DNA Damage Signaling/Repair Proteins & DNA Ligases</i>										
M30938	XRCC5	3.28	2.21	6.32	4.83	3.08	17.72	5.91	3.30	5.83
M74524	UBE2A	26.36	40.04	33.66	39.26	32.64	52.22	58.52	41.09	40.47
M36089	XRCC1	5.57	4.68	12.32	12.60	8.90	9.32	4.70	4.32	7.80
<i>Other DNA Synthesis, Recombination & Repair Proteins</i>										
X90392	DNASE1L1	2.46	2.38	6.80	5.40	1.96	12.21	2.42	2.60	4.53
M60974	GADD45A	6.81	9.90	35.59	14.14	9.07	13.50	40.49	10.48	17.47
X74262	RBBP4	25.38	30.23	73.31	49.64	42.54	54.08	62.28	49.55	48.37



Appendix I : Gene expression in 8 cancer subjects. One subject (SYN-39) was excluded from all further analysis as the level of gene expression in this subject was much higher than other cancer subjects and was considered an outlier. For a full list of gene names see **Appendix III**

Accession	Gene Name	Cancer Subjects								
Number		Syn05	Syn13	Syn15	Syn30	Syn38	Syn39	Syn58	Syn77	Ave
	Angiogenic factors									
M32977	VEGF	11.63	15.72	42.10	13.83	23.92	16.86	30.06	29.32	22.93
U43142	VEGFC	2.80	2.23	9.97	3.57	2.61	16.93	2.61	2.65	5.42
U48801	VEGFB	7.63	3.21	8.30	6.04	7.86	14.90	8.94	8.25	8.14
J05081	endothelin 3	3.10	1.39	7.09	5.50	2.63	11.33	3.31	2.26	4.57
M65199	endothelin 2	3.31	1.96	10.53	6.53	3.09	20.00	3.60	3.02	6.50
U01134	FLT1	21.61	51.33	10.27	19.36	23.49	34.04	30.55	25.21	26.98
	Functionally Unclassified									
U32907	LRRC17	1.74	2.75	7.14	5.48	2.85	25.42	8.04	2.95	7.04
U37688	RATS1	3.25	14.24	5.33	2.87	1.80	12.84	4.65	3.98	6.12
D25216	LRRC14	2.66	3.59	7.85	8.47	4.11	12.37	5.94	3.36	6.04



Appendix 1 : Gene expression in 8 cancer subjects. One subject (SYN-39) was excluded from all further analysis as the level of gene expression in this subject was much higher than other cancer subjects and was considered an outlier. For a full list of gene names see *Appendix III*

Appendix II

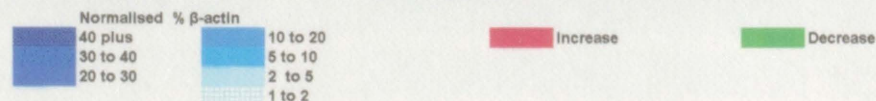
Accession Number	Gene Name	Normal Subjects							Cancer Subjects							Polyp Subjects							Canc vs Norm	Polyp vs Norm	Canc vs Polyp	
		1	3	4	5	6	7	Ave	Syn05	Syn13	Syn15	Syn30	Syn38	Syn58	Syn77	Ave	Syn40	Syn65	Syn70	Syn42	Syn49	Syn74	Ave			
Transcription Activators & Repressors																										
U76638	BARD1	7.31	4.55	3.10	4.02	0.36	1.07	3.40	1.73	1.80	8.09	3.75	1.74	5.51	2.52	3.59	2.16	3.43	3.68	1.42	5.55	4.61	3.47			
M96577	E2F1	4.89	4.42	2.74	3.82	0.85	2.15	3.14	2.76	3.66	4.06	4.46	4.37	4.56	2.18	3.72	2.44	3.46	3.28	5.51	3.54	10.08	4.72		1.50	
Y10479	E2F3	5.28	7.29	8.12	9.87	1.72	7.67	6.65	7.06	4.35	13.15	13.97	9.51	15.37	13.41	10.97	8.47	5.55	5.50	11.66	15.07	12.86	9.85	1.65	1.48	
U15642	E2F5	5.50	4.83	6.32	5.02	0.89	3.24	4.30	2.81	1.37	10.44	4.47	6.20	7.70	3.97	5.28	2.26	3.36	2.54	3.54	7.49	11.76	5.16			
M62829	EGR1	19.86	10.81	10.47	20.41	16.45	9.24	14.37	12.88	7.79	42.73	21.78	22.20	27.01	16.30	21.52	24.42	12.64	16.18	11.79	19.95	22.64	17.93	1.50		
D11117	homeo box D3	6.09	5.73	4.15	2.92	2.93	2.32	4.02	4.00	1.94	15.19	5.07	4.15	10.80	2.96	6.30	3.13	5.00	3.49	5.23	9.60	11.04	6.25	1.57	1.56	
M91196	ICSBP1	3.60	7.82	3.22	3.70	2.63	3.64	4.10	4.54	2.78	8.12	3.95	3.27	2.30	1.06	3.72	1.69	3.41	3.01	6.63	7.91	9.14	5.29			1.42
X14454	IRF1	13.72	13.42	10.54	28.59	27.74	28.61	20.43	18.42	7.13	49.50	23.79	24.75	36.27	20.49	25.76	16.75	15.50	13.74	26.87	41.17	49.03	27.17			
X17620	NME1	8.02	10.06	5.63	6.21	1.46	10.91	7.05	6.18	2.65	9.23	9.39	9.58	10.81	9.06	8.13	3.83	5.95	5.98	5.54	16.75	11.93	8.33			
L16785	NME2	133.26	147.38	136.83	118.68	124.56	184.91	141.27	135.46	120.95	243.35	137.11	154.67	229.89	144.70	166.58	86.44	59.63	112.61	162.18	248.84	208.04	152.95			
M96684	PURA	5.48	29.45	23.22	16.71	2.77	6.67	14.05	6.53	32.25	15.42	15.05	6.81	11.72	12.89	14.38	8.81	6.68	6.47	9.68	14.66	12.11	9.73		1.44	1.48
X06538	RARA	10.82	15.95	10.41	16.96	14.68	16.43	14.21	14.83	5.88	20.90	12.29	16.84	21.41	18.78	15.85	13.14	9.85	5.85	15.80	22.10	19.64	14.39			
X07282	RARB	4.57	6.58	5.17	1.28	2.46	2.00	3.68	2.08	2.26	8.22	2.42	2.99	3.56	3.33	3.55	1.32	2.22	4.46	2.31	4.87	10.20	4.23			
M24857	RARG	3.99	6.18	3.49	1.88	5.39	4.35	4.21	3.37	4.71	6.66	4.33	3.23	6.56	2.54	4.48	2.37	3.38	2.71	4.41	5.47	10.00	4.72			
M97935	STAT1	2.79	5.28	2.34	2.70	0.33	1.17	2.43	2.77	1.53	5.10	3.86	3.09	2.73	1.50	2.94	1.93	2.88	3.29	3.42	7.57	11.30	5.06		2.08	1.72
M97934	STAT2	17.08	29.69	35.18	20.77	13.45	22.82	23.16	19.27	28.12	9.70	27.70	15.32	30.27	23.82	22.03	20.15	16.57	6.48	24.30	41.62	37.00	24.35			
U47686	STAT 5B	7.50	12.20	9.77	11.06	2.15	7.51	8.36	4.54	8.30	17.64	7.16	9.34	13.14	16.62	10.96	5.58	5.79	5.79	8.95	19.97	14.25	10.05			
U59863	TANK	6.92	7.01	5.46	4.83	1.18	7.38	5.46	3.28	2.48	9.86	4.85	4.42	9.95	2.14	5.28	2.59	4.27	3.98	9.50	5.75	12.97	6.51			
L23959	TFDP1	11.73	7.47	11.82	16.72	10.69	17.43	12.64	11.28	5.56	21.38	10.13	17.29	12.63	15.54	13.40	9.04	8.00	9.58	13.38	27.37	15.74	13.85			
U18422	TFDP2	7.44	3.58	6.71	5.66	3.48	2.82	4.95	3.48	3.31	7.70	2.27	5.67	5.96	5.17	4.79	2.59	3.63	5.20	6.79	5.92	16.08	6.70			1.40
Cell Cycle																										
U33286	CSE1L	4.96	6.21	3.94	1.47	0.55	1.26	3.06	3.23	1.83	2.75	5.01	1.90	3.60	0.80	2.73	0.76	4.20	2.66	5.25	6.13	5.07	4.01			1.47
L34075	FRAP1	4.24	7.31	3.91	2.96	1.60	4.50	4.08	3.84	2.78	8.66	6.16	3.74	3.32	2.26	4.38	2.33	3.55	2.83	2.39	6.00	6.33	3.90			
Z12020	Mdm2	13.47	4.19	5.41	5.73	0.74	2.32	5.31	2.87	3.34	7.87	3.78	2.12	6.00	2.63	4.09	2.33	3.37	2.93	4.19	8.14	10.64	5.26			
AF007111	Mdm4	6.74	4.75	4.50	2.20	0.46	1.61	3.37	2.76	1.37	4.90	3.30	2.88	2.34	2.63	2.88	1.31	1.61	3.32	3.56	5.53	7.30	3.77			
U63295	SIAH1	0.64	4.21	3.21	2.71	0.55	0.88	2.03	1.81	1.62	7.93	2.09	1.54	4.27	2.31	3.08	1.12	1.88	2.38	1.86	4.64	7.69	3.26	1.52	1.60	
Y11416	tumor protein p73	10.72	8.50	5.58	5.16	0.69	3.48	5.69	3.73	8.03	4.83	3.28	4.02	7.62	6.90	5.49	3.71	3.60	3.05	4.15	7.49	7.76	4.96			
Cyclins																										
X51688	cyclin A2	7.54	3.76	4.81	2.88	2.19	9.13	5.05	3.38	2.37	5.82	4.65	2.01	6.32	3.07	3.94	2.16	3.71	2.96	5.60	10.22	10.57	5.87			1.49
M25753	cyclin B1	6.65	17.16	9.52	6.90	4.39	24.88	11.58	4.33	5.13	11.44	7.45	7.27	11.69	6.82	7.59	4.44	5.59	4.04	11.66	10.67	14.26	8.42		1.53	
M74091	cyclin C	1.09	3.73	4.84	2.22	1.40	1.62	2.48	3.82	1.53	6.10	3.69	3.04	3.70	1.48	3.34	1.16	3.20	3.27	3.83	8.01	5.36	4.13			1.67
X59798	cyclin D1	4.07	8.35	4.02	9.95	3.39	7.28	6.18	2.70	5.19	14.35	6.53	6.93	12.73	7.31	7.96	6.21	4.90	5.65	9.43	9.63	10.35	7.69			
D13639	cyclin D2	26.82	14.23	25.07	39.52	25.10	45.99	29.47	8.17	6.78	56.84	8.28	37.88	70.62	46.36	33.56	17.08	17.30	13.62	59.74	56.33	28.44	32.08			
M92287	cyclin D3	6.23	6.08	4.31	7.38	4.27	4.44	5.45	5.86	3.15	9.11	7.91	5.47	7.73	2.70	5.99	5.96	4.46	7.98	8.07	7.43	9.69	7.26			
M73812	cyclin E1	18.10	4.49	8.89	5.42	5.67	3.85	7.70	4.01	3.93	9.10	7.90	4.04	4.57	1.49	5.00	1.92	5.27	2.86	6.31	9.78	7.56	5.62		1.54	
U47413	cyclin G1	18.05	6.80	15.51	8.94	6.59	6.60	10.41	4.19	4.91	7.71	7.11	4.82	6.83	3.08	5.52	3.91	4.79	5.32	9.65	10.79	8.92	7.23		1.89	1.44
U47414	cyclin G2	13.00	5.25	5.02	4.97	1.86	1.87	5.33	2.13	1.75	13.08	5.73	3.54	5.41	1.35	4.71	1.26	3.77	5.77	5.34	2.91	11.61	5.11			
U11791	cyclin H	13.25	10.78	8.16	8.06	4.09	10.41	9.12	6.23	3.34	17.88	10.16	7.27	17.90	4.77	9.65	5.32	6.69	4.05	13.09	9.53	13.53	8.70			
Cell cycle-regulating kinases																										
L29222	CDC-like kinase 1	2.89	4.95	8.17	2.31	1.59	2.58	3.75	2.83	3.19	10.52	10.58	4.57	6.64	3.87	6.03	2.92	5.38	3.11	6.86	6.38	12.74	6.23		1.61	1.66
L29216	CDC-like kinase 2	3.88	7.52	7.41	6.91	2.36	5.04	5.52	2.72	3.61	16.63	9.41	4.83	7.83	4.87	7.13	3.61	4.46	3.78	4.14	10.37	16.62	7.16			
L29220	CDC-like kinase 3	16.79	5.74	11.13	9.29	7.43	6.35	9.45	4.97	3.06	11.26	17.33	7.78	11.59	2.23	8.32	6.51	4.74	7.34	12.79	8.56	11.16	8.51			
M68520	CDK 2	17.20	4.84	13.34	5.03	2.29	3.24	7.65	2.95	3.44	11.09	8.95	4.72	5.67	2.38	5.60	2.71	3.43	3.90	5.64	8.23	7.91	5.30			1.44
X66357	CDK 3	16.20	6.96	8.27	5.72	0.84	3.25	6.87	3.73	4.10	9.85	8.96	4.04	4.30	1.58	5.22	1.67	3.16	4.00	7.36	8.03	10.42	5.77			
M14505	CDK 4	24.22	9.06	13.44	13.53	5.11	11.92	12.73	5.94	2.94	16.70	7.49	8.41	10.72	5.33	8.22	4.15	6.36	5.57	5.88	8.87	9.93	6.79		1.55	1.87
X66364	CDK 5	11.99	11.34	13.71	13.87	12.73	14.13	12.96	6.84	5.23	14.79	11.11	12.85	11.47	7.19	9.92	6.30	6.95	5.93	12.08	15.72	17.00	10.66			



Appendix II The ratios of difference of each gene for cancer versus normal, polyp versus normal and cancer versus polyp subjects. Genes with a ratio of difference of 1.4 or above are highlighted. All genes are normalised to the housekeeping gene β -actin and expressed as % of β -actin.

For a full list of gene names see **Appendix III**

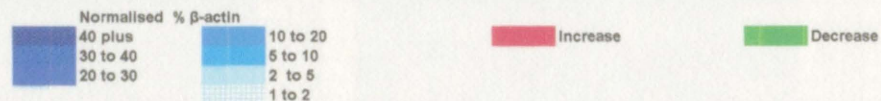
Accession	Gene Name	Normal Subjects							Cancer Subjects							Polyp Subjects								Canc	Polyp	Canc	
Number		1	3	4	5	6	7	Ave	Syn05	Syn13	Syn15	Syn30	Syn38	Syn58	Syn77	Ave	Syn40	Syn65	Syn70	Syn42	Syn49	Syn74	Ave		vs Norm	vs Norm	vs Polyp
Cell cycle-regulating kinases																											
X66365	CDK 6	87.07	107.53	116.29	107.12	107.58	140.41	111.00	74.73	73.57	156.78	100.69	111.67	193.92	126.05	119.63	71.77	57.04	54.34	138.09	166.93	119.45	101.27				
L20320	CDK 7	10.17	5.47	3.29	6.00	2.74	2.40	5.01	2.76	2.23	5.85	7.85	3.21	5.79	1.70	4.17	2.28	3.26	3.68	3.16	8.25	9.95	5.09				
L25676	CDK 9	15.39	8.97	6.36	13.11	11.29	12.05	11.19	8.43	4.58	12.95	15.32	12.42	15.46	10.70	11.41	12.07	10.39	6.35	17.92	25.50	17.09	14.88				
L33264	CDK 10	18.02	10.81	8.00	15.29	7.20	6.83	11.02	6.21	5.79	15.61	10.15	12.34	10.28	9.73	10.01	10.84	9.50	6.77	12.98	32.47	13.30	14.31				1.43
X66358	CDKL1	15.88	5.87	10.44	5.09	3.35	2.25	7.14	4.20	3.77	11.95	6.02	2.76	5.27	1.98	5.13	1.62	2.82	4.74	6.26	4.16	11.01	5.10		1.40		
X05360	CDC2	19.09	5.67	8.19	5.55	5.56	6.90	8.49	5.60	3.37	10.49	11.10	4.98	5.90	1.75	6.17	3.05	4.13	12.73	9.38	13.67	10.96	8.99				1.46
M80629	CDC2L5	8.52	6.46	6.17	6.79	6.93	10.49	7.56	2.83	5.03	16.41	6.64	4.62	18.24	5.78	8.50	5.74	6.87	4.41	11.89	11.81	14.25	9.16				
X60188	MAPK3	37.43	35.87	21.28	35.42	41.81	41.17	35.49	31.60	20.72	79.20	30.90	43.92	45.01	34.93	40.89	28.96	26.52	25.26	47.48	63.94	35.43	37.93				
U02687	FLT3	4.62	9.05	6.30	4.39	0.83	3.95	4.86	2.98	4.98	6.93	4.98	6.07	4.94	3.72	4.94	2.70	3.16	5.93	5.86	9.99	12.26	6.65				
X59727	MAPK4	1.67	6.09	2.47	1.81	0.92	0.74	2.28	2.68	2.39	7.14	2.86	1.55	2.62	1.93	3.02	1.50	3.44	3.83	5.48	2.56	6.44	3.87		1.70		
X80692	MAPK6	2.17	7.68	7.26	5.00	3.44	4.00	4.92	4.60	2.92	11.55	5.90	3.62	7.99	3.05	5.66	3.07	4.80	3.59	6.44	8.28	14.05	6.70				
U25278	MAPK7	5.55	5.98	4.34	5.50	3.48	1.55	4.40	3.80	3.09	8.71	8.54	3.60	6.84	4.32	5.56	5.26	5.48	4.07	7.19	11.65	9.77	7.24		1.65		
X79483	MAPK12	7.92	5.95	6.70	4.16	1.49	1.81	4.67	3.59	3.72	10.83	8.30	5.19	5.04	4.79	5.92	3.31	3.38	5.84	4.99	10.85	7.43	5.96				
L35253	MAPK14	8.26	17.03	11.91	14.38	9.83	15.12	12.75	6.73	9.76	15.06	11.54	13.06	18.95	14.40	12.78	9.86	9.12	7.11	16.01	18.65	18.19	13.16				
L05624	MAP2K1	19.14	9.01	6.12	10.58	4.63	4.87	9.06	4.75	3.17	9.34	7.47	6.90	7.74	2.97	6.05	8.30	4.78	8.29	5.20	12.83	10.34	8.29		1.50		
U25265	MAP2K5	27.73	9.65	11.89	16.30	12.10	15.73	15.56	7.15	8.06	23.35	14.52	10.87	22.09	2.37	12.63	11.31	5.61	9.92	14.00	11.80	11.83	10.74			1.45	
U39657	MAP2K6	18.38	11.67	12.16	11.39	4.30	7.30	10.86	4.30	5.90	11.39	7.34	4.09	6.66	3.67	6.19	3.46	4.30	3.23	8.94	6.16	13.45	6.59		1.76	1.65	
U78876	MAP3K3	11.37	4.41	6.94	7.25	3.70	3.13	6.13	3.94	1.93	12.24	4.06	4.78	5.12	3.05	5.02	4.21	3.29	3.60	5.33	7.59	8.22	5.37				
U01038	PLK1	3.87	8.62	4.91	5.41	2.55	13.28	6.44	3.39	3.39	9.19	5.04	3.39	5.58	2.81	4.68	2.43	3.39	2.69	6.07	10.15	11.64	6.06				
CDK inhibitors																											
U09579	CDKN1A	35.29	56.97	64.80	58.27	57.68	69.69	57.11	51.31	53.17	117.82	71.72	60.47	103.34	67.90	75.10	55.82	51.26	26.38	76.31	102.42	71.40	64.26				
U22398	CDKN1C	7.68	3.71	5.33	6.82	3.50	1.95	4.83	3.29	1.44	6.54	4.43	5.95	8.94	4.44	5.00	2.51	5.20	5.28	6.32	7.92	10.34	6.26				
L27211	CDKN2A	7.15	33.88	34.00	11.56	8.47	6.56	16.95	5.28	34.72	5.33	9.37	8.25	8.54	12.63	12.02	4.86	6.09	4.99	8.32	10.94	12.28	7.91		1.41	2.14	1.52
U17075	CDKN2B	6.81	4.92	4.13	6.04	5.40	1.46	4.79	4.14	2.59	8.46	6.29	2.69	4.84	4.07	4.72	6.32	7.48	6.64	9.21	11.19	7.26	8.01			1.67	1.70
U40343	CDKND	14.50	9.69	6.93	13.75	11.97	12.90	11.62	11.67	5.21	14.57	23.62	16.31	11.35	12.47	13.60	14.21	12.39	9.40	14.41	22.75	16.20	14.89				
U10564	WEE1	4.86	14.92	12.33	8.94	4.40	17.62	10.51	5.56	3.77	21.92	8.14	11.40	48.41	14.35	16.22	11.18	6.75	5.05	16.64	20.51	20.24	13.39		1.54		
Other Cell Cycle Controls																											
U77949	CDC6	8.03	6.61	4.31	3.44	2.01	1.21	4.26	2.65	2.40	5.52	6.25	3.37	5.01	1.91	3.87	2.19	2.34	2.96	2.96	6.84	4.58	3.64				
S72008	CDC10	9.73	24.07	20.74	17.71	10.98	43.13	21.06	8.35	19.98	52.43	14.57	17.83	37.65	21.83	24.66	15.70	14.48	8.20	34.21	30.02	30.63	22.20				
U18291	CDC16	14.53	7.55	10.72	10.24	7.23	7.60	9.64	4.54	2.31	11.81	9.63	5.73	7.70	4.08	6.54	8.04	8.96	3.22	15.71	10.76	14.44	10.19		1.47		1.56
U63131	CDC37	38.67	20.09	17.07	35.09	28.60	36.56	29.34	18.82	10.04	38.28	24.97	31.00	31.45	22.35	25.27	20.79	15.18	13.91	30.31	42.95	21.37	24.08				
S57153	ARID4A	4.10	4.59	7.30	4.48	0.97	3.03	4.08	3.90	4.21	16.41	3.27	4.20	9.55	1.44	6.14	2.99	4.84	2.99	9.89	6.30	15.40	7.07		1.51	1.73	
M81933	CDC25A	7.45	4.37	5.39	4.45	3.09	4.39	4.86	3.59	2.24	9.48	6.15	2.39	5.18	2.65	4.52	2.26	4.03	5.00	2.87	4.75	10.45	4.89				
M81934	CDC25B	12.07	8.28	9.90	8.46	6.42	15.44	10.09	4.88	2.35	12.59	11.43	7.19	13.09	7.75	8.47	3.66	4.73	6.10	8.61	15.04	16.80	9.15				
M34065	CDC25C	3.27	14.23	10.35	5.24	2.80	3.30	6.53	2.85	9.33	7.31	7.27	3.08	8.07	3.12	5.86	2.87	6.41	3.60	5.88	8.58	7.78	5.85				
U00001	CDC27	28.01	15.67	25.26	23.09	15.30	25.72	22.17	8.31	11.17	30.48	20.06	15.24	29.57	4.65	17.07	13.30	10.09	6.43	28.17	21.09	18.18	16.21				
X80343	CDK5R1	7.10	5.70	5.94	6.17	4.34	3.75	5.50	1.84	2.19	6.50	8.77	3.06	5.30	3.16	4.40	2.04	2.10	3.71	3.35	6.95	10.63	4.79				
U34051	CDK5R2	9.51	7.17	6.53	5.38	3.13	2.81	5.75	2.23	1.59	4.21	5.92	3.93	9.60	1.12	4.08	1.76	3.20	3.71	3.81	4.49	12.76	4.95		1.41		
U66469	CGRRF1	3.63	3.50	3.58	2.82	0.46	1.48	2.58	2.27	1.98	7.35	2.79	2.40	5.58	1.37	3.39	1.31	1.34	6.14	3.07	6.52	8.96	4.55		1.72		
L13698	GAS1	12.51	5.32	5.02	5.30	0.62	3.15	5.32	2.04	1.47	7.30	5.49	3.32	3.31	2.95	3.70	1.56	3.38	5.41	5.65	9.24	11.54	6.13		1.44		1.66
S66431	JARID1A	5.40	6.27	6.07	4.80	1.37	3.63	4.59	4.61	3.35	7.91	7.84	4.14	6.96	3.19	5.43	3.35	2.54	4.44	5.96	5.40	8.95	5.10				
D13365	MT3	16.26	14.03	19.85	45.83	48.16	11.43	25.89	13.47	9.37	16.48	30.21	31.84	31.35	45.94	25.52	34.05	25.68	24.42	67.03	43.37	66.23	43.46		1.68		1.70
D63878	NEDD5	11.72	10.51	17.87	15.05	9.43	27.12	15.28	6.93	7.06	20.04	17.66	10.45	21.11	10.15	13.34	13.13	10.88	8.05	18.10	23.69	21.12	15.83				
U49070	PIN1	13.43	6.96	6.26	13.00	9.56	16.67	10.98	5.21	6.40	14.85	13.67	12.29	17.12	10.31	11.40	9.46	8.25	6.66	12.47	15.16	18.21	11.70				
X85134	RBBP5	9.35	13.47	6.37	8.90	5.39	15.25	9.79	6.73	8.43	15.01	5.32	7.40	13.06	9.77	9.39	5.72	6.00	6.65	10.11	19.35	15.34	10.53				



Appendix II The ratios of difference of each gene for cancer versus normal, polyp versus normal and cancer versus polyp subjects. Genes with a ratio of difference of 1.4 or above are highlighted. All genes are normalised to the housekeeping gene β -actin and expressed as % of β -actin.

For a full list of gene names see **Appendix III**

Accession Number	Gene Name	Normal Subjects							Cancer Subjects							Polyp Subjects							Canc vs Norm	Polyp vs Norm	Canc vs Polyp	
		1	3	4	5	6	7	Ave	Syn05	Syn13	Syn15	Syn30	Syn38	Syn58	Syn77	Ave	Syn40	Syn65	Syn70	Syn42	Syn49	Syn74	Ave			
Other Cell Cycle Controls																										
X85133	RBBP6	11.85	10.49	8.19	9.91	3.98	11.07	9.25	3.09	3.51	10.92	8.77	6.63	6.65	8.42	6.85	5.36	3.68	4.14	8.20	6.53	12.04	6.66			
U58334	TP53BP2	6.80	5.00	4.45	3.50	1.05	2.23	3.84	2.41	1.41	9.56	7.47	3.57	5.18	1.22	4.40	1.60	1.90	3.30	3.30	4.47	8.47	3.84			
Cell Adhesion Receptors/Proteins																										
M81104	CD34	3.34	5.06	4.67	3.64	1.82	1.15	3.28	3.34	2.10	10.31	8.38	3.93	9.54	2.85	5.78	1.73	2.66	2.96	4.95	11.31	12.01	5.94	1.76	1.81	
Z24680	GARP	3.37	4.83	3.57	2.03	1.19	1.74	2.79	2.35	0.91	5.10	3.33	2.12	7.89	1.28	3.28	1.07	2.15	2.56	1.83	5.65	7.21	3.41			
Matrix Adhesion Receptors																										
M24795	CD36	4.48	8.06	5.78	2.19	1.90	1.88	4.04	2.46	4.59	6.42	2.20	3.27	5.86	2.86	3.95	1.91	2.45	1.82	5.53	5.78	9.92	4.57			
M14219	decorin	12.73	10.35	19.31	13.42	11.64	12.40	13.31	5.77	5.02	12.32	12.73	10.71	10.56	11.52	9.80	12.60	12.66	6.96	12.30	13.72	16.06	12.38			
X56654	desmoglein 1	4.21	6.40	4.81	1.74	1.52	1.15	3.30	2.10	1.62	10.12	8.15	3.28	3.80	0.87	3.99	1.87	1.32	2.85	3.74	7.80	9.89	4.54			
Z26317	desmoglein 2	19.13	10.07	9.93	11.48	13.28	19.24	13.85	11.84	5.91	15.22	9.60	15.46	11.30	8.91	11.18	16.13	12.07	14.74	23.91	34.57	17.74	19.91	1.44	1.78	
U43901	LAMR1	26.61	21.60	13.63	26.53	28.68	42.97	26.66	25.21	6.41	28.73	12.89	31.33	36.38	22.24	23.31	10.71	19.09	18.15	41.87	47.85	34.72	28.73			
U14588	paxillin	39.93	46.81	38.59	45.13	29.68	52.41	42.07	31.66	36.97	55.76	36.13	45.02	49.45	40.65	42.65	33.16	26.11	26.06	42.22	59.90	52.22	39.94			
L12350	thrombospondin 2	7.08	7.50	6.61	2.86	4.96	4.87	5.65	3.57	2.32	8.62	6.99	2.65	6.19	2.80	4.73	2.13	2.98	4.98	3.15	11.24	13.44	6.32			
Cell-Cell Adhesion Receptors																										
M34064	cadherin 2	3.94	4.79	5.49	2.43	0.08	1.05	2.96	2.15	1.02	4.77	3.66	4.56	4.59	1.91	3.24	2.11	2.07	6.13	3.16	5.98	7.58	4.50		1.52	
X63629	cadherin 3	2.76	8.16	5.95	2.89	0.26	1.75	3.62	2.56	2.09	3.53	3.83	3.38	9.67	2.02	3.87	1.32	2.73	3.85	4.06	6.82	13.03	5.30		1.48	
L34059	cadherin 4	5.57	7.14	4.28	2.60	0.86	1.83	3.71	3.61	3.07	8.94	3.14	3.09	8.53	2.86	4.75	1.18	4.59	5.39	4.11	9.28	6.94	5.25		1.41	
X79981	cadherin 5	95.94	132.52	133.69	91.11	88.59	127.46	111.55	87.11	79.42	198.09	106.22	129.79	174.64	136.80	130.58	69.12	73.17	70.81	139.40	186.05	164.89	117.24			
D31784	cadherin 6	8.00	9.69	6.49	3.89	3.78	3.75	5.93	2.56	3.70	10.03	3.66	4.64	5.27	4.98	4.97	2.92	3.50	5.80	3.43	6.37	10.12	5.35			
L34060	cadherin 8	2.17	6.61	7.71	1.87	2.17	1.60	3.67	2.76	4.82	6.50	2.84	3.23	5.48	1.72	3.90	1.45	1.68	3.07	1.59	7.04	7.42	3.71			
L34056	cadherin 11	1.18	7.21	6.32	2.78	2.04	3.07	3.76	1.77	3.60	7.12	6.02	4.28	6.69	1.73	4.46	1.64	2.68	3.37	3.17	8.32	9.00	4.70			
L34057	cadherin 12	1.08	5.48	6.02	1.78	1.21	2.07	2.94	2.47	2.62	8.39	4.79	1.56	7.35	1.80	4.14	1.33	1.34	2.86	4.91	4.70	7.18	3.72	1.41		
L34058	cadherin 13	2.37	5.93	4.93	2.27	1.52	2.75	3.29	2.96	3.01	9.20	5.78	2.95	5.62	3.35	4.69	2.46	3.88	3.66	4.45	9.23	2.91	4.43	1.43		
D83542	cadherin 15	1.32	6.41	5.60	2.43	1.16	1.92	3.14	2.03	0.89	9.27	3.36	3.29	3.32	2.20	3.48	1.34	3.55	2.78	2.41	6.95	6.07	3.85			
D13866	catenin alpha 1	15.78	13.84	14.44	12.90	7.94	11.68	12.76	9.70	12.01	9.96	18.13	13.24	15.45	13.35	13.12	10.47	5.29	7.88	9.32	20.20	12.71	10.98			
M94151	catenin alpha 2	12.90	12.80	8.42	5.32	2.24	4.56	7.70	3.91	11.35	8.53	3.67	4.84	4.74	4.70	5.96	1.43	2.51	5.43	5.59	12.82	10.20	6.33			
X87838	catenin beta 1	31.21	25.72	12.65	33.39	25.35	38.75	27.84	18.88	14.20	42.19	20.32	26.15	33.92	26.95	26.08	18.98	19.03	18.42	37.60	49.46	44.60	31.35			
X72925	desmocollin 1	4.82	3.92	2.36	2.54	0.42	0.75	2.47	1.81	1.14	5.14	2.53	2.57	2.51	1.76	2.49	2.06	1.09	4.75	3.05	7.29	10.89	4.82	1.95	1.93	
X56807	desmocollin 2	4.88	4.53	4.98	3.46	0.70	1.29	3.30	2.47	1.74	9.05	3.44	1.25	3.95	1.42	3.33	2.46	2.37	2.69	3.56	7.59	5.06	3.95			
X83929	desmocollin 3	3.10	7.59	4.44	2.45	1.68	2.36	3.60	2.46	5.57	8.10	3.24	1.59	4.28	3.13	4.05	2.16	3.15	1.75	3.18	6.81	6.79	3.97			
M77830	desmoplakin	28.78	35.19	18.20	33.65	38.43	50.39	33.77	17.02	19.04	52.89	21.53	31.81	44.33	32.02	31.20	24.53	25.72	23.50	35.85	54.86	34.15	33.10			
U53786	envoplakin	7.07	6.93	6.29	5.09	7.81	5.66	6.47	4.38	3.39	7.73	6.00	7.49	4.96	6.04	5.71	4.67	6.64	4.80	6.48	12.85	8.23	7.28			
M74387	L1CAM	2.94	4.19	4.76	3.12	1.89	1.87	3.13	3.20	2.01	9.27	5.99	4.91	8.82	3.00	5.03	2.45	3.64	3.54	6.64	8.94	10.40	5.93	1.61	1.90	
X16841	NCAM1	2.16	3.53	5.07	1.75	1.88	0.58	2.50	2.54	0.82	8.49	3.09	2.90	6.11	1.04	3.57	1.59	1.48	3.01	2.43	3.86	9.06	3.57	1.43	1.43	
U72661	ninjurin 1	2.24	4.73	11.28	2.87	2.21	1.37	4.11	3.98	3.03	7.40	3.89	5.25	8.88	3.19	5.09	2.17	4.01	2.24	8.35	6.38	11.81	5.82		1.42	
L34774	OPCML	0.79	5.70	3.33	2.34	1.40	1.64	2.53	4.90	1.63	7.19	3.79	2.29	5.98	1.92	3.96	1.65	3.13	3.45	3.06	5.44	8.88	4.27	1.56	1.69	
U52111	plexin B3	4.89	4.55	6.43	2.33	1.46	1.61	3.54	2.25	4.45	5.20	3.55	3.26	7.22	2.78	4.10	1.62	3.24	6.44	3.64	7.78	8.09	5.13		1.45	
AAA36418	protocadherin 1	20.23	16.30	9.88	23.05	14.54	19.15	17.19	22.99	8.74	15.00	22.35	20.89	15.93	24.03	18.56	21.04	16.85	21.08	20.93	44.02	27.29	25.20		1.47	
L11373	PCDHGC3	4.77	7.72	6.74	3.69	5.38	5.81	5.68	3.72	2.94	14.27	6.50	6.90	15.00	9.32	8.39	2.64	3.71	5.15	7.61	9.87	15.26	7.37	1.48		
U60800	SEMA4	4.32	9.00	4.98	3.51	2.41	3.84	4.67	3.82	1.92	9.34	3.33	2.86	3.95	3.13	4.05	2.81	3.24	5.27	5.81	9.81	11.89	6.47			1.60
X85978	TAX1	1.50	4.54	5.31	2.95	1.18	1.71	2.86	1.76	1.38	8.83	3.29	2.08	3.74	2.05	3.30	2.13	3.56	2.67	4.63	4.86	5.29	3.85			
Integrins																										
X68742	integrin, alpha 1	4.57	8.66	3.31	2.63	1.56	4.46	4.20	2.56	2.63	11.10	7.38	3.75	7.99	2.47	5.41	3.76	4.24	2.22	7.57	6.96	9.46	5.70			
M28249	integrin, alpha 2	4.25	8.03	5.29	3.29	2.06	2.61	4.25	3.86	3.20	4.75	3.54	3.32	5.31	2.50	3.78	3.01	2.73	5.83	3.86	5.74	7.13	4.71			
M59911	integrin, alpha 3	17.70	33.23	25.01	31.43	21.93	25.58	25.79	15.79	16.64	46.68	19.27	28.88	17.26	29.38	24.85	18.73	17.01	15.40	24.81	8.05	20.59	17.43		1.48	1.43
L12002	integrin, alpha 4	7.97	11.85	5.98	6.16	1.79	2.85	6.10	3.20	7.56	9.27	6.84	2.95	6.23	5.66	5.93	2.39	3.73	4.91	7.86	5.78	11.40	6.01			



Appendix II The ratios of difference of each gene for cancer versus normal, polyp versus normal and cancer versus polyp subjects. Genes with a ratio of difference of 1.4 or above are highlighted. All genes are normalised to the housekeeping gene β -actin and expressed as % of β -actin.

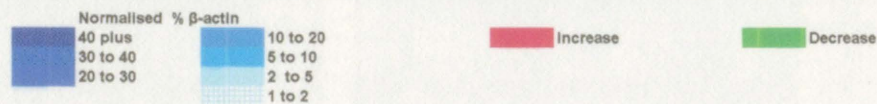
For a full list of gene names see **Appendix III**

Accession	Gene Name	Normal Subjects							Cancer Subjects							Polyp Subjects							Canc	Polyp	Canc	
Number		1	3	4	5	6	7	Ave	Syn05	Syn13	Syn15	Syn30	Syn38	Syn58	Syn77	Ave	Syn40	Syn65	Syn70	Syn42	Syn49	Syn74	Ave	vs	vs	vs
	Integrins																							Norm	Norm	Polyp
X06256	integrin, alpha 5	5.40	4.30	5.47	3.30	2.71	3.24	4.07	3.84	2.30	6.47	4.60	2.84	11.14	2.44	4.80	2.78	5.24	2.94	5.75	5.35	14.52	6.10			1.50
X53586	integrin, alpha 6	26.09	32.57	30.62	41.27	35.09	43.26	34.80	23.80	24.48	78.59	29.99	40.68	67.01	35.90	42.92	29.26	23.79	24.77	57.18	60.67	53.66	41.55			
X74295	integrin, alpha 7	4.53	3.99	3.77	3.78	2.15	3.23	3.57	2.56	2.72	6.68	4.87	3.44	6.82	3.60	4.38	3.83	3.09	3.47	5.37	6.61	8.28	5.11			1.43
L36531	integrin, alpha 8	7.15	6.33	3.93	3.22	2.63	2.94	4.36	2.87	0.90	11.48	8.12	4.76	7.15	1.50	5.25	3.35	5.24	3.84	4.97	7.83	11.82	6.17			1.41
D25303	integrin, alpha 9	3.89	5.28	4.06	2.40	3.78	3.25	3.78	2.59	1.70	7.27	4.37	3.90	6.09	4.23	4.30	3.37	3.16	5.17	5.70	5.18	11.02	5.60			1.48
L25851	integrin, alpha E	4.26	9.69	9.29	4.16	4.47	6.00	6.31	3.93	2.14	7.62	5.48	3.41	8.66	2.94	4.88	2.71	4.84	4.28	7.77	9.17	8.22	6.16			
M34189	integrin, beta 1	3.36	4.26	4.04	1.96	2.88	2.14	3.10	2.31	0.73	10.70	6.60	1.34	8.92	1.90	4.64	1.35	2.31	2.20	5.25	5.26	9.35	4.29	1.50		
J02703	integrin, beta 3	4.61	6.55	5.11	4.87	4.85	3.78	4.96	3.84	2.25	8.05	20.89	5.05	6.70	3.97	7.25	4.83	4.38	4.56	5.70	10.64	12.28	7.06	1.46	1.42	
U37139	integrin beta 3 (BP)	5.08	6.65	3.66	2.95	1.75	2.79	3.64	2.00	2.57	7.03	3.82	2.51	4.90	1.37	3.45	2.22	3.16	2.93	3.65	4.85	11.49	4.71			
X53587	integrin, beta 4	32.63	31.58	19.42	56.30	26.37	26.40	32.12	24.93	16.99	61.43	35.26	43.44	37.59	53.03	38.95	22.79	18.20	31.76	26.89	44.67	31.31	29.27			
J05633	integrin, beta 5	3.86	5.87	5.46	3.27	2.52	1.43	3.73	4.92	4.13	11.14	18.79	3.37	7.67	3.37	7.63	2.77	5.27	2.57	3.73	4.82	12.04	5.20	2.04		1.47
M35198	integrin, beta 6	2.81	5.34	4.43	5.45	1.46	3.69	3.86	4.58	3.94	11.61	7.15	5.03	8.59	5.08	6.57	4.33	3.06	5.40	8.84	4.40	10.80	6.14	1.70	1.59	
M62880	integrin, beta 7	12.68	16.12	14.52	12.71	11.02	9.52	12.76	8.44	8.60	50.57	21.59	18.78	19.87	19.60	20.78	12.96	13.06	6.45	22.26	20.21	20.68	15.94	1.63		
M73780	integrin, beta 8	9.21	21.46	38.51	13.80	12.40	18.81	19.03	8.69	12.64	21.79	14.83	9.61	28.20	8.45	14.88	14.86	11.98	4.00	39.47	19.09	34.91	20.71			
Immune System Proteins																										
M34671	CD59	61.36	99.02	136.79	81.44	94.53	95.91	94.84	102.63	104.61	221.03	97.81	98.46	197.48	101.19	131.91	83.68	73.03	59.33	140.24	184.56	164.79	117.80			
M11886	HLA-C	118.61	50.38	39.12	109.75	82.36	48.18	74.73	89.99	45.04	60.99	98.63	64.12	72.15	97.87	78.40	91.56	112.44	102.00	120.09	61.37	104.68	98.69			
M21626	TRAV14DV4	1.07	6.91	3.68	2.20	1.10	1.35	2.72	2.94	1.56	10.48	4.81	1.76	5.53	1.68	4.11	1.47	2.65	2.37	2.15	5.23	6.89	3.46	1.51		
Oncogenes & Tumor Suppressors																										
U23435	abl-interactor 2	5.96	6.70	5.97	4.67	1.30	2.73	4.55	3.51	1.26	8.30	5.34	4.03	5.72	1.38	4.22	1.15	1.67	5.85	4.99	3.64	7.70	4.16			
M74088	APC	7.12	14.18	12.96	11.88	1.87	12.80	10.13	8.17	8.56	27.89	5.48	11.51	18.26	16.53	13.77	5.87	8.60	6.49	17.07	17.63	34.22	14.98		1.48	
U43746	BRCA2	7.28	29.02	12.31	3.57	0.37	2.38	9.15	3.14	24.32	7.01	3.81	2.05	4.15	2.06	6.65	1.60	4.73	3.10	4.47	5.72	6.56	4.36		2.10	1.52
X03663	CSF1R	21.88	9.83	7.65	17.02	5.57	8.70	11.77	8.20	4.42	10.09	11.32	12.15	10.57	12.02	10.54	6.54	7.74	8.74	12.08	31.24	23.70	15.00			1.42
X76132	DCC	4.70	5.17	4.96	2.48	6.08	1.67	4.17	4.03	2.79	16.87	6.97	4.27	7.10	5.06	6.72	2.42	6.28	3.72	6.39	5.17	9.72	5.61	1.61		
U49089	DLG3	19.87	21.21	20.44	32.17	21.90	38.44	25.67	16.89	14.61	70.72	29.31	37.66	73.31	42.17	40.67	22.61	21.72	17.28	33.95	46.99	53.35	32.63	1.58		
AF010314	ENC1	3.93	4.93	3.32	2.04	0.09	0.96	2.54	2.52	1.48	6.57	3.09	1.23	4.24	1.64	2.97	1.39	3.13	2.37	3.83	4.09	6.89	3.61		1.42	
X00588	EGFR	9.81	18.31	8.18	17.47	14.84	14.12	13.79	9.31	11.31	33.36	18.74	19.26	12.25	20.66	17.84	12.30	11.55	7.30	13.75	19.76	13.45	13.02			
M99487	FOLH1	6.57	6.75	4.32	2.48	0.27	1.48	3.64	3.66	2.39	9.61	2.81	1.81	5.72	1.24	3.89	1.65	2.92	2.09	3.80	5.83	10.21	4.41			
M19722	FGR	6.72	5.86	5.30	4.50	1.72	2.26	4.39	5.79	2.21	4.75	7.53	1.79	5.79	2.46	4.33	2.81	2.74	2.28	2.57	8.96	9.54	4.82			
L29511	GRB2	59.05	49.09	47.90	62.16	45.76	80.64	57.43	45.40	29.08	149.49	61.80	66.71	112.04	64.44	75.56	45.83	41.08	43.03	80.59	106.65	91.88	68.18			
AF001954	ING1	11.63	7.62	16.76	10.93	4.88	6.97	9.79	6.12	2.90	12.66	13.30	7.30	6.82	2.58	7.38	8.02	6.60	6.50	6.70	10.98	8.54	7.55			
M29039	JUNB	4.62	6.29	6.60	9.79	3.72	1.71	5.45	5.50	2.58	6.68	5.90	4.80	5.28	3.39	4.87	4.51	4.95	11.65	2.33	9.62	9.68	7.12			1.46
U43527	KISS1	5.15	5.11	3.41	1.87	0.59	1.43	2.93	2.93	1.85	4.67	3.01	2.12	4.96	1.14	2.95	1.69	1.55	3.16	3.61	4.97	8.88	3.97			
X06182	KIT	6.69	5.99	5.39	4.79	2.71	2.83	4.73	3.02	1.43	5.97	7.88	2.81	3.14	1.59	3.69	2.39	2.31	4.39	5.07	4.25	8.63	4.50			
X13967	LIF	1.12	6.77	5.47	3.78	1.76	4.20	3.85	3.80	4.06	12.90	3.93	4.06	7.68	3.42	5.69	5.08	3.13	3.33	6.73	3.46	10.85	5.40	1.48	1.40	
AF010312	LITAF	51.88	57.01	40.26	59.82	48.18	71.29	54.74	36.74	29.52	91.03	41.01	62.59	96.05	56.22	58.16	35.61	32.60	37.11	62.98	78.36	82.46	54.86			
J02958	MET	6.62	9.55	3.72	10.78	3.36	8.94	7.16	5.68	4.24	14.88	4.40	6.96	11.71	4.59	7.49	4.95	4.81	5.12	3.24	13.52	21.41	8.84			
U35113	MTA1	10.67	6.46	4.37	9.20	1.94	6.16	6.47	5.81	3.61	11.86	5.43	6.12	9.13	6.60	6.94	4.74	6.13	6.95	5.05	12.63	7.65	7.19			
U68162	MPL	3.84	5.58	3.53	2.30	1.40	1.63	3.04	2.86	2.58	8.07	2.40	2.69	2.54	2.28	3.34	1.67	3.40	3.42	2.99	7.41	8.48	4.56			1.50
AF010315	p53-induced protein	3.04	2.57	3.07	1.91	0.13	0.86	1.93	3.02	2.08	6.83	2.68	1.73	7.79	1.69	3.69	1.07	1.42	3.60	3.85	6.43	6.85	3.87	1.91	2.01	
X02811	PDGFB	5.64	6.69	3.34	2.03	2.19	2.37	3.71	3.53	1.52	12.05	2.08	4.12	3.93	1.31	4.08	2.12	3.31	3.51	1.39	5.85	7.00	3.86			
D89667	prefoldin 5	32.78	47.99	61.10	47.87	49.65	56.75	49.35	43.52	44.16	83.18	40.83	61.75	69.84	92.98	55.18	36.84	52.38	41.63	73.79	85.29	68.90	63.16			
AF010310	PRODH	4.58	3.68	3.44	2.88	0.64	0.92	2.69	2.75	2.56	4.95	3.46	2.33	4.24	1.20	3.07	1.76	2.19	2.81	2.30	3.68	7.23	3.33			
AF010316	PTGES	2.74	7.11	3.01	3.76	0.91	1.79	3.22	3.50	1.83	6.51	4.02	2.23	3.98	1.19	3.32	1.69	1.73	2.86	2.01	6.00	10.46	4.12			
AF010309	TP5313	13.31	6.35	2.33	10.62	2.12	4.91	6.60	4.19	4.18	12.61	7.82	6.29	7.32	2.09	6.32	14.10	3.19	3.20	4.01	10.48	9.26	7.37			
L25080	RHOA	137.04	184.48	180.62	147.62	125.15	192.49	161.23	122.79	128.04	296.75	149.57	150.36	256.09	185.46	178.29	96.09	92.41	89.63	157.12	240.29	200.55	146.01			



Appendix II The ratios of difference of each gene for cancer versus normal, polyp versus normal and cancer versus polyp subjects. Genes with a ratio of difference of 1.4 or above are highlighted. All genes are normalised to the housekeeping gene β -actin and expressed as % of β -actin. For a full list of gene names see **Appendix III**

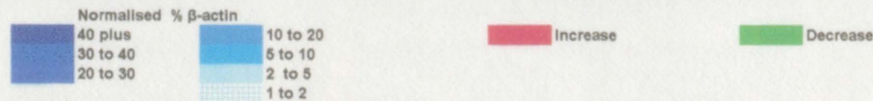
Accession Number	Gene Name	Normal Subjects						Cancer Subjects							Polyp Subjects						Canc vs Norm	Polyp vs Norm	Canc vs Polyp			
		1	3	4	5	6	7	Ave	Syn05	Syn13	Syn15	Syn30	Syn38	Syn58	Syn77	Ave	Syn40	Syn65	Syn70	Syn42	Syn49	Syn74	Ave	Norm	Norm	Polyp
Oncogenes & Tumor Suppressors																										
L25081	RHOC	34.79	45.07	40.29	45.50	45.16	69.79	46.76	58.05	39.56	105.63	37.14	66.10	77.67	61.00	63.59	43.98	44.53	46.43	85.03	103.35	88.65	68.66			1.47
D85815	RHOD	3.45	4.50	3.85	1.99	1.30	1.07	2.69	2.36	1.51	4.98	2.39	2.83	3.27	1.79	2.73	2.11	2.32	4.06	4.05	5.54	8.15	4.37			1.62
X95282	RHOE	7.05	13.64	15.71	16.09	7.30	11.32	11.85	7.81	7.52	27.92	10.03	21.53	39.89	24.70	19.91	12.71	11.88	14.34	21.73	30.00	30.44	20.18	1.68		1.70
X61587	RHOE	5.15	6.34	6.53	3.83	3.27	4.76	4.98	4.85	3.98	7.84	6.96	5.44	8.48	4.83	6.05	3.23	5.59	4.27	10.67	7.50	9.19	6.79			
Z35227	RHOH	3.98	7.04	4.08	2.25	0.99	1.31	3.27	3.06	4.98	4.57	1.71	3.14	4.49	1.57	3.36	0.90	4.25	4.66	1.62	10.22	8.90	5.09		1.55	1.52
M31213	oncogene RET	4.74	5.65	2.17	3.86	1.59	1.86	3.31	2.27	1.27	6.64	5.35	2.67	6.00	0.94	3.59	1.27	2.81	2.79	2.10	4.56	5.42	3.16			
M15400	RBI1	3.68	7.18	4.02	4.46	1.16	5.90	4.40	1.45	4.17	11.92	4.85	5.48	6.94	0.90	5.10	3.29	3.30	3.66	3.77	6.47	14.91	5.90			
X74594	RBL2	17.90	7.58	12.83	12.49	6.13	11.95	11.48	4.44	2.95	16.53	12.18	7.19	10.76	2.65	8.10	5.55	4.27	7.20	12.73	10.81	19.17	9.95	1.42		
U16296	TIAM1	2.67	8.50	3.92	2.84	2.30	2.66	3.81	3.34	2.05	8.63	3.91	3.02	8.03	2.97	4.56	1.99	3.42	2.35	4.27	5.84	13.57	5.21			
Z29083	TPBG	6.53	5.62	7.19	5.05	4.34	3.29	5.33	3.93	1.43	8.91	6.50	3.43	1.47	1.48	3.88	2.35	2.88	6.08	2.31	8.36	8.19	5.02			
M14694	tumor protein p53	7.36	5.63	2.72	5.22	0.56	1.31	3.80	3.42	1.60	10.91	6.65	2.28	4.44	1.44	4.39	1.86	3.47	2.41	3.88	5.99	4.87	3.75			
M35296	ABL2	11.49	5.40	3.35	5.98	0.34	2.80	4.89	3.60	3.20	5.30	7.31	4.41	4.32	3.11	4.46	2.20	2.31	5.22	4.13	4.47	6.43	4.13			
M63167	AKT1	6.08	6.17	10.37	2.34	0.69	1.83	4.58	2.43	1.02	7.05	4.24	2.11	4.23	1.24	3.19	1.41	1.75	2.31	2.16	4.72	10.00	3.72	1.44		
M77198	AKT2	4.30	5.86	2.37	3.02	0.44	1.24	2.87	3.00	2.08	8.58	5.59	1.59	4.19	1.08	3.73	0.82	1.47	2.41	2.77	4.35	9.36	3.53			
M11730	ERBB2	9.51	8.41	5.48	9.03	4.18	8.83	7.24	8.28	1.34	13.69	7.35	8.82	9.85	9.07	8.34	7.91	7.56	4.52	6.50	18.33	13.82	9.77			
M29366	ERBB3	33.32	61.97	56.41	43.40	35.30	43.90	45.71	29.73	51.95	34.19	16.77	27.15	43.68	32.87	33.76	22.81	19.11	16.18	39.81	42.49	46.57	31.16		1.47	
L07868	ERBB4	4.02	3.19	2.73	2.09	3.21	1.45	2.78	3.84	2.07	8.50	3.08	2.27	3.76	1.73	3.61	2.14	3.80	3.91	4.95	3.97	11.34	5.01		1.80	
K00650	FOS	7.21	5.33	3.17	4.24	2.90	1.64	4.06	2.63	1.96	7.97	5.21	3.36	3.40	2.68	3.89	3.42	2.25	5.27	3.02	12.96	10.51	6.24		1.54	1.61
M54968	KRAS2	5.39	5.51	2.21	4.52	1.02	2.62	3.54	3.74	1.40	6.24	4.33	1.83	6.21	1.04	3.54	2.15	2.75	3.48	4.11	10.92	9.13	5.42		1.53	1.53
M13228	MYCN	3.10	5.18	5.63	3.59	1.83	0.68	3.33	2.16	1.87	3.41	2.94	3.60	3.18	2.11	2.75	2.19	3.18	4.73	2.56	7.39	10.43	5.08		1.52	1.85
M95712	BRAF	15.42	13.88	15.97	17.32	6.90	17.87	14.56	8.39	9.00	30.71	7.92	13.94	32.99	19.42	17.48	8.53	12.09	6.77	22.30	23.09	25.39	16.36			
X03484	RAF1	15.97	10.06	8.43	14.24	5.15	13.37	11.20	7.04	4.93	19.72	11.59	9.53	10.46	10.66	10.56	8.26	8.63	6.50	8.42	14.43	16.35	10.43			
NM_00541	SRC	14.57	6.63	4.55	9.40	4.16	4.84	7.36	3.43	2.49	7.18	12.26	4.89	8.20	2.93	5.91	6.93	5.75	2.86	3.04	6.12	5.66	5.06		1.46	
M15990	YES1	8.91	6.35	2.75	12.31	3.09	13.87	7.88	6.46	2.82	22.81	8.22	8.69	20.15	7.25	10.91	7.18	7.52	4.14	7.62	13.22	17.02	9.45			
Extracellular Matrix Proteins																										
M55172	aggrecan 1	23.58	29.75	17.10	23.21	15.89	20.63	21.69	9.80	61.42	77.91	83.71	34.81	21.15	33.54	41.76	27.18	20.62	19.75	33.72	35.39	23.99	26.77		1.93	1.56
J04599	biglycan	3.13	5.46	7.09	3.77	2.85	3.01	4.22	3.35	2.30	11.41	8.30	4.49	5.48	2.60	5.42	2.55	3.13	4.82	3.81	4.63	11.43	5.06			
U16306	CSPG2	2.91	5.18	4.86	3.62	1.38	2.78	3.45	1.78	3.00	7.21	3.36	2.17	3.14	1.46	3.16	1.08	3.92	2.00	5.52	5.98	5.87	4.06			
X02761	fibronectin 1	41.48	56.77	82.16	51.27	55.82	65.11	58.77	25.96	51.11	57.98	29.82	45.91	73.38	67.72	50.26	37.41	35.58	30.35	52.96	56.58	63.76	46.10			
M85289	HSPG2	3.69	7.72	7.99	7.12	4.57	8.44	6.59	3.91	2.34	12.17	8.35	8.18	10.23	6.90	7.44	3.86	3.65	4.03	7.78	9.02	11.61	6.66			
X78565	tenascin C	6.99	5.32	5.68	2.40	2.29	3.54	4.37	3.94	1.89	13.34	5.86	3.57	4.94	3.09	5.23	2.11	3.59	2.93	4.79	6.32	9.28	4.83			
U86759	netrin 2-like	7.43	7.16	4.88	4.34	2.55	3.06	4.90	4.10	3.45	11.99	4.11	3.03	4.98	2.38	4.86	1.79	3.36	4.86	4.28	5.88	14.00	5.69			
M30269	nidogen	11.92	5.95	3.12	4.60	4.72	5.76	6.01	6.77	2.10	9.57	6.96	4.52	9.65	3.35	6.13	3.58	4.80	4.19	7.00	7.04	10.93	6.25			
J03040	SPARC	12.38	12.59	13.80	15.87	21.94	23.95	16.75	15.08	10.45	14.31	25.31	22.41	40.74	22.59	21.55	12.70	11.33	11.08	18.88	19.63	36.70	18.38			
X98085	tenascin R	11.12	9.81	7.05	4.31	3.81	4.28	6.73	4.66	3.53	23.03	17.38	5.26	4.52	5.24	10.51	4.04	4.01	4.49	7.08	7.97	11.74	6.55	1.56		1.60
X14787	thrombospondin 1	1.39	2.93	6.47	2.85	1.11	1.75	2.75	2.53	1.63	10.45	4.60	2.74	5.14	1.85	4.13	1.26	1.52	4.29	2.62	2.91	7.90	3.41	1.50		
X03168	vitronectin	10.14	9.70	6.89	8.29	9.11	10.33	9.07	5.31	5.46	31.76	21.00	12.27	10.89	14.48	14.42	6.27	7.20	6.41	10.41	8.97	12.79	8.67	1.59		1.66
Trafficking/Targeting Proteins																										
X55525	COL1A2	6.43	4.93	8.77	3.47	2.85	2.48	4.82	3.13	2.42	6.35	9.32	2.30	5.43	3.06	4.57	2.36	3.35	2.54	4.58	4.52	6.28	3.94			
X16468	COL2A1	11.07	12.12	14.59	7.34	5.41	14.10	10.77	4.91	10.49	15.95	11.45	7.78	17.06	6.02	10.52	6.20	5.50	3.67	10.27	14.49	15.85	9.29			
X14420	COL3A1	7.14	6.04	5.17	3.38	2.16	2.57	4.41	2.79	2.78	10.49	6.98	1.48	4.72	2.58	4.54	1.65	3.52	4.76	5.65	6.56	8.91	5.17			
X05610	COL4A2	4.70	5.61	2.68	2.61	1.23	1.86	3.11	3.58	1.90	9.99	5.23	2.34	5.87	2.45	4.48	1.66	2.21	2.34	3.37	10.30	12.42	5.38	1.44		1.73
M92993	COL4A3	7.89	9.49	6.85	3.93	3.52	3.33	5.80	3.42	3.47	12.23	12.77	4.23	8.19	3.50	6.83	1.67	4.46	5.34	4.88	5.88	6.28	4.75			1.44
D21337	COL4A6	3.28	4.31	7.46	2.13	2.01	1.91	3.52	5.11	4.12	12.00	11.33	2.44	8.99	4.24	6.89	1.52	3.03	2.89	3.46	5.14	8.32	4.06	1.96		1.70
X15879	COL6A1	21.25	6.97	6.62	13.53	16.47	15.34	13.36	13.41	4.25	19.32	18.71	13.22	17.97	15.81	14.67	12.99	10.05	8.89	15.33	27.87	23.33	16.41			
M34570	COL6A2	13.62	10.74	9.20	7.36	5.75	7.21	8.98	4.59	6.31	6.92	16.15	6.51	8.64	5.08	7.74	2.93	6.16	4.22	7.66	8.55	8.66	6.36		1.41	



Appendix II The ratios of difference of each gene for cancer versus normal, polyp versus normal and cancer versus polyp subjects. Genes with a ratio of difference of 1.4 or above are highlighted. All genes are normalised to the housekeeping gene β -actin and expressed as % of β -actin.

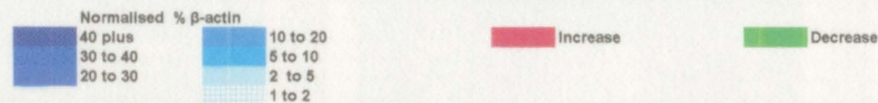
For a full list of gene names see **Appendix III**

Accession	Gene Name	Normal Subjects							Cancer Subjects							Polyp Subjects							Canc	Polyp	Canc	
Number		1	3	4	5	6	7	Ave	Syn05	Syn13	Syn15	Syn30	Syn38	Syn58	Syn77	Ave	Syn40	Syn65	Syn70	Syn42	Syn49	Syn74	Ave	vs Norm	vs Norm	vs Polyp
Trafficking/Targeting Proteins																										
X52022	COL6A3	14.40	8.85	12.23	7.97	9.72	21.36	12.42	7.65	3.05	17.03	11.15	8.69	12.91	12.84	10.47	3.23	5.72	5.42	8.73	7.33	14.22	7.44		1.67	1.41
X57527	COL8A1	2.67	3.38	5.78	2.45	1.97	2.51	3.13	3.03	1.59	10.71	4.54	1.93	6.31	2.04	4.30	1.44	2.06	3.79	4.85	4.85	7.13	4.02			
J04177	COL11A1	2.81	4.89	2.95	1.87	1.04	1.81	2.56	3.68	3.38	7.89	3.87	3.38	7.97	2.45	4.66	1.90	2.43	3.67	4.70	4.74	7.27	4.12	1.82	1.61	
U32169	Col11A2	2.23	4.65	4.83	3.24	1.53	1.84	3.05	3.53	1.30	8.68	2.62	3.23	7.38	1.38	4.02	1.40	2.05	4.33	3.80	4.87	7.83	4.04			
M92642	COL16A1	5.14	7.73	11.51	10.01	6.02	3.37	7.29	5.53	2.20	16.71	8.17	8.73	12.62	9.60	9.08	4.69	5.98	8.16	8.82	9.34	18.65	9.27			
L22548	COL18A1	4.74	6.71	6.34	4.06	3.98	1.61	4.57	4.65	2.90	9.03	6.49	2.97	5.53	3.43	5.00	2.76	3.33	2.80	7.61	11.16	11.48	6.52		1.43	
M61916	laminin, beta 1	10.21	8.86	6.71	9.93	8.75	6.40	8.47	7.58	5.18	10.63	12.56	8.29	12.38	10.28	9.55	9.89	8.97	10.72	10.68	11.64	12.94	10.80			
S77512	laminin, beta 2	4.68	5.28	6.30	3.77	2.52	1.55	4.01	2.95	2.15	8.52	4.19	7.00	4.61	3.12	4.65	2.09	3.31	3.13	6.06	8.81	7.43	5.14			
X70904	laminin, alpha 4	3.34	4.57	11.33	1.68	2.46	1.64	4.17	4.11	2.24	7.72	4.41	3.54	7.07	2.41	4.50	1.51	4.86	3.47	3.42	9.47	10.94	5.61			
J03202	laminin, gamma 1	3.48	4.96	6.47	3.36	0.86	0.97	3.35	4.65	4.05	6.64	4.08	3.28	5.58	1.81	4.30	1.27	3.06	3.06	4.54	8.35	7.68	4.66			
X97442	TMP21	19.93	18.58	18.45	20.10	22.84	38.81	23.12	7.66	13.84	37.32	22.72	20.01	35.77	20.61	22.56	16.07	9.36	7.11	23.95	29.01	17.45	17.16			
Metabolism																										
X01677	GAPD	224.43	261.87	361.37	194.96	193.30	242.93	246.47	363.82	306.93	689.19	198.86	370.81	619.95	342.96	413.22	244.42	249.95	195.60	177.54	173.21	479.67	253.40	1.68		1.63
U90313	GSTO1	53.21	46.74	37.92	48.66	49.76	64.58	50.14	24.06	38.61	73.14	51.25	56.82	76.40	45.23	52.21	33.88	21.61	24.49	50.20	62.94	38.17	38.55			
V00530	HPRT1	5.89	5.07	7.64	2.86	2.83	5.05	4.89	3.21	3.00	11.27	4.05	3.04	7.75	4.00	5.19	2.10	4.70	4.02	8.25	3.67	10.00	5.45			
Y07604	NME4	12.99	12.11	12.06	15.04	13.26	15.07	13.42	11.51	6.67	20.09	17.69	19.20	13.60	17.89	15.23	8.17	10.95	13.70	13.08	20.44	20.41	14.46			
M13267	SOD1	33.79	49.39	36.05	47.48	42.72	77.30	47.77	42.44	49.12	84.75	62.96	48.09	78.91	37.83	57.73	40.87	34.63	38.91	69.63	104.41	77.88	61.05			
Ribosomal Proteins																										
X65923	FAU	52.43	25.24	14.35	48.33	38.63	44.98	37.33	39.01	20.17	45.33	46.18	45.16	33.44	46.21	39.44	38.07	45.09	45.06	38.81	83.35	53.54	50.62			
X56932	ribosomal protein L13a	205.15	159.82	169.98	173.92	167.67	179.60	176.02	164.63	160.62	272.06	175.14	187.00	285.09	154.22	195.53	169.16	171.81	161.61	169.65	164.43	241.55	179.71			
U14971	ribosomal protein S9	81.57	64.75	60.74	59.80	58.95	61.74	71.26	52.19	80.33	74.30	55.92	71.94	83.03	52.21	67.13	57.50	60.76	57.10	79.44	40.12	85.79	63.45			
Apoptosis Associated Proteins																										
Death Receptor-Associated Proteins & Adaptors																										
U84388	CRADD	2.53	5.54	3.13	3.41	0.41	1.47	2.75	2.59	2.16	7.16	2.59	4.31	8.79	2.96	4.36	1.82	2.84	6.96	2.97	6.58	8.60	4.96	1.59	1.81	
U82938	SIVA	5.49	6.25	2.98	2.62	0.97	0.97	3.21	4.24	1.08	7.04	3.17	2.23	8.29	1.74	3.68	1.55	2.59	2.85	3.42	2.91	5.28	3.10			
AF015956	DAXX	8.92	5.11	5.90	6.16	3.24	4.06	5.56	5.37	2.12	3.37	8.13	3.76	6.88	6.30	5.13	5.06	3.97	3.90	6.52	8.31	11.25	6.50			
L41690	TRADD	6.12	7.71	3.67	10.21	2.43	5.17	5.88	6.42	3.74	6.21	4.56	5.94	9.98	8.91	6.54	5.61	5.48	5.75	7.09	15.51	14.51	8.99		1.53	
U78798	TRAF6	5.08	9.32	6.35	8.30	2.05	5.90	6.17	4.64	8.30	12.13	3.69	3.39	12.24	3.69	6.86	3.60	5.43	3.56	9.43	9.88	11.80	7.28			
U90875	TNFRSF10A	2.02	4.86	3.20	2.79	0.44	1.23	2.42	2.52	0.65	7.85	1.64	2.67	2.45	1.36	2.73	1.56	2.09	5.04	5.01	5.47	9.92	4.85		2.00	1.77
Caspases																										
U13699	caspase 1	16.48	8.67	23.69	13.04	8.48	25.51	15.98	5.88	8.15	20.96	10.82	10.06	12.79	3.77	10.34	7.55	6.72	5.21	24.11	10.98	18.09	11.77		1.54	
U13021	caspase 2	14.59	10.98	15.62	15.71	9.87	19.94	14.38	7.80	6.26	12.86	12.43	10.78	15.69	9.29	10.73	11.50	5.71	5.96	21.52	22.40	10.27	12.89			
U13737	caspase 3	20.41	19.01	14.34	20.49	12.48	21.15	17.98	12.13	18.52	32.55	18.44	20.70	31.06	20.00	21.91	15.39	17.41	9.17	26.34	30.47	23.12	20.32			
U28014	caspase 4	20.68	24.46	23.72	26.88	17.50	31.55	24.46	12.14	19.65	44.52	15.83	20.31	28.25	25.25	23.71	14.03	17.12	8.49	41.53	31.91	47.45	26.76			
U20537	caspase 6	7.98	5.12	3.10	5.77	1.81	5.96	4.95	4.19	2.68	10.59	4.18	2.16	7.41	2.90	4.87	1.67	3.04	3.45	7.91	6.40	10.90	5.56			
U37448	caspase 7	6.31	4.46	5.89	9.06	2.37	5.33	5.57	4.05	4.67	13.41	12.58	3.40	6.48	4.42	7.00	6.05	7.41	7.96	7.46	17.08	8.57	9.09			1.63
X98176	caspase 8	1.82	3.72	1.78	2.94	0.61	0.74	1.93	2.27	1.19	8.19	2.66	1.97	5.24	1.72	3.32	0.85	2.76	2.74	2.16	6.96	6.33	3.63		1.72	1.88
U56390	caspase 9	9.96	4.62	3.36	10.37	2.18	5.06	5.92	6.16	1.77	19.53	15.72	7.21	7.19	8.34	9.41	8.09	11.52	6.38	11.05	11.54	15.82	10.73		1.59	1.81
U60519	caspase 10	14.84	9.57	4.74	31.09	6.00	4.80	11.84	5.21	2.09	7.49	8.34	8.47	15.76	16.24	9.08	2.62	5.82	3.86	5.69	8.79	10.68	6.24		1.90	1.46
BCL2 Family Proteins																										
U23765	BAK1	8.58	8.07	8.37	14.15	9.33	18.21	11.11	12.53	5.98	9.95	12.20	13.49	15.26	19.59	12.71	9.00	8.79	5.96	20.58	34.78	28.49	17.93		1.61	1.41
U66879	BAD	3.33	6.81	8.90	5.96	1.21	2.51	4.78	5.12	8.58	8.10	3.63	4.25	5.25	4.95	5.70	3.26	3.84	4.77	4.02	7.01	10.44	5.56			
S83171	BAG1	10.26	23.16	20.67	28.43	11.25	31.05	20.80	15.70	19.80	26.37	19.61	22.20	41.26	26.31	24.46	20.25	15.15	10.16	36.84	49.45	28.90	26.79			
L22474	BAX	10.97	8.42	3.81	11.86	8.08	13.45	9.43	9.04	4.55	15.11	11.92	13.81	7.74	9.37	10.22	6.90	5.32	7.81	13.45	17.57	14.59	10.94			
M14745	BCL2	13.64	7.75	8.13	6.56	1.41	4.55	7.00	3.29	2.11	16.18	8.24	3.99	5.49	2.14	5.78	2.28	3.57	5.26	6.58	8.71	8.45	5.81			
U29680	BCL2A1	2.27	4.85	2.71	5.21	2.36	2.58	3.33	1.83	1.21	4.62	6.51	3.80	4.52	1.32	3.40	2.21	3.49	4.84	4.72	6.33	10.44	5.34		1.60	1.57



Appendix II The ratios of difference of each gene for cancer versus normal, polyp versus normal and cancer versus polyp subjects. Genes with a ratio of difference of 1.4 or above are highlighted. All genes are normalised to the housekeeping gene β -actin and expressed as % of β -actin.

Accession Number	Gene Name	Normal Subjects							Cancer Subjects							Polyp Subjects							Canc vs Norm	Polyp vs Norm	Canc vs Polyp	
		1	3	4	5	6	7	Ave	Syn05	Syn13	Syn15	Syn30	Syn38	Syn58	Syn77	Ave	Syn40	Syn65	Syn70	Syn42	Syn49	Syn74	Ave			
BCL2 Family Proteins																										
U59747	BCL2L2	3.47	6.11	4.60	4.82	2.56	1.60	3.86	4.78	1.86	7.88	3.88	4.67	4.11	3.18	4.31	3.51	3.77	6.59	5.70	7.68	12.53	6.63		1.72	1.54
U34584	BIK	2.46	4.44	2.83	4.30	1.61	2.47	3.02	3.44	1.96	8.77	3.60	4.22	3.75	2.58	4.04	2.28	2.52	5.22	6.52	9.70	10.88	6.18		2.05	1.53
S82185	BRAG-1	1.10	4.50	4.26	3.01	0.38	0.91	2.36	3.22	2.27	8.17	2.50	2.16	3.18	1.43	3.27	0.86	2.81	3.45	2.64	3.69	9.16	3.77		1.60	
L08246	MCL1	17.87	30.84	28.28	35.39	28.20	42.05	30.44	24.68	14.02	58.29	24.96	28.04	66.85	39.64	36.63	26.93	18.96	10.32	37.22	44.70	35.10	28.87			
Death Kinases																										
X76104	DAPK1	11.19	11.78	4.43	17.33	9.43	7.75	10.32	8.41	7.07	17.68	10.07	15.22	14.19	17.57	12.88	7.70	7.97	8.01	11.37	19.86	20.04	12.49			
Y10256	MAP3K14	1.99	6.06	3.11	5.39	0.28	2.95	3.29	3.79	1.69	10.77	10.20	8.01	7.32	6.20	6.85	6.01	4.73	4.06	7.90	5.28	14.38	7.06	2.08	2.14	
X66363	PCTK1	26.55	8.18	5.67	18.00	7.35	12.68	13.07	6.48	3.64	11.63	15.28	9.05	9.33	11.40	9.54	9.30	7.64	4.34	8.70	21.09	9.78	10.14			
X66360	PCTK2	12.36	6.27	5.20	4.81	2.33	1.95	5.48	2.48	1.53	11.14	4.80	2.97	5.23	1.20	4.19	1.61	3.78	4.40	4.20	5.10	7.18	4.37			
X66362	PCTK3	10.12	8.87	12.87	14.84	14.87	8.09	11.61	3.20	3.54	11.03	8.84	13.67	10.78	7.55	8.34	4.72	5.56	7.08	9.90	9.86	11.46	8.09			
Other Apoptosis-Associated Proteins																										
U45879	BIRC2	1.52	4.82	6.31	2.73	0.93	0.83	2.85	2.87	1.36	5.06	2.35	5.32	2.94	1.86	3.11	1.05	2.74	4.42	3.23	5.25	5.13	3.63			
U45878	BIRC3	2.86	5.14	2.22	3.03	0.38	3.54	2.86	3.00	1.84	4.81	4.60	4.35	5.72	2.00	3.76	1.20	2.58	2.72	6.90	5.74	18.17	6.22	2.17	1.85	
U45880	BIRC4	3.19	4.10	2.88	4.30	0.23	1.66	2.72	3.79	1.64	11.33	2.28	2.81	5.07	1.97	4.13	4.97	2.42	4.28	5.29	5.72	9.24	5.32	1.51	1.95	
U75285	BIRC5	7.48	5.34	3.66	3.16	0.57	1.85	3.67	2.51	1.48	6.46	2.78	2.60	4.56	2.08	3.21	2.86	2.61	2.63	4.21	3.56	11.23	4.52			1.41
AF010127	CFLAR	8.29	7.64	6.39	7.21	1.54	4.98	6.01	2.50	2.02	7.21	2.43	6.33	8.46	4.31	4.75	2.40	4.21	7.84	6.86	8.89	13.89	7.35			1.55
U18321	DAP3	4.11	5.24	2.08	7.39	2.45	5.37	4.44	4.51	1.75	11.30	8.69	7.73	7.86	5.55	6.77	5.35	6.04	5.48	5.70	12.12	14.24	8.15	1.53	1.84	
U91985	DFFA	2.54	8.87	2.30	2.36	0.60	0.96	2.94	2.82	1.74	3.66	4.47	2.64	4.07	1.88	3.04	2.10	2.29	3.12	4.81	5.01	4.56	3.65			
S40706	DDIT3	14.54	43.64	37.34	26.17	14.10	21.04	26.14	10.89	45.55	30.84	23.00	28.72	37.29	29.19	29.07	18.40	17.95	9.82	27.84	25.46	37.01	22.75			
S78085	PDCD2	9.23	13.12	7.59	12.60	3.62	17.51	10.61	9.00	13.40	21.49	8.58	9.96	20.26	10.66	13.34	10.81	9.72	10.45	18.62	30.78	21.53	16.98		1.60	
AF022385	PDCD10	5.69	5.21	3.56	2.81	0.38	0.99	3.10	2.01	0.79	3.83	3.77	5.33	5.21	2.21	3.30	1.32	2.78	7.30	2.30	6.43	7.18	4.55		1.47	
U25994	RIPK1	10.09	5.83	9.17	11.61	4.27	12.21	8.86	4.47	5.56	11.75	10.63	9.78	8.99	2.99	7.45	5.57	5.66	5.54	11.06	11.54	7.74	7.85			
RNA processing, turnover, and transport																										
U12431	ELACL2	3.18	28.63	22.05	2.86	2.46	1.76	10.15	3.64	32.73	6.03	10.04	3.22	4.56	3.11	9.05	2.09	3.23	4.71	6.80	5.76	7.43	5.00		2.03	1.81
Cell Receptors																										
Growth Factor & Chemokine Receptors																										
L17075	ACVRL1	4.43	6.09	6.07	8.51	8.08	5.58	6.46	5.30	2.42	8.06	6.96	10.20	9.43	5.29	6.81	5.81	5.48	4.17	4.20	9.83	12.64	6.99			
M60459	EPOR	3.74	3.76	5.21	1.74	2.79	1.89	3.19	3.72	2.83	3.96	4.25	1.97	6.69	1.82	3.60	2.01	4.56	2.29	3.69	5.07	5.84	3.91			
X68203	FLT4	3.21	5.51	4.07	1.49	2.33	1.78	3.06	4.38	3.32	13.61	4.26	3.86	4.52	4.01	5.42	2.04	3.72	4.74	5.71	8.57	10.19	5.83		1.77	1.90
L37882	FZD2	5.03	6.13	6.82	3.16	2.26	1.38	4.13	1.56	1.68	9.20	6.38	2.26	4.17	2.08	3.90	2.69	2.11	3.67	4.11	8.06	10.37	5.17			
U28811	GLG1	16.43	11.56	12.61	18.37	10.01	21.69	15.11	8.75	6.94	23.08	19.52	17.11	16.45	16.32	15.45	14.55	11.66	11.45	17.31	23.49	17.68	16.02			
X04434	IGF1R	7.05	5.87	7.53	5.18	5.91	3.66	5.86	3.38	3.26	8.15	4.09	3.79	4.28	3.04	4.28	3.79	3.89	3.47	5.82	7.82	7.80	5.43			
Y00285	IGF2R	23.41	26.80	13.41	23.18	20.37	31.29	23.04	13.86	13.82	24.98	24.50	19.20	24.10	20.11	20.08	17.49	11.75	8.74	19.77	24.58	25.73	18.01			
M35410	IGFBP2	13.42	16.75	5.98	8.84	12.23	9.43	11.11	25.38	4.38	9.72	19.76	16.25	11.87	4.57	13.13	11.66	9.42	6.15	20.03	17.97	20.57	14.30			
U12140	NTRK2	5.00	10.94	6.41	9.71	7.88	4.87	7.47	2.93	12.16	30.94	6.97	9.61	9.97	6.02	11.23	2.73	5.83	3.71	7.30	8.57	12.53	6.78	1.50		1.66
U05012	NTRK3	3.42	6.73	2.89	6.55	2.16	2.69	4.07	3.60	0.81	10.77	1.84	2.40	2.14	2.54	3.44	0.94	2.41	3.93	3.91	5.20	10.96	4.55			
M99437	Notc2 homolog 2	21.40	18.64	15.63	19.13	9.88	23.64	18.05	12.74	10.49	31.95	18.09	19.75	18.71	19.79	18.79	18.21	10.99	7.92	17.60	28.80	38.25	20.29			
U77493	Notch homolog 2	3.20	7.89	5.98	5.01	1.19	4.70	4.66	3.14	5.69	4.14	5.99	5.37	3.53	3.05	4.41	2.32	2.82	3.80	3.65	7.66	7.44	4.61			
U95299	Notch homolog 4	11.54	15.18	4.71	6.51	2.52	2.68	7.19	4.51	9.06	7.74	10.26	6.66	5.52	4.83	6.94	8.22	8.96	7.00	4.84	13.27	9.91	8.70			
U43148	PTCH	4.29	6.34	6.47	4.12	0.62	1.46	3.88	2.65	2.59	4.19	3.00	2.49	5.24	2.55	3.24	1.13	3.25	5.04	2.36	8.21	7.39	4.56			1.41
M21574	PDGFRA	15.66	25.94	30.51	13.96	13.42	33.86	22.22	4.64	11.68	9.08	5.12	10.29	28.63	12.84	11.72	10.36	5.64	5.22	26.51	18.04	32.26	16.34	1.90		
M21616	PDGFRB	9.31	6.38	3.85	4.17	3.08	2.72	4.92	2.70	1.46	9.45	4.67	2.89	3.40	2.87	3.92	2.47	4.28	3.40	3.43	8.06	10.72	5.39			
L07594	TGFR3	9.07	5.80	14.01	4.50	5.17	4.59	7.19	2.60	2.11	12.00	6.75	5.79	4.13	2.18	5.08	2.63	2.26	6.41	3.92	8.95	8.41	5.43	1.42		
X85960	TRK-fused gene	13.76	23.85	16.00	24.29	26.48	56.00	26.73	13.28	19.57	62.05	21.47	18.42	66.51	20.08	31.62	16.03	16.29	6.42	38.78	39.38	39.52	25.90			
AF016268	TNFRSF10B	3.90	13.87	3.62	6.11	0.85	2.16	5.08	3.74	12.17	9.85	3.44	3.27	5.11	3.48	5.86	2.92	4.10	2.91	3.20	6.95	5.68	4.29			
Y09392	TNFRSF25	15.13	11.70	4.74	21.51	1.13	15.41	11.60	7.13	4.05	25.35	14.20	10.55	19.40	11.25	13.13	8.22	9.23	6.51	8.64	10.49	12.79	9.29			



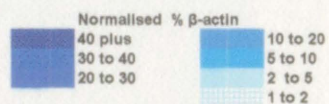
Appendix II The ratios of difference of each gene for cancer versus normal, polyp versus normal and cancer versus polyp subjects. Genes with a ratio of difference of 1.4 or above are highlighted. All genes are normalised to the housekeeping gene β -actin and expressed as % of β -actin.

For a full list of gene names see **Appendix III**

Accession Number	Gene Name	1	3	Normal Subjects								Cancer Subjects									Polyp Subjects							Canc vs Norm	Polyp vs Norm	Canc vs Polyp
		4	5	6	7	Ave		Syn05	Syn13	Syn15	Syn30	Syn38	Syn58	Syn77	Ave		Syn40	Syn65	Syn70	Syn42	Syn49	Syn74	Ave							
Growth Factor & Chemokine Receptors																														
M33294	TNFRSF1A	10.67	9.42	5.49	12.26	14.53	15.62	11.33	9.87	5.68	15.44	14.19	13.67	14.94	10.98	12.10	10.08	5.41	6.84	7.57	19.09	21.89	11.81							
M32315	TNFRSF1B	5.37	12.09	11.30	8.63	5.81	9.96	8.86	4.12	10.89	13.19	4.22	8.01	5.80	6.16	7.48	4.51	3.94	3.30	3.38	10.24	8.36	5.62		1.58					
X60592	TNFRSF5	9.55	7.01	4.20	5.28	5.44	7.25	6.45	3.76	3.37	8.76	8.77	3.87	4.08	2.95	5.08	3.14	5.37	4.34	7.21	6.70	11.56	6.38							
Z70519	TNFRSF6	13.22	8.38	13.56	7.17	4.00	6.30	8.77	3.82	6.39	11.86	8.33	4.21	4.57	2.23	5.91	3.31	3.04	2.36	5.31	10.03	14.56	6.43	1.48						
FGF receptors																														
M37722	FGFR1	7.43	9.10	5.66	6.00	5.48	5.94	6.60	3.85	4.06	9.94	6.90	8.82	9.67	9.65	7.55	5.19	4.33	5.06	10.45	11.37	17.62	9.00							
U11814	FGFR2	2.64	4.03	4.21	4.10	3.31	2.74	3.50	3.01	3.13	10.89	4.17	5.97	5.51	2.57	5.03	2.44	4.16	3.17	5.36	7.94	10.50	5.59	1.44	1.60					
M58051	FGFR3	2.73	5.38	5.97	2.44	1.27	1.77	3.26	2.70	2.22	9.14	3.90	1.92	3.44	2.16	3.64	2.21	2.47	3.62	3.01	7.10	9.88	4.70		1.44					
Ephrin receptors																														
M59371	EphA2	5.87	14.84	7.96	8.17	4.64	5.12	7.77	7.31	9.91	14.98	9.58	8.40	22.78	12.80	12.25	13.09	7.68	5.78	7.84	17.55	11.17	10.52	1.58						
L40636	EphB1	1.70	5.21	3.27	0.86	1.10	2.46	2.43	1.62	1.17	9.63	2.48	1.84	3.90	2.09	3.24	2.24	2.56	3.81	4.73	3.43	7.72	4.08		1.68					
L41939	EphB2	3.08	4.85	3.82	1.51	1.38	2.62	2.87	3.61	2.32	6.89	2.11	1.31	6.63	1.42	3.47	3.62	1.55	1.93	5.19	3.69	6.32	3.72							
U07695	EphB4	2.39	5.45	6.83	1.87	1.49	1.61	3.27	2.57	0.92	8.44	2.09	2.23	3.80	1.37	3.06	1.87	1.96	5.72	3.55	4.95	3.81	3.64							
Interleukin Receptors																														
M63099	interleukin 1 receptor	4.70	5.68	2.68	2.65	2.11	1.79	3.27	3.47	1.97	4.91	3.77	3.96	8.19	3.19	4.21	2.64	3.68	3.23	2.62	5.56	7.05	4.13							
M20566	interleukin 6 receptor	5.84	7.32	5.33	9.13	6.78	11.96	7.73	6.02	5.77	13.44	7.11	5.33	8.76	3.95	7.19	7.94	6.72	4.49	12.38	10.82	16.77	9.85							
Interferon receptors																														
J03171	IFNAR1	4.28	6.70	5.08	6.48	4.40	7.64	5.76	3.97	2.69	16.42	2.77	4.00	5.98	4.22	5.72	2.27	3.85	2.39	10.93	10.08	11.17	6.78							
X77722	IFNAR2	4.55	7.28	4.59	4.59	2.05	5.37	4.74	2.70	1.58	9.13	2.80	1.98	12.45	2.05	4.67	2.87	4.07	4.57	8.17	7.55	9.19	6.07							
A09781	IFNGR	3.15	3.88	5.16	3.31	1.03	4.11	3.44	2.18	1.64	10.04	4.31	2.33	3.58	1.44	3.64	2.66	2.89	4.43	2.84	5.80	7.78	4.40							
J03143	IFNGR1	5.36	6.79	6.59	3.26	2.35	7.73	5.18	3.38	2.36	15.61	4.68	3.81	7.05	3.00	5.70	3.20	3.83	2.21	6.07	9.30	12.58	6.20							
U05875	IFNGR2	8.39	11.11	10.95	20.77	18.02	22.51	15.29	7.98	6.08	36.01	7.86	17.69	31.41	16.57	17.66	11.14	10.05	7.04	8.76	16.94	24.64	13.09							
Hormone Receptors																														
X52773	RXRA	9.03	8.48	7.09	8.99	7.03	5.08	7.61	8.41	3.71	7.82	7.76	7.90	5.94	4.27	6.54	7.21	10.51	4.79	6.06	15.36	10.05	8.99							
M84820	RXRB	20.11	26.81	16.43	20.21	15.96	19.06	19.76	13.24	24.47	22.82	26.07	19.10	17.31	19.04	20.29	18.79	14.09	8.04	18.83	24.71	23.99	18.07							
Protein Kinases																														
X61656	KDR	1.79	4.65	7.66	1.16	1.84	1.36	3.07	3.46	1.49	9.22	4.44	3.69	5.40	2.37	4.29	1.52	4.91	3.19	6.84	4.25	6.08	4.43	1.40	1.44					
U33635	PTK7	6.08	4.02	3.02	3.83	2.43	2.28	3.61	3.69	1.41	5.79	2.83	2.25	7.16	2.76	3.70	2.72	3.38	2.92	6.67	7.81	5.85	4.89							
D17517	TYRO3	9.74	11.38	2.87	5.87	2.15	2.62	5.77	4.17	3.36	9.89	6.72	4.12	7.13	3.59	5.57	2.55	2.87	3.78	3.46	8.08	8.89	4.94							
Y09028	NTRK1	9.72	34.63	24.05	12.24	14.04	9.94	17.44	6.13	29.89	9.54	9.22	9.03	9.53	8.56	11.70	8.66	6.39	5.91	13.07	10.95	6.10	8.51	1.49	2.05					
Other Receptors																														
X95425	EPHA5	2.76	3.89	3.21	2.88	0.73	0.94	2.40	2.47	1.35	3.68	3.16	1.70	3.89	1.53	2.54	1.41	2.13	2.26	4.85	8.75	4.20	3.93		1.64	1.55				
U43318	FZD5	3.38	5.18	6.56	1.69	1.18	0.89	3.15	1.59	2.34	5.00	2.06	1.72	2.46	2.80	2.56	1.72	1.71	4.31	2.66	5.36	12.29	4.67		1.49	1.82				
U82169	FZD9	4.09	9.17	4.79	1.82	0.93	1.33	3.69	1.79	2.49	5.15	2.54	2.19	8.45	1.67	3.47	1.70	2.93	5.35	1.75	5.66	8.80	4.36							
X02492	GIP3	4.71	5.19	4.98	9.90	7.31	12.74	7.47	4.46	3.45	12.49	6.04	6.42	7.60	6.08	6.65	3.27	5.40	4.18	4.43	7.42	13.29	6.33							
X13916	LRP1	12.78	28.83	26.14	11.75	8.14	12.38	16.67	6.15	11.91	15.57	6.26	12.69	15.27	13.24	11.58	9.23	4.93	5.06	11.91	12.41	10.82	9.06	1.44	1.84					
U04441	LRP2	3.67	3.52	4.56	3.74	0.38	1.30	2.86	1.92	2.02	6.31	1.93	3.14	5.66	1.97	3.26	1.58	2.04	5.81	2.67	4.96	5.87	3.82							
M14764	NGFR	6.27	4.85	2.10	2.51	2.20	3.46	3.56	3.66	3.07	11.30	3.21	2.96	5.19	1.64	4.43	2.36	1.92	1.77	3.40	5.64	8.02	3.85							
U08839	PLAUR	4.08	6.39	8.20	3.46	3.53	2.59	4.71	3.14	2.22	9.51	3.04	4.32	5.88	3.64	4.53	4.13	3.72	5.56	4.73	6.58	5.66	5.06							
X87852	SEX gene	14.13	18.12	26.14	16.23	12.45	17.66	17.45	5.43	11.18	12.59	7.92	9.74	20.51	14.61	11.71	10.86	9.06	4.08	20.83	13.99	32.60	15.23	1.49						
X60957	TIE	3.89	4.92	3.68	2.55	2.08	5.16	3.71	3.20	2.10	7.42	3.27	4.26	4.63	4.02	4.13	2.11	2.46	3.67	4.42	8.28	11.28	5.37		1.45					
Cell Signaling, Extracellular Communication Proteins																														
Neuropeptides																														
L19063	GDNF	1.71	6.61	5.44	2.67	0.54	1.86	3.14	2.52	1.56	6.33	5.01	2.23	6.88	2.84	3.91	2.06	3.83	3.58	2.90	7.18	6.40	4.32							
X52599	NGFB	2.39	7.91	4.81	2.54	1.23	1.58	3.41	2.16	2.69	9.84	3.15	2.19	8.70	2.09	4.40	1.80	2.74	3.67	2.62	6.82	8.32	4.33							
M73980	Notch homolog 1	7.63	13.32	7.28	7.01	3.90	4.84	7.33	5.33	8.10	5.95	7.10	6.03	9.00	6.97	6.92	6.74	5.40	2.96	6.96	10.34	13.71	7.68							
S41541	NTF6G	10.37	5.00	7.22	4.20	4.61	4.01	5.90	4.03	1.06	9.08	9.59	5.16	5.27	2.99	5.31	4.58	3.84	3.82	5.92	10.32	7.68	6.03							
X53655	neurotrophin 3	10.58	4.99	4.23	3.31	2.21	1.97	4.55	4.34	2.00	3.41	4.65	5.04	3.55	4.22	3.88	3.36	3.72	3.39	4.42	12.46	15.41	7.12		1.57	1.83				



Accession	Gene Name	Normal Subjects							Cancer Subjects							Polyp Subjects							Canc vs	Polyp vs	Canc vs	
Number		1	3	4	5	6	7	Ave	Syn05	Syn13	Syn15	Syn30	Syn38	Syn58	Syn77	Ave	Syn40	Syn65	Syn70	Syn42	Syn49	Syn74	Ave	Norm	Norm	Polyp
Growth Factors, Cytokines & Chemokines																										
M30704	amphiregulin	1.25	5.45	4.01	1.49	0.98	0.76	2.32	1.95	2.62	4.61	2.62	2.41	4.41	2.19	2.97	1.35	1.71	3.09	3.89	6.91	5.23	3.69			1.59
M15530	B-cell growth factor 1	2.53	6.22	3.67	2.98	1.60	1.41	3.07	2.63	1.29	5.42	5.03	2.16	5.88	2.43	3.55	2.20	2.49	2.10	3.58	4.58	7.18	3.69			
M61176	BDNF	2.17	5.02	4.29	2.49	1.92	1.66	2.92	2.65	1.63	8.39	5.19	1.94	3.49	2.80	3.73	1.99	1.69	2.14	1.09	5.31	6.37	3.09			
M73238	CNTFR	1.80	4.51	4.60	1.59	1.21	1.28	2.49	2.18	2.38	7.00	3.29	2.64	3.21	2.94	3.37	1.50	3.23	3.61	1.16	6.61	9.02	4.19			1.68
M92934	CTGF	2.13	4.38	3.10	1.75	0.19	0.68	2.04	2.24	2.27	8.49	3.79	2.16	5.10	2.31	3.76	2.25	2.21	2.33	3.25	6.63	9.34	4.33	1.85	2.13	
U15979	DLK1	6.68	4.91	4.14	3.54	1.51	1.75	3.75	2.22	1.76	6.50	2.64	3.01	3.14	2.75	3.14	2.85	4.56	1.33	2.78	5.82	5.62	3.83			
M60278	DTR	2.01	5.68	1.88	4.19	1.79	2.83	3.06	4.58	2.80	11.70	3.91	4.52	13.04	6.86	6.77	4.40	2.63	2.90	6.79	11.27	10.53	6.42	2.21	2.09	
U49262	DVL3	2.73	6.59	2.72	2.18	0.86	0.98	2.67	2.28	1.98	3.90	3.71	2.89	2.73	1.61	2.73	1.02	2.82	2.55	2.82	4.08	6.39	3.28			
X04571	EGF	2.47	6.04	3.59	2.29	0.35	1.06	2.63	2.77	1.35	9.72	3.45	1.65	5.65	2.49	3.87	1.91	1.18	2.64	3.43	6.57	8.83	4.09	1.47	1.58	
U04806	FLT3LG	8.23	5.48	3.48	5.80	2.39	2.22	4.60	3.98	1.18	8.25	4.48	3.09	4.65	3.32	4.13	3.62	3.22	3.61	5.99	5.91	9.65	5.33			
M86492	GMFB	7.02	41.18	29.18	12.96	5.50	34.28	21.68	7.86	37.28	23.04	8.70	7.48	41.45	19.56	20.77	10.48	11.07	3.53	17.54	22.27	29.81	15.78			
D49493	GDF10	4.42	8.47	5.60	1.49	1.12	1.86	3.83	2.07	7.94	11.15	2.10	2.66	5.90	1.23	4.72	2.35	3.17	2.16	3.24	8.40	9.93	4.54			
U46010	HGF	0.59	3.46	3.09	1.47	0.55	1.58	1.79	3.50	1.01	10.85	3.58	1.75	6.11	1.82	4.08	1.46	2.74	2.62	3.88	6.32	5.01	3.67	2.28	2.06	
D16431	HDGF	9.89	11.97	6.13	17.04	10.63	23.96	13.27	16.29	6.52	32.62	14.20	22.39	19.05	16.68	18.25	13.97	13.65	11.45	18.59	22.13	15.92	15.95			1.14
D14012	HGF activator	4.88	4.50	2.95	3.19	2.73	1.17	3.23	2.16	1.47	6.99	3.19	2.25	7.68	2.55	3.75	2.12	2.17	3.71	5.23	11.18	6.95	5.23		1.62	
A25270	IK cytokine	10.54	24.25	17.36	23.95	15.07	34.90	21.01	11.86	14.06	63.01	19.07	29.47	50.95	35.67	32.01	18.72	19.53	10.98	27.10	36.64	39.91	25.48	1.52		
M59964	KITLG	3.48	7.79	8.17	3.26	3.32	13.37	6.56	4.18	4.74	11.54	6.36	3.56	15.48	4.69	7.22	3.99	4.28	5.02	10.59	13.24	17.16	9.04			
M74178	MST1	6.09	10.06	5.79	13.52	2.88	4.49	7.14	3.98	3.15	25.91	8.42	11.79	21.91	8.89	12.01	2.77	4.30	8.94	8.72	10.67	11.64	7.84	1.68		1.53
X72755	CXCL9	2.79	4.97	4.12	1.63	0.99	2.07	2.76	2.27	2.64	7.03	2.81	2.92	3.55	1.32	3.22	1.61	2.16	2.10	6.93	6.38	4.52	3.95		1.43	
L12280	neuregulin 1	0.91	7.13	5.86	2.92	2.76	5.00	4.10	4.01	1.98	8.97	4.66	3.89	9.52	3.08	5.16	2.93	3.83	3.73	3.67	10.82	9.61	5.76		1.41	
X54936	PGF	6.58	23.21	25.69	5.84	3.56	5.34	11.70	3.69	31.18	5.95	10.31	4.70	8.14	6.89	10.12	3.40	5.48	6.10	4.27	10.66	7.51	6.24		1.88	1.62
X06374	PDGFA	5.79	6.62	4.06	11.59	5.96	7.68	6.95	6.88	6.25	8.52	8.45	10.77	10.20	9.59	8.59	11.70	10.14	5.96	10.93	14.66	13.73	11.18		1.81	
M57399	pleiotrophin	14.58	15.25	20.77	17.91	11.86	18.50	16.47	3.99	6.90	43.63	14.56	34.98	57.33	34.53	27.99	18.31	22.84	18.26	41.07	30.69	36.36	28.92	1.70	1.78	
M54995	PPBP	7.35	15.94	11.11	10.17	2.14	3.53	8.37	8.61	9.98	16.45	7.34	10.23	9.51	8.52	10.09	6.63	5.93	7.85	5.99	13.73	8.78	8.15			
L42379	quiescin Q6	1.72	4.52	3.44	2.78	1.01	1.07	2.42	2.58	1.61	6.56	2.96	2.21	3.02	2.69	3.07	2.55	3.40	3.19	2.25	4.47	9.25	4.18		1.73	
L26081	SEMA3A	4.46	10.26	9.54	3.18	1.46	2.75	5.28	2.68	5.93	7.88	4.28	3.73	5.45	2.07	4.57	2.10	2.77	7.98	3.93	5.70	8.02	5.08			
AB000220	SEMA3C	2.38	4.84	3.62	1.93	0.82	2.80	2.73	3.14	1.21	5.51	3.34	2.66	5.90	1.71	3.35	1.99	2.48	6.36	3.69	7.59	11.14	5.54		2.03	1.65
U33920	SEMA3F	11.65	5.77	4.70	7.57	6.61	5.50	6.96	3.92	2.64	8.26	7.09	8.26	7.42	6.71	6.33	6.81	6.74	3.79	7.43	11.65	9.37	7.59			
X02530	CXCL10	3.20	6.99	3.48	2.52	1.20	3.02	3.40	3.19	1.23	14.50	3.17	1.92	5.71	1.37	4.44	1.66	1.62	3.55	3.81	4.05	7.82	3.75			
L38518	SHH	8.75	11.63	8.36	7.29	3.70	3.36	7.18	3.36	10.32	7.28	8.20	5.21	5.44	5.03	6.40	2.99	3.98	2.40	5.93	7.78	13.22	6.05			
L36034	CXCL12	7.71	7.34	7.04	9.06	5.28	8.18	7.43	5.46	7.01	10.51	3.28	10.85	5.56	11.48	7.73	11.11	10.77	8.96	7.20	20.01	9.81	11.28		1.52	1.46
M96956	TDGF3	2.02	4.59	4.01	4.64	4.64	9.57	4.91	2.76	3.08	9.04	3.84	3.18	9.57	5.70	5.31	2.56	2.97	3.05	4.09	5.03	5.61	3.88			
L36052	thrombopoietin	2.88	4.14	2.23	2.96	2.43	2.43	2.84	2.35	1.23	11.21	2.67	2.68	5.96	1.18	3.89	1.99	2.45	3.33	4.61	7.81	6.69	4.48		1.57	
K03222	TGFA	4.88	5.21	7.17	4.14	2.19	3.90	4.58	5.09	2.78	8.59	5.25	5.22	8.47	4.40	5.40	3.51	5.84	5.05	5.50	11.55	9.13	6.76		1.48	
X02812	TGFB1	5.29	5.96	3.59	4.67	3.14	4.11	4.46	3.57	1.64	11.60	4.11	4.78	5.22	4.14	5.01	1.22	3.85	3.01	5.64	4.35	11.27	4.89			
M19154	TGFB2	1.91	23.37	22.96	2.47	1.30	2.71	9.12	3.60	22.87	10.02	4.74	3.02	6.40	3.23	7.69	2.94	3.32	1.80	4.23	5.68	10.00	4.66		1.96	1.65
J03241	TGFB3	2.19	3.99	3.72	2.33	2.24	2.05	2.75	2.48	2.49	5.09	3.27	1.65	3.32	1.74	2.86	3.08	2.55	2.84	3.49	5.58	6.19	3.95		1.44	
L07414	TNFSF5	2.11	19.23	13.97	3.14	4.10	2.90	7.57	3.74	27.08	6.91	2.98	1.58	4.97	1.37	6.95	3.11	2.97	2.47	3.75	6.66	9.09	4.67		1.62	1.49
D38122	TNFSF6	5.88	4.23	4.93	2.99	2.06	2.15	3.70	4.68	1.24	4.73	7.52	2.58	6.61	2.65	4.28	2.64	2.19	3.36	5.85	5.07	9.39	4.75			
L08096	TNFSF7	4.77	5.83	5.32	4.00	5.11	2.83	4.64	3.18	1.43	6.34	2.64	2.24	2.93	3.48	3.17	2.10	2.56	3.85	3.71	9.09	10.11	5.23	1.46		1.65
L09753	TNFSF8	2.38	22.81	20.33	2.43	2.94	2.48	8.89	3.69	28.20	7.20	2.67	2.56	5.78	1.06	7.31	2.08	2.06	2.83	2.28	6.36	8.78	4.06		2.19	1.80
U57059	TNFSF10	5.89	5.81	3.73	7.24	1.56	4.96	4.86	4.52	4.88	11.17	7.09	5.86	5.13	3.78	6.06	4.86	6.35	6.53	9.01	7.63	14.65	8.17		1.68	
Fibroblast Growth Factor																										
X51943	FGF1	1.28	3.93	3.33	1.93	1.92	0.82	2.20	1.78	2.06	5.46	1.84	1.25	1.94	3.18	2.50	1.48	2.58	2.49	3.79	6.87	7.96	4.19		1.91	1.68
M27968	FGF2	1.69	4.72	3.91	1.94	0.83	2.09	2.53	2.76	3.22	6.91	4.09	2.28	10.37	1.91	4.50	2.41	2.05	3.03	6.02	5.73	10.69	4.99		1.78	1.97
X14445	FGF3	1.12	8.41	9.18	1.37	1.19	2.44	3.95	7.32	9.63	9.78	3.82	1.81	6.42	4.53	6.19	1.85	3.51	2.90	3.76	9.42	5.84	4.55		1.57	

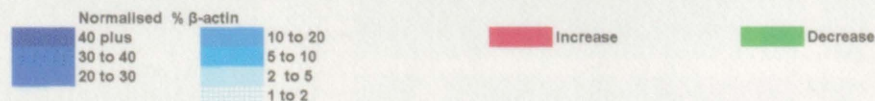


Increase

Decrease

Appendix II The ratios of difference of each gene for cancer versus normal, polyp versus normal and cancer versus polyp subjects. Genes with a ratio of difference of 1.4 or above are highlighted. All genes are normalised to the housekeeping gene β -actin and expressed as % of β -actin. For a full list of gene names see **Appendix III**

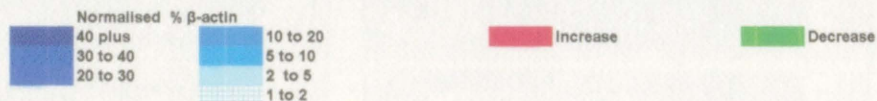
Accession	Gene Name	Normal Subjects							Cancer Subjects							Polyp Subjects							Canc vs	Polyp vs	Canc vs	
Number		1	3	4	5	6	7	Ave	Syn05	Syn13	Syn15	Syn30	Syn38	Syn58	Syn77	Ave	Syn40	Syn65	Syn70	Syn42	Syn49	Syn74	Ave	Norm	Norm	Polyp
Fibroblast Growth Factor																										
M37825	FGF5	1.43	6.17	6.09	1.30	0.30	1.50	2.80	2.96	2.19	8.40	3.74	2.45	5.21	1.92	3.84	1.71	1.41	4.30	3.24	5.06	6.50	3.70			
X63454	FGF6	2.17	4.88	3.15	2.11	0.86	1.88	2.50	4.15	1.24	10.58	4.59	3.31	3.03	3.42	4.33	2.00	2.21	3.12	3.15	4.14	4.97	3.26	1.73		
M60828	FGF7	1.37	9.66	10.38	4.07	2.74	6.02	5.71	4.50	3.42	20.80	5.59	6.42	14.20	5.63	8.65	5.31	6.27	3.33	15.74	7.01	12.61	8.38	1.52	1.47	
U36223	FGF8	8.65	18.08	9.97	9.68	3.75	6.91	9.50	4.50	12.20	12.55	6.36	8.47	11.06	12.18	9.62	6.60	5.49	4.64	6.62	18.19	15.91	9.57			
D14838	FGF9	1.56	4.91	5.65	2.91	0.63	1.24	2.81	2.44	1.50	6.54	2.62	3.84	4.31	2.21	3.35	1.21	3.94	2.60	3.59	5.64	8.52	4.25		1.51	
U66197	FGF12	0.95	4.23	3.53	1.78	1.08	1.68	2.21	2.26	0.87	4.66	3.15	1.60	5.61	2.61	2.96	1.34	2.79	2.65	1.81	7.66	9.83	4.34		1.97	1.47
L03840	FGFR4	3.87	3.93	4.73	2.92	1.90	1.84	3.20	3.80	5.33	10.80	3.88	2.40	7.92	4.38	5.50	2.27	3.54	3.55	6.64	8.23	6.60	5.14	1.72	1.61	
Bone Morphogenetic Protein																										
M22488	BMP1	4.24	10.17	7.02	9.21	5.16	7.84	7.27	7.09	4.66	15.45	5.21	9.42	8.11	11.38	8.76	8.63	6.42	6.04	6.15	18.55	14.08	9.98			
M22489	BMP2	2.66	8.34	4.67	6.02	2.47	3.09	4.54	4.03	2.97	10.21	7.67	6.41	9.00	7.19	6.78	6.45	5.19	3.97	6.00	12.53	16.04	8.36	1.49	1.84	
M22491	BMP3	2.32	16.71	10.35	4.04	1.44	10.44	7.55	10.37	4.14	25.96	2.56	5.24	6.30	1.95	8.07	7.26	5.37	2.55	18.72	19.53	21.66	12.51		1.66	1.55
D30751	BMP4	6.13	11.49	4.54	9.94	2.15	5.65	6.65	5.63	3.68	16.68	8.52	5.48	12.58	9.69	8.89	10.84	12.87	9.42	11.22	14.88	12.50	11.95		1.80	
M60314	BMP5	9.58	11.95	6.19	7.78	2.68	10.77	8.16	4.98	5.60	11.34	4.38	5.85	17.00	5.01	7.74	4.33	9.11	3.07	5.83	12.93	14.14	8.23			
M60315	BMP6	4.08	4.00	2.43	2.18	1.62	2.43	2.79	5.51	2.03	11.00	1.87	2.58	5.11	3.34	4.49	2.35	2.49	1.95	3.74	9.38	4.52	4.07	1.61	1.46	
M60316	BMP7	1.31	6.42	5.70	1.85	0.47	2.74	3.08	1.43	2.61	5.97	2.98	2.53	5.23	1.43	3.17	2.22	2.82	4.27	4.41	8.00	5.41	4.52		1.47	1.43
M97016	BMP8A	3.90	3.50	5.36	3.34	0.82	1.87	3.13	2.88	1.17	8.50	3.99	2.37	7.14	3.10	4.16	2.42	3.10	2.72	2.54	7.91	7.15	4.31			
Ephrins																										
M57730	ephrin-A1	2.63	5.61	4.04	1.87	1.60	0.54	2.71	2.33	1.32	6.19	3.68	2.45	3.23	1.85	3.00	1.77	2.18	5.47	3.24	4.54	4.09	3.55			
U26403	ephrin-A5	4.86	5.45	4.47	1.63	1.02	1.01	3.07	2.13	2.44	3.65	2.74	1.91	4.98	2.24	2.87	0.92	2.41	2.80	4.94	7.33	5.85	4.01			1.40
U09304	ephrin-B1	5.18	5.76	3.84	1.85	0.79	0.77	3.03	2.15	1.83	7.53	2.56	2.45	2.44	1.42	2.91	1.93	1.41	2.29	3.70	5.48	7.03	3.64			
L38734	ephrin-B2	6.61	8.47	3.37	6.24	1.49	2.70	4.81	3.54	3.31	14.10	6.99	5.41	10.71	8.62	7.52	9.03	7.45	6.34	10.13	13.43	13.24	9.94	1.56	2.07	
U66406	ephrin-B3	2.44	5.32	2.36	0.98	0.66	0.68	2.07	2.15	1.89	8.09	2.53	2.48	5.61	1.59	3.48	1.54	1.87	1.59	5.36	4.30	5.94	3.43	1.68	1.66	
Interleukins																										
X02851	interleukin 1, alpha	1.48	2.91	3.73	1.57	0.43	1.13	1.87	2.88	2.24	10.17	4.59	3.59	2.94	1.57	3.99	2.42	3.39	3.73	2.30	4.47	3.73	3.34	2.13	1.78	
K02770	interleukin 1, beta	22.33	60.98	52.71	39.01	37.12	56.10	44.70	32.48	51.99	34.28	25.91	33.20	60.90	44.73	40.50	29.65	26.98	20.53	44.96	45.97	50.44	36.42			
A14844	interleukin 2	3.07	19.43	11.27	3.12	1.38	1.56	6.64	3.20	25.33	5.27	3.24	4.53	4.58	1.82	6.85	2.50	2.59	3.88	3.77	8.70	7.45	4.81			1.42
M14743	interleukin 3	4.13	6.53	6.60	2.23	0.94	1.54	3.66	4.14	4.78	6.73	2.36	2.12	3.67	2.38	3.74	1.32	2.23	2.87	3.18	8.19	5.60	3.89			
M13982	interleukin 4	1.56	5.11	2.95	2.52	0.83	0.96	2.32	2.90	2.38	8.25	1.69	2.22	4.53	1.49	3.29	1.73	1.60	4.38	1.33	8.06	5.33	3.74	1.42	1.61	
X04688	interleukin 5	1.17	4.87	1.76	1.42	0.87	2.07	2.02	2.87	1.66	9.00	5.17	2.18	4.22	1.23	3.76	1.69	1.46	3.93	2.85	5.33	7.28	3.75	1.86	1.85	
X04602	interleukin 6	13.92	13.99	13.04	10.04	9.04	11.69	11.95	6.37	10.84	39.40	17.35	14.49	11.95	14.12	16.36	10.77	11.30	4.97	15.30	14.03	15.46	11.97			
J04156	interleukin 7	1.84	9.36	7.20	1.88	1.08	1.89	3.87	3.11	3.74	5.40	2.75	2.39	4.48	1.38	3.32	1.39	2.92	2.49	4.82	5.08	5.22	3.65			
X17543	interleukin 9	0.91	5.32	3.33	1.45	0.32	2.02	2.22	2.24	1.02	7.62	4.09	2.20	4.99	1.33	3.35	2.58	2.00	5.13	4.25	8.47	12.53	5.49	1.51	2.47	1.64
M57627	interleukin 10	7.58	10.48	8.86	7.67	4.31	6.89	7.26	3.76	3.67	5.40	6.17	5.36	5.40	7.82	5.37	4.79	5.50	3.64	8.36	10.34	10.82	7.24			
M57765	interleukin 11	5.96	3.49	4.02	2.54	2.04	2.67	3.45	3.86	3.09	6.19	2.60	2.88	3.27	3.54	3.63	2.03	4.04	4.14	5.59	5.47	5.43	4.45			
M65291	interleukin 12A	2.89	5.03	3.18	2.42	1.29	1.60	2.73	3.26	2.39	8.76	3.53	1.89	2.35	2.22	3.48	1.81	3.20	2.67	1.22	4.09	7.80	3.46			
M65290	interleukin 12B	3.37	27.68	10.83	2.21	2.64	3.05	8.29	3.78	24.88	6.54	3.72	2.37	5.81	2.10	7.03	1.71	4.92	2.47	0.85	4.87	9.45	4.04			1.74
L06801	interleukin 13	25.62	6.37	7.19	15.38	12.58	17.18	14.05	12.65	3.60	23.34	26.44	17.65	6.80	13.41	14.84	15.14	16.02	13.15	15.34	30.13	14.72	17.41			
L15344	interleukin 14	3.26	5.73	2.71	3.02	1.74	3.57	3.34	2.20	2.02	6.12	3.58	2.90	6.41	2.21	3.63	3.53	2.31	4.10	3.05	4.89	12.21	5.01		1.50	
U14407	interleukin 15	4.02	5.02	4.58	1.95	1.92	2.99	3.41	4.35	1.25	7.52	2.31	4.88	3.63	1.85	3.68	1.46	3.11	5.16	2.06	4.98	7.24	4.00			
U32659	interleukin 17	2.63	3.88	5.42	1.33	1.71	1.68	2.77	2.77	1.83	7.31	3.38	2.63	8.04	1.55	3.93	1.22	2.27	2.75	3.88	3.82	10.08	4.00	1.42	1.44	
Interferon																										
M28622	IFNB1	1.32	4.64	6.50	1.44	1.93	3.62	3.24	4.40	3.09	6.63	3.83	1.56	5.63	2.17	3.90	1.88	2.95	3.94	4.30	4.89	3.78	3.62			
J00209	interferon, alpha 10	1.01	6.96	4.43	1.13	1.68	1.84	2.84	1.82	5.02	8.78	6.36	3.16	3.82	2.64	4.51	1.18	3.27	2.18	2.66	4.19	7.35	3.47	1.59		
X01992	interferon gamma	1.54	4.76	3.17	2.77	1.00	2.11	2.56	2.77	1.68	8.09	5.07	2.50	11.43	2.55	4.87	2.76	2.67	3.33	3.45	5.52	7.41	4.19	1.90	1.64	



Appendix II The ratios of difference of each gene for cancer versus normal, polyp versus normal and cancer versus polyp subjects. Genes with a ratio of difference of 1.4 or above are highlighted. All genes are normalised to the housekeeping gene β -actin and expressed as % of β -actin.

For a full list of gene names see **Appendix III**

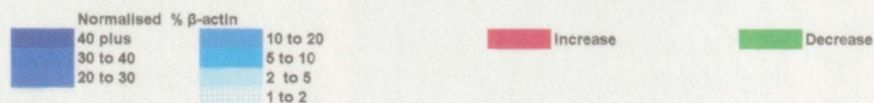
Accession Number	Gene Name	Normal Subjects							Cancer Subjects							Polyp Subjects							Canc vs Norm	Polyp vs Norm	Canc vs Polyp		
		1	3	4	5	6	7	Ave	Syn05	Syn13	Syn15	Syn30	Syn38	Syn58	Syn77	Ave	Syn40	Syn65	Syn70	Syn42	Syn49	Syn74	Ave				
Insulin-like Growth Factor Binding Protein																											
M27544	IGF1	9.13	4.24	1.78	15.32	3.42	2.05	5.99	6.00	1.75	11.53	9.38	4.17	3.93	7.81	6.37	4.89	7.83	8.17	3.15	13.36	5.48	7.15				
M31159	IGFBP3	17.57	10.36	9.02	13.52	15.14	12.01	12.93	6.01	3.88	10.45	7.00	16.84	12.27	14.56	10.11	13.08	5.68	7.25	11.65	13.18	13.02	10.64				
M62403	IGFBP4	6.71	6.11	3.00	7.27	6.82	3.33	5.54	6.79	3.15	9.33	9.44	4.50	5.42	2.89	5.93	9.49	11.02	6.64	9.54	12.14	8.11	9.49		1.71	1.60	
M65062	IGFBP5	7.26	4.43	8.81	5.11	4.89	3.77	5.71	3.05	1.76	7.25	6.45	3.59	2.93	3.90	4.13	3.00	3.67	5.38	5.21	10.29	8.37	5.98			1.45	
M62402	IGFBP6	6.83	6.61	3.44	3.90	3.50	2.62	4.48	2.09	1.73	4.20	4.33	2.53	4.99	2.03	3.13	3.42	2.78	5.53	4.19	7.06	12.41	5.90		1.43	1.89	
Other Extracellular Communication Proteins																											
U83508	angiopoietin 1	2.27	2.96	7.17	2.39	0.60	1.70	2.85	3.31	3.05	7.05	4.83	2.56	3.42	2.92	3.88	1.58	2.57	3.79	3.35	7.32	10.66	4.88			1.71	
M38690	CD9 antigen (p24)	73.12	109.40	132.81	99.65	101.13	149.87	110.99	102.90	112.85	189.12	94.81	126.17	236.04	123.40	140.77	98.59	83.93	79.19	151.45	135.03	162.84	126.84				
U24163	FRZB	9.67	7.74	5.63	8.16	9.61	9.80	8.43	4.21	5.63	14.28	11.49	7.05	7.41	6.26	8.05	14.03	12.15	6.59	12.08	7.11	20.70	12.11		1.44	1.50	
AF028593	jagged 1	19.91	17.65	10.85	19.06	6.67	23.69	16.30	9.32	13.20	41.37	22.70	17.41	38.97	21.55	23.50	20.76	10.07	7.09	16.70	21.12	33.83	19.93		1.44		
AF003521	jagged 2	8.71	8.82	4.75	7.16	2.46	3.87	5.96	4.01	5.94	6.16	6.40	3.70	4.89	3.69	4.97	3.29	3.55	4.12	1.86	7.64	8.79	4.87				
U94354	LIFNG	4.71	9.89	6.62	4.78	1.61	8.73	6.06	3.23	6.83	10.45	4.00	3.48	4.26	3.62	5.12	2.26	3.96	3.35	3.17	10.37	5.00	4.68				
M25639	MIF	2.83	5.04	3.24	8.38	2.94	10.39	5.47	4.93	1.57	10.88	14.03	7.87	8.00	4.62	7.41	5.88	5.40	4.66	8.06	10.44	17.45	8.65			1.58	
U94352	MFNG	6.41	6.64	2.90	3.97	1.46	1.55	3.82	3.70	1.83	7.19	6.16	3.94	8.63	4.35	5.11	3.39	4.55	5.50	4.23	16.26	11.73	7.61		1.99	1.49	
U41745	PDAP1	6.44	9.13	7.90	11.12	6.71	16.92	9.70	7.19	3.86	14.65	6.79	12.30	14.88	12.71	10.34	7.94	7.81	3.94	8.98	14.99	18.16	10.30				
AF017986	SFRP2	2.31	4.71	3.11	4.16	0.35	0.60	2.54	3.68	2.27	4.09	4.06	2.60	7.62	2.50	3.83	2.95	2.57	3.33	3.74	7.97	8.81	4.89		1.51	1.93	
AF017988	SFRP5	5.25	4.68	3.56	2.94	0.38	1.22	3.00	3.19	1.81	4.98	2.55	3.32	6.84	2.30	3.57	0.85	1.47	6.16	3.19	3.88	5.95	3.58				
X07876	WNT2	4.55	6.05	7.92	3.09	3.20	2.57	4.56	2.71	2.63	8.12	3.74	2.22	4.34	1.74	3.64	2.00	1.94	7.97	2.22	2.11	5.47	3.62				
Z71621	WNT2B	5.11	8.44	4.89	4.01	4.40	3.70	5.09	3.57	4.42	6.92	8.24	4.67	6.93	4.48	5.60	4.04	4.12	3.30	4.24	6.10	10.36	5.36				
L20861	WNT5A	12.47	8.96	6.94	9.44	6.35	8.52	8.78	4.36	7.68	11.69	10.38	10.71	4.81	5.76	7.90	4.96	5.12	7.34	4.06	13.81	10.24	7.59				
X91940	WNT8B	9.84	19.63	16.11	9.68	6.48	13.10	12.47	5.72	16.50	10.16	12.72	4.76	7.50	9.80	9.59	7.68	7.50	4.13	10.27	11.53	11.72	8.80		1.42		
X97057	WNT10B	2.80	5.86	6.84	1.59	1.14	1.95	3.36	2.23	1.93	5.58	3.39	1.13	4.37	0.53	2.74	1.18	2.05	2.63	4.37	4.29	4.58	3.18				
Intracellular Transducers/Effectors/Modulators																											
M76125	AXL	14.49	6.74	5.89	9.12	9.30	8.44	9.00	5.82	3.55	15.02	9.22	12.40	8.30	11.59	9.41	5.47	6.69	5.00	7.61	19.11	11.21	9.18				
U24166	MAPRE1	26.10	25.03	24.07	30.29	20.37	48.75	28.27	21.98	24.88	49.35	40.44	34.78	49.28	37.72	36.92	30.48	34.28	23.31	50.71	81.70	64.64	47.52			1.68	
L11353	neurofibromin 2	3.18	4.57	4.16	2.75	3.03	3.14	3.47	4.70	3.98	12.71	3.96	4.13	4.94	3.84	5.46	2.85	2.85	4.86	4.08	4.33	10.09	4.84		1.58	1.40	
Y00815	PTPRF	3.28	5.82	7.32	4.27	3.53	3.87	4.68	5.72	3.41	10.95	5.89	3.78	9.12	7.16	6.57	6.63	5.82	6.67	6.05	11.25	8.26	7.44		1.41	1.59	
L06139	TEK	2.51	4.71	3.97	3.32	1.82	3.26	3.26	2.74	3.59	4.62	3.04	2.92	7.97	3.09	3.99	1.97	3.55	5.88	4.16	7.44	10.82	5.63		1.73	1.41	
U77845	TRIP	2.01	4.30	3.93	2.20	0.33	1.25	2.34	1.81	2.66	4.53	3.18	2.96	3.10	0.94	2.74	1.08	2.37	3.45	2.89	7.62	10.30	4.62			1.98	
Adaptor & Receptor-Associated Proteins																											
U07707	EPS15	15.57	13.90	9.65	13.24	10.15	15.69	13.03	6.41	6.08	8.75	7.31	6.92	18.03	11.22	9.24	7.89	7.33	5.62	15.33	10.33	20.98	11.24		1.41		
U12535	EPS8	3.49	12.60	5.03	6.83	3.12	10.36	6.90	4.87	6.46	11.68	9.08	8.99	11.08	8.74	8.70	6.97	10.27	4.29	6.56	7.33	18.89	9.05				
U69276	GRB10	3.93	5.18	6.69	4.12	1.45	3.05	4.07	2.89	1.70	7.50	4.56	4.91	6.76	2.48	4.37	2.28	3.39	4.08	4.35	4.82	10.12	4.84				
AK026432	HCK	1.94	4.98	3.22	1.85	0.62	2.29	2.48	2.37	1.01	7.33	2.69	1.17	6.66	2.38	3.37	2.14	2.08	3.12	5.50	7.58	5.31	4.29			1.73	
U40282	ILK	2.71	8.75	6.00	12.06	12.64	14.88	9.50	8.61	3.58	20.39	8.42	13.58	17.54	13.63	12.25	15.33	12.77	9.90	23.93	11.13	24.96	16.33			1.72	
M63959	LRPAP1	10.67	10.04	30.85	13.21	1.58	2.72	11.51	6.54	4.35	8.38	13.49	9.56	5.23	7.88	7.92	6.47	12.21	10.38	11.76	14.38	14.23	11.57		1.45	1.46	
U84401	SMO	8.28	5.17	3.56	3.56	1.71	2.59	4.14	3.93	3.46	6.06	5.23	4.96	6.90	4.76	5.04	3.87	4.30	5.85	5.19	11.45	9.25	6.65			1.61	
U12597	TRAF2	2.58	3.50	6.13	2.98	0.88	1.35	2.90	2.51	1.42	6.83	4.04	2.83	4.49	1.80	3.41	2.23	3.96	3.29	4.56	4.35	7.93	4.38			1.51	
U21092	TRAF3	5.48	7.06	5.88	11.54	3.03	5.91	6.48	4.44	1.41	11.82	6.92	6.53	10.73	8.40	6.89	5.60	5.12	5.21	8.18	10.08	13.78	7.99				
U69108	TRAF5	2.08	4.83	3.33	3.37	0.37	1.81	2.63	3.11	1.45	3.91	4.69	2.60	3.47	1.81	3.00	1.89	2.16	2.79	3.96	5.97	10.75	4.58		1.74	1.53	
L05148	ZAP70	3.30	4.53	3.41	2.44	0.90	1.08	2.81	2.67	2.14	4.81	2.05	2.02	2.40	2.54	2.66	1.46	1.82	4.89	4.32	10.55	5.80	4.80		1.84	1.81	
AF000974	TRIP6	2.64	3.60	3.11	2.23	1.50	2.81	2.64	3.82	2.15	6.84	2.73	3.18	5.06	1.77	3.65	1.22	2.47	6.97	4.73	4.29	9.47	4.86		1.84		



Appendix II The ratios of difference of each gene for cancer versus normal, polyp versus normal and cancer versus polyp subjects. Genes with a ratio of difference of 1.4 or above are highlighted. All genes are normalised to the housekeeping gene β -actin and expressed as % of β -actin.

For a full list of gene names see **Appendix III**

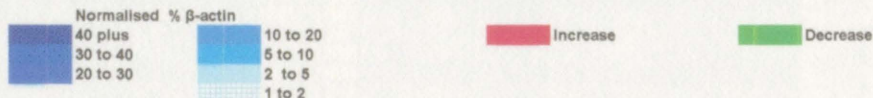
Accession	Gene Name	Normal Subjects							Cancer Subjects							Polyp Subjects							Canc	Polyp	Canc	
Number		1	3	4	5	6	7	Ave	Syn05	Syn13	Syn15	Syn30	Syn38	Syn58	Syn77	Ave	Syn40	Syn65	Syn70	Syn42	Syn49	Syn74	Ave	vs	vs	vs
																								Norm	Norm	Polyp
	Intracellular Kinase Network Members (non-receptor protein kinases)																									
X58957	BTK	5.48	6.89	1.85	5.89	0.37	1.79	3.71	3.12	1.38	7.58	2.07	2.82	4.01	3.01	3.43	1.84	2.35	3.02	4.57	7.92	9.33	4.84			1.41
X86779	FAST kinase	21.92	12.26	6.00	26.43	13.59	17.81	16.33	16.84	6.46	31.49	17.78	23.51	26.54	25.65	21.18	13.06	15.89	15.28	20.01	27.49	26.82	19.75			
L26318	MAPK8	5.01	8.55	5.77	6.52	2.06	2.96	5.14	3.08	2.43	9.15	3.88	3.85	7.02	2.41	4.54	3.92	3.01	4.98	4.94	5.61	10.90	5.56			
L31951	MAPK9	6.36	7.10	6.42	5.33	1.34	2.57	4.85	3.01	2.80	9.96	4.04	3.48	6.19	4.16	4.80	1.85	3.47	3.28	8.37	5.74	7.98	5.11			
U34619	MAPK10	2.78	6.29	4.73	3.22	1.15	2.30	3.41	2.06	1.57	6.67	3.42	2.78	5.36	2.08	3.42	1.81	2.96	4.92	5.05	5.20	6.27	4.37			
U24152	PAK1	8.25	10.70	15.69	15.33	4.83	18.77	12.26	7.02	8.47	33.05	10.37	18.70	23.50	16.66	16.82	13.61	10.13	10.63	17.74	21.21	19.25	15.43			
U24153	PAK2	10.40	7.57	5.02	11.95	1.20	6.07	7.03	4.63	3.38	13.45	7.80	14.37	14.39	14.72	10.39	9.41	10.67	11.10	11.76	28.84	18.26	15.00	1.48	2.13	1.44
U43522	PAK2B	2.16	9.83	9.56	7.28	7.57	7.32	7.28	4.60	5.34	16.30	4.78	7.23	8.70	7.41	7.76	3.68	4.64	8.26	8.39	9.57	14.27	7.80			
L13616	PTK2	9.85	6.74	8.35	7.73	7.98	8.25	8.15	5.87	4.30	13.77	5.05	9.64	11.83	6.16	8.09	10.98	9.34	4.78	15.23	15.61	13.49	11.57	1.42		1.43
U43195	ROCK1	4.19	5.89	5.50	2.61	1.26	2.26	3.62	1.86	0.76	9.28	3.29	2.08	5.61	2.51	3.63	2.02	4.52	4.22	7.00	6.59	13.37	6.28	1.74		1.73
	G-Proteins																									
M35543	cell division cycle 42	15.56	12.54	4.08	18.80	8.55	16.80	12.55	9.47	4.85	26.57	16.99	15.50	19.78	19.62	16.11	13.40	13.60	9.96	14.82	30.28	21.83	17.31			
Y07923	GTP-binding protein	3.37	4.49	10.13	3.01	1.07	1.06	3.85	3.29	2.07	10.81	3.30	5.22	4.08	2.33	4.44	1.66	1.91	6.51	2.06	6.48	9.75	4.73			
X06820	RHOB	37.50	16.15	12.09	42.85	38.92	53.16	33.44	43.38	17.83	53.86	47.52	49.70	57.22	45.37	44.98	47.99	39.72	36.97	71.10	81.41	72.66	59.98		1.79	
X95456	RHON	3.45	6.59	5.78	2.87	0.70	0.55	3.32	2.91	1.18	7.55	3.22	3.63	4.89	2.41	3.68	1.26	1.55	6.76	4.85	4.07	14.24	5.45		1.64	1.46
M31470	RHOQ	3.35	6.64	3.89	3.50	1.76	4.72	3.97	3.80	2.97	9.79	6.51	5.92	8.05	3.69	5.82	3.07	5.76	5.54	9.80	10.80	12.76	7.95	1.46		2.00
M29870	RAC1	21.05	42.68	34.74	32.16	25.68	54.82	35.19	27.31	31.75	78.78	26.37	30.68	88.12	33.80	45.26	25.18	23.80	20.82	65.04	64.43	74.05	45.52			
M64595	RAC2	14.40	15.47	12.57	18.97	9.54	13.29	14.04	10.99	5.12	22.93	9.38	21.24	18.04	20.19	15.35	8.10	12.67	15.85	25.10	32.01	46.94	23.39		1.67	1.52
L24564	RRAD	6.93	5.26	3.21	2.78	0.99	1.26	3.40	3.06	0.61	4.76	5.43	2.32	4.63	1.90	3.24	5.10	2.96	4.00	2.51	7.43	8.93	5.16		1.52	1.59
	GTP/GDP Exchangers & G-Protein GTPase Activity Modulators																									
Z18951	caveolin 1	3.87	5.42	4.29	3.60	4.81	2.90	4.14	4.56	4.00	10.57	6.85	8.17	7.30	5.88	6.76	3.08	6.28	4.00	13.65	10.28	10.52	7.97	1.63		1.92
U32114	caveolin 2	1.33	4.95	6.41	1.63	2.12	1.70	3.02	2.22	3.83	12.05	6.40	5.28	8.15	3.82	5.68	2.43	3.78	3.22	5.15	5.17	10.11	4.97	1.88		1.65
U11690	faciogenital dysplasia	1.56	5.47	4.13	2.21	1.58	1.39	2.72	3.99	2.57	11.58	2.27	2.70	4.73	2.00	4.26	2.00	3.07	1.76	4.17	6.59	11.54	4.85	1.57		1.78
U59752	PSCD2	23.83	25.37	22.54	32.48	21.30	33.05	26.43	20.07	26.21	53.63	24.85	33.73	47.06	35.10	34.06	27.83	19.88	17.29	34.65	38.26	46.39	30.71			
U02570	ARHGAP1	2.21	4.30	2.58	1.64	0.83	0.85	2.07	1.92	1.88	6.08	4.75	2.45	6.06	2.53	3.86	1.49	3.69	4.90	3.53	5.16	6.75	4.25	1.77		2.06
X78817	ARHGAP4	5.68	9.82	8.61	16.86	10.01	12.45	10.57	12.52	6.56	30.03	14.38	14.20	14.41	15.49	15.37	7.07	12.10	9.00	18.78	22.62	37.58	17.85	1.45		1.69
X69550	ARHGDIA	88.53	93.05	111.68	105.76	81.92	133.67	102.43	86.45	76.54	208.01	108.25	122.54	181.19	148.03	133.00	71.74	72.48	67.80	148.31	175.42	176.36	118.68			
L20688	ARHGDIB	9.08	6.66	8.13	11.05	6.82	11.36	8.84	8.30	3.35	11.62	10.80	9.62	11.18	14.17	9.86	5.10	8.93	7.15	18.41	26.49	49.41	19.25		2.18	1.95
U82532	ARHGDIG	1.88	5.06	2.51	1.66	1.08	1.34	2.25	2.52	2.06	6.52	2.28	2.22	7.13	1.87	3.51	1.00	3.96	2.76	3.11	5.92	9.90	4.44	1.56		1.97
	Phospholipases & Phosphoinositid Kinases																									
U38545	phospholipase D1	6.29	11.62	12.17	12.41	4.24	11.70	9.74	7.46	2.91	15.47	5.20	7.10	12.07	5.63	7.98	4.94	5.73	4.63	11.53	11.99	15.34	9.03			
M86400	YWHAZ	6.68	23.35	15.88	7.11	7.90	6.97	11.31	5.08	22.02	14.17	9.00	5.89	8.19	8.70	10.43	7.46	7.45	6.46	12.65	10.92	10.67	9.27			
	Other Intracellular Transducers/Effectors/Modulators																									
L22005	cell division cycle 34	43.63	31.59	34.46	38.32	31.75	32.54	35.38	21.53	27.99	46.53	31.51	36.62	44.20	35.42	34.84	28.74	17.72	15.83	32.87	50.03	15.90	26.85			
U46461	DVL1L1	32.76	26.65	7.61	29.02	20.81	23.06	23.32	13.24	34.81	66.31	38.25	29.99	22.74	24.74	32.87	21.62	19.31	18.12	22.64	30.69	28.83	23.53	1.41		1.40
U56976	PDE1B	11.10	7.69	7.73	4.18	2.65	2.51	5.97	3.19	6.09	6.18	4.97	2.52	3.78	1.60	4.05	1.19	2.21	4.53	5.03	3.28	9.40	4.27	1.48		1.40
	Protein Turnover																									
	Serine Proteases																									
D49742	HABP2	9.41	3.74	10.19	5.26	2.91	3.06	5.76	4.63	2.77	9.72	6.19	2.68	2.36	3.45	4.54	2.03	3.52	6.84	2.31	8.72	7.18	5.10			
X05199	plasminogen	3.64	6.13	3.76	2.07	1.84	2.13	3.26	2.23	1.95	4.91	2.36	3.70	5.38	2.03	3.22	1.49	2.73	3.97	2.87	4.14	8.44	3.94			
M15518	PLAT	28.54	17.96	18.61	21.88	18.85	24.57	21.73	17.92	9.49	38.92	27.84	25.36	29.95	24.60	24.87	18.62	18.06	16.56	29.29	33.30	36.88	25.45			
M15476	PLAU	5.12	4.92	5.06	4.37	1.92	2.04	3.91	4.13	1.16	12.24	7.61	4.44	4.59	3.04	5.31	3.06	3.38	3.37	3.31	12.85	11.70	6.28		1.61	
	Metalloproteinases																									
X05231	MMP1	2.35	5.34	3.42	2.09	0.65	1.60	2.57	1.78	2.57	7.69	2.65	3.00	4.10	2.47	3.46	1.24	2.09	3.84	2.38	5.75	6.99	3.71		1.44	
J03210	MMP2	3.31	5.21	8.39	5.14	1.67	2.95	4.44	2.78	1.15	9.76	2.53	3.43	5.61	3.65	4.13	0.98	3.28	5.58	4.27	4.30	9.47	4.64			
X05232	MMP3	3.14	2.67	3.52	3.33	0.89	1.37	2.49	3.05	2.78	5.33	4.31	5.30	6.87	3.35	4.43	2.37	2.83	4.52	2.67	7.87	9.37	4.94	1.78		1.99



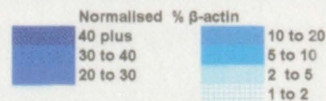
Appendix II The ratios of difference of each gene for cancer versus normal, polyp versus normal and cancer versus polyp subjects. Genes with a ratio of difference of 1.4 or above are highlighted. All genes are normalised to the housekeeping gene β -actin and expressed as % of β -actin.

For a full list of gene names see **Appendix III**

Accession Number	Gene Name	1	3	4	5	6	7	Ave	Syn05	Syn13	Syn15	Syn30	Syn38	Syn58	Syn77	Ave	Syn40	Syn65	Syn70	Syn42	Syn49	Syn74	Ave	Canc vs Norm	Polyp vs Norm	Canc vs Polyp
Metalloproteinases																										
X07819	MMP7	4.86	5.01	4.39	3.59	0.80	2.12	3.46	3.10	1.91	4.49	2.71	2.51	3.49	1.39	2.80	2.27	2.52	3.85	3.87	4.89	7.25	4.11			1.47
J05556	MMP8	6.25	7.36	7.79	3.13	1.24	2.67	4.57	2.31	2.28	7.76	3.35	3.48	4.31	3.63	3.87	2.04	2.05	4.76	6.32	7.95	7.08	5.03		1.85	2.19
J05070	MMP9	8.14	6.65	5.39	9.53	6.60	6.71	7.17	5.81	5.91	7.75	6.60	4.53	6.72	5.04	6.05	2.60	7.21	8.08	10.72	24.04	26.70	13.22			
X07820	MMP10	10.37	4.81	2.96	4.54	1.23	1.72	4.27	2.02	1.32	5.96	2.20	1.81	4.91	1.88	2.87	1.85	1.73	4.53	3.69	6.50	8.03	4.39	1.49		1.53
X57766	MMP11	22.89	24.67	22.32	20.55	15.50	27.08	22.13	6.83	15.81	12.69	22.31	18.75	18.92	22.91	16.88	10.69	15.68	11.90	22.16	24.04	33.51	19.66			
L23808	MMP12	7.90	7.31	6.00	2.70	7.28	3.73	5.82	6.78	2.92	8.10	4.36	3.85	4.88	2.37	4.75	1.90	4.21	3.60	7.44	7.12	7.22	5.25			
X75308	MMP13	3.31	4.90	3.72	1.47	0.74	1.28	2.57	4.30	2.20	8.31	3.02	2.70	7.59	2.11	4.32	1.09	3.25	3.25	2.48	6.83	8.34	4.20	1.68	1.84	
D26512	MMP14	18.45	32.68	32.34	28.70	40.09	39.24	31.91	20.07	16.06	34.80	27.21	40.42	36.78	34.41	29.96	20.53	19.40	17.45	32.78	47.43	42.29	29.98			
Z48482	MMP15	3.09	9.03	6.44	7.01	7.65	5.91	6.52	8.75	2.91	11.77	12.01	11.48	11.38	9.86	9.73	12.49	9.08	8.92	8.72	24.35	9.95	12.25	1.49	1.88	
D85511	MMP16	1.73	10.70	7.50	1.55	3.14	3.70	4.72	3.02	5.72	8.88	4.95	2.93	7.18	3.57	5.18	2.49	4.26	4.86	2.83	8.11	12.71	5.87			
X89576	MMP17	2.21	5.37	4.65	2.75	2.05	2.05	3.18	3.49	3.96	7.97	4.06	3.63	6.77	2.47	4.62	1.95	4.36	2.18	4.89	6.06	5.24	4.11	1.49		
X92521	MMP19	3.59	5.67	2.13	2.71	0.43	0.66	2.53	1.87	2.71	5.61	2.01	3.33	3.52	1.79	2.98	1.48	1.90	5.04	2.38	6.71	6.94	4.08		1.61	
U41766	ADAM9	6.12	11.02	6.75	3.48	2.15	9.41	6.49	4.34	6.72	13.12	7.26	6.95	9.88	6.53	7.83	3.61	7.08	5.93	6.84	7.61	12.14	7.20			
U69611	ADAM17	7.27	14.27	6.53	14.93	3.50	14.95	10.24	6.62	9.22	15.22	10.81	11.17	18.29	12.05	11.91	8.60	8.89	4.57	12.59	18.87	17.23	11.76			
Protease Inhibitors																										
M11313	A2M	11.28	7.84	7.02	12.14	3.32	3.88	7.58	5.85	2.07	7.10	10.70	12.57	11.70	14.60	9.22	9.72	13.33	12.67	11.58	25.69	15.60	14.76		1.95	1.60
M68516	SERPINA5	3.09	5.21	6.36	2.52	3.04	4.18	4.06	2.25	0.87	6.39	3.24	1.44	4.58	2.50	3.04	1.17	2.17	4.88	4.72	7.70	10.16	5.13			1.69
M18082	SERPINE2	4.33	5.86	3.52	2.81	1.90	3.10	3.59	3.67	2.69	7.76	7.72	4.44	4.08	3.42	4.82	4.68	3.55	3.97	4.74	13.68	14.31	7.49		2.09	1.55
X04429	SERPINE1	2.61	4.31	4.56	1.51	2.16	2.38	2.92	2.83	1.02	6.49	1.27	3.39	4.76	1.59	3.05	1.79	4.06	4.28	2.69	4.94	7.85	4.27		1.46	1.40
X03124	TIMP1	40.83	17.66	10.87	38.88	29.95	34.59	28.46	17.17	13.43	57.43	37.84	34.77	28.80	34.45	31.99	20.65	23.55	25.24	31.11	44.42	58.15	33.52			
J05593	TIMP2	7.27	6.91	4.46	7.37	3.11	2.35	5.24	5.52	2.38	10.56	4.81	7.38	7.22	9.10	6.71	6.16	5.63	14.38	5.34	11.92	12.32	9.29			1.77
Z30183	TIMP3	15.14	16.31	18.41	18.89	11.91	16.50	16.19	16.77	13.30	30.92	16.08	25.22	39.72	40.16	26.02	22.66	14.04	20.37	37.86	43.24	41.64	29.95	1.61	1.85	
U76456	TIMP4	2.74	5.90	2.96	6.60	0.46	1.43	3.35	3.41	1.13	8.68	5.98	2.66	4.89	2.60	3.90	2.00	2.27	3.85	2.79	3.15	8.73	3.80			
Others Involved in Protein Turnover																										
L20471	BSG	28.13	58.02	19.72	38.48	30.68	42.93	35.66	56.64	44.78	38.41	34.93	42.75	61.82	38.38	45.39	38.91	32.26	31.68	47.69	97.89	60.30	51.45		1.44	
M26880	ubiquitin C	157.78	207.93	216.86	152.40	174.72	184.92	182.43	159.41	169.08	364.18	154.85	201.18	261.18	193.00	214.69	147.92	165.96	155.61	176.26	108.86	251.82	167.75			
Cytoskeleton/Motility Proteins																										
X00351	beta actin	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00			
U29343	RHAMM	0.67	3.16	3.61	1.35	1.51	1.75	2.01	2.33	1.15	6.88	4.04	2.44	4.78	1.33	3.27	1.39	2.77	3.77	2.50	7.96	6.69	4.18	1.63	2.08	
M23410	junction plakoglobin	53.19	59.36	50.86	56.34	47.26	64.29	55.21	51.49	41.30	110.89	63.79	76.78	94.13	65.15	70.50	40.19	31.99	42.74	58.70	103.60	84.29	56.91			
M77349	TGF-β1	8.11	9.29	8.75	11.25	8.58	12.90	9.48	9.07	4.87	14.32	13.41	14.90	16.41	19.95	13.27	8.17	8.13	7.27	8.05	34.31	18.44	14.06	1.40	1.49	
K00558	alpha tubulin	100.87	84.85	76.10	77.18	71.93	98.12	84.84	75.87	78.52	80.33	86.91	58.48	77.50	73.08	76.67	66.98	65.43	72.60	96.81	32.88	78.29	69.33			
X51521	villin 2	39.79	30.70	12.78	32.18	24.97	31.39	27.97	28.58	24.81	55.52	40.11	46.70	65.92	43.82	43.63	38.59	28.58	28.81	39.41	76.91	34.26	41.09	1.58	1.47	
X94991	zyxin	10.12	8.24	6.97	13.98	5.68	5.86	8.47	4.04	3.00	10.74	9.01	10.78	9.38	13.65	8.65	6.52	4.82	11.38	7.63	18.27	12.37	10.16			
Intermediate Filament Proteins																										
M63618	DST	3.43	7.17	3.94	2.21	2.11	3.40	3.71	4.39	3.01	9.72	4.90	2.81	8.50	3.68	5.29	2.73	4.77	3.30	4.60	5.76	9.42	5.09	1.43		
M99063	cytokeratin 2	4.67	3.99	7.57	2.52	1.01	1.94	3.61	3.22	1.25	7.71	3.51	5.10	2.97	0.60	3.48	1.14	2.99	6.33	4.18	4.28	7.27	4.36			
U59167	desmin	29.38	6.80	38.33	21.40	87.91	30.42	28.79	19.08	12.53	24.42	29.38	62.44	30.12	68.46	33.77	35.27	26.90	29.23	57.24	38.89	69.17	42.46		1.45	
M98776	keratin 1	12.59	7.75	8.73	4.14	3.02	4.29	6.75	5.43	4.34	9.36	7.98	8.24	4.65	3.42	6.20	3.41	4.96	7.51	5.25	7.87	10.40	6.57			
M99061	keratin 2A	6.59	3.47	2.51	3.84	0.46	2.00	3.14	4.14	2.20	4.57	4.71	5.18	5.79	3.50	4.30	3.28	3.66	6.59	3.73	9.08	8.18	5.75		1.83	
X67683	keratin 4	4.37	5.98	4.78	3.63	1.77	2.35	3.81	3.05	2.30	8.52	3.62	4.23	3.91	2.27	3.98	1.70	3.12	9.11	5.05	8.04	8.90	5.99		1.57	1.50
M21389	keratin 5	5.69	3.84	4.24	3.54	1.30	0.78	3.23	2.58	0.51	7.45	3.13	2.93	4.33	2.21	3.30	1.19	1.99	4.26	2.25	4.19	8.06	3.65			
J00269	keratin 6A	3.52	3.89	5.91	3.37	0.62	0.74	3.01	2.88	0.98	6.73	3.04	2.49	5.29	1.85	3.32	1.23	3.78	3.45	2.93	4.45	8.45	4.05			
L42592	keratin 6B	1.55	4.15	4.02	3.26	0.69	2.79	2.74	2.48	3.04	10.18	3.43	2.32	5.42	1.74	4.09	1.19	2.78	2.53	3.37	4.03	7.12	3.50	1.49		
X03212	keratin 7	2.45	4.39	4.27	1.99	0.67	1.45	2.53	2.99	1.11	8.17	3.28	3.39	3.75	2.04	3.53	0.81	1.69	6.09	2.78	6.34	7.99	4.21		1.66	
M34225	keratin 8	62.06	80.81	31.38	70.06	60.25	83.57	64.69	54.34	49.80	93.10	59.06	68.22	78.92	70.68	67.73	55.50	40.15	59.30	47.42	121.37	66.68	65.07			
Z29074	keratin 9	2.65	4.49	4.50	3.04	1.64	1.27	2.93	4.44	1.48	4.47	3.53	2.84	3.65	1.86	3.18	2.55	2.98	4.83	4.14	6.50	6.23	4.54		1.55	1.43



Accession	Gene Name	Normal Subjects							Cancer Subjects							Polyp Subjects							Canc vs Norm	Polyp vs Norm	Canc vs Polyp	
Number		1	3	4	5	6	7	Ave	Syn05	Syn13	Syn15	Syn30	Syn38	Syn58	Syn77	Ave	Syn40	Syn65	Syn70	Syn42	Syn49	Syn74	Ave			
Intermediate Filament Proteins																										
M19156	keratin 10	2.66	7.93	4.83	1.43	1.82	1.18	3.31	2.96	1.26	5.27	3.35	2.55	2.78	2.81	2.99	1.67	2.67	1.87	2.85	6.19	5.89	3.52			
D78367	keratin 12	3.00	7.12	5.86	2.00	0.47	0.75	3.20	3.18	0.88	8.41	3.29	3.69	8.00	1.48	4.13	1.28	2.48	4.42	4.72	9.15	8.45	5.08		1.59	
X52426	keratin 13	3.48	3.35	3.21	1.84	1.67	0.91	2.41	2.94	2.53	6.32	2.60	4.02	3.42	0.73	3.22	1.36	2.38	2.97	5.21	6.17	7.42	4.25		1.77	
J00124	keratin 14	3.78	4.46	2.14	2.27	0.80	1.03	2.41	3.04	1.41	11.42	3.79	1.86	3.47	2.61	3.94	0.85	2.05	4.45	3.52	6.19	5.01	3.68	1.64	1.53	
M26326	keratin 18	39.77	28.03	20.51	49.22	33.15	53.05	37.29	44.36	28.98	50.21	52.22	46.26	39.52	42.48	46.07	53.23	30.88	36.93	64.33	121.47	16.02	53.81		1.44	
Y00503	keratin 19	103.53	62.66	46.81	72.09	61.33	111.45	76.31	67.71	61.26	88.23	140.54	91.49	98.22	64.72	87.45	107.93	67.87	64.98	75.67	149.16	85.96	91.93			
M21772	K16:CK16	12.15	7.62	4.81	6.67	1.09	3.42	5.96	3.41	1.94	8.26	4.39	4.78	9.49	0.76	4.72	2.00	3.45	12.20	4.44	5.92	9.51	6.25			
X56134	vimentin	52.71	82.49	164.13	57.72	64.70	99.21	86.83	62.10	58.32	207.40	44.73	57.66	171.03	84.66	98.00	64.15	63.56	43.69	161.05	155.76	232.02	120.03			
DNA Synthesis, Recombination & Repair																										
DNA Replication: Polymerases, Replication Factors & Topoisomerases																										
M15796	PCNA	26.84	43.77	18.78	34.10	41.32	74.35	39.86	34.93	37.67	98.20	52.35	47.51	71.28	46.19	55.47	28.68	30.65	30.29	67.95	84.80	64.56	51.14			
X06745	POLA	4.53	5.35	3.27	3.08	1.79	4.65	3.78	3.75	8.35	9.91	6.29	2.53	6.51	2.08	5.63	4.79	3.37	5.29	7.65	8.88	11.67	6.94	1.49	1.84	
M63488	RPA1	8.62	8.53	9.52	7.07	4.18	11.39	8.22	5.94	3.45	9.97	5.96	4.60	7.74	3.10	5.82	3.04	6.05	7.73	6.34	6.75	9.35	6.54	1.41		
M87338	RFC2	7.71	25.59	17.34	4.03	0.90	2.23	9.63	3.92	31.96	12.17	4.76	4.19	4.46	3.81	9.32	2.71	6.13	3.16	5.03	7.80	9.14	5.66		1.70	1.65
L07541	RFC3	12.49	34.64	14.15	10.08	5.68	7.20	14.04	8.16	32.74	9.96	11.58	7.00	9.18	11.83	12.92	7.75	7.59	3.40	8.03	15.46	10.48	8.78	1.60	1.47	
M87339	RFC4	5.91	16.10	6.23	4.77	2.32	6.95	7.04	4.32	10.90	12.26	5.90	5.22	6.32	5.02	7.13	4.14	3.85	4.50	4.65	13.12	17.88	7.98			
L07640	RFC5	4.61	4.55	5.36	2.81	1.03	0.50	3.14	3.49	1.92	6.40	2.81	2.52	4.54	1.87	3.36	1.41	2.47	3.79	3.34	6.61	7.00	4.10			
J03250	TOP1	7.70	7.97	9.08	2.61	2.37	1.91	5.27	4.88	3.10	11.28	6.20	2.94	7.32	4.26	5.71	8.40	11.13	2.63	8.89	16.64	9.37	9.48	1.80		
J04088	TOP2A	6.07	12.34	4.25	3.18	1.46	15.31	7.10	3.74	3.05	7.26	6.89	3.78	7.23	5.15	5.30	3.62	3.08	2.51	4.93	8.07	9.56	5.29			
DNA Damage Signaling/Repair Proteins & DNA Ligases																										
U33841	ATM	10.25	26.35	12.88	10.25	7.08	12.41	13.20	5.47	36.32	6.48	10.26	3.63	13.28	10.19	12.09	8.06	2.90	3.75	7.46	18.13	8.76	8.17	1.61	1.48	
M13194	ERCC1	13.42	12.12	9.85	15.03	7.24	11.05	11.45	7.52	4.83	20.83	12.58	18.85	20.23	15.96	14.40	9.11	8.84	8.89	14.16	18.41	23.28	13.75			
X52221	ERCC2	5.78	5.83	3.84	4.82	1.77	1.36	3.90	2.78	4.56	7.43	6.33	2.44	7.51	2.54	4.80	2.81	2.81	3.74	3.52	3.89	5.86	3.77			
M31899	ERCC3	9.36	10.88	6.90	7.95	4.39	4.10	7.26	3.93	6.54	10.72	7.56	5.24	7.30	2.88	6.31	4.97	2.87	6.64	6.79	7.49	11.10	6.64			
L20046	ERCC5	16.37	11.70	7.94	17.57	11.07	17.93	13.76	8.02	6.44	25.25	21.09	17.66	21.98	17.65	16.87	15.44	10.25	9.17	24.37	23.14	33.35	19.28	1.40		
X84740	LIG3	3.73	6.22	2.91	3.58	1.05	2.80	3.38	3.02	1.84	7.69	4.67	2.22	2.42	3.17	3.57	1.37	1.88	3.73	4.45	3.91	10.31	4.27			
X83441	LIG4	3.77	6.82	3.61	1.59	1.94	2.55	3.38	1.20	1.11	10.48	4.53	2.67	3.26	2.61	3.69	1.55	3.21	3.20	4.06	4.53	5.16	3.62			
U07418	MLH1	4.98	4.39	3.89	1.76	0.91	1.21	2.86	1.90	1.58	6.50	5.63	1.86	3.35	1.13	3.13	4.91	3.95	3.65	4.17	5.35	7.58	4.93	1.73		
U04045	MSH2	7.57	6.39	4.18	7.63	3.08	11.50	6.72	3.36	2.60	10.38	8.73	3.47	9.00	4.77	6.04	5.22	3.31	3.09	7.71	12.87	8.22	6.74			
U54777	MSH6	7.51	8.44	4.53	7.44	2.89	8.46	6.54	1.49	4.96	10.90	7.51	3.27	9.44	4.78	6.05	3.89	3.84	2.76	8.15	9.40	13.87	6.98			
M29971	MGMT	6.82	9.69	2.74	3.19	4.02	2.40	4.81	3.46	4.96	8.62	4.56	3.81	7.72	2.99	5.16	2.96	4.87	4.48	4.74	7.82	7.62	5.41			
U35635	PRKDC	9.83	11.42	7.96	10.64	7.72	23.20	11.79	5.60	6.46	12.64	9.39	5.61	9.39	3.95	7.58	16.04	5.20	5.81	19.18	20.40	15.62	13.71	1.56		
D21235	RAD23A	26.60	28.66	18.11	29.71	18.78	29.26	25.19	19.73	21.89	25.01	33.10	33.25	28.34	33.38	27.81	20.80	16.73	21.51	26.30	44.04	27.94	26.22			
U63139	RAD50	6.61	4.91	2.67	2.63	2.02	3.33	3.69	2.22	2.21	9.34	2.44	1.85	9.63	1.76	4.20	2.56	3.29	3.37	3.74	4.35	8.51	4.30			
D13804	RAD51	8.78	6.15	3.24	4.80	2.93	6.99	5.48	2.95	2.48	12.63	4.85	2.52	5.29	3.18	4.83	3.14	2.84	3.70	4.65	10.93	6.39	5.27			
U12134	RAD52	4.90	5.45	2.00	1.97	0.88	1.17	2.73	1.80	1.13	9.04	2.97	2.80	4.01	0.81	3.22	1.48	3.24	2.14	3.10	10.43	8.33	4.78	1.75		
M32865	G22P1	12.91	13.69	6.13	15.31	10.82	19.49	13.06	7.53	4.02	13.35	11.66	11.56	11.35	7.92	9.62	5.28	8.12	7.44	5.36	17.39	18.80	10.39			
DNA Damage Signaling/Repair Proteins & DNA Ligases																										
M30938	XRCC5	5.68	6.53	3.29	4.68	0.85	8.83	4.97	3.28	2.21	6.32	4.83	3.08	5.91	3.30	4.13	1.65	2.08	2.59	2.70	8.34	9.13	4.41			
M74524	UBE2A	31.03	47.67	40.04	33.66	26.24	63.81	38.74	26.36	40.04	33.66	39.26	32.64	59.52	41.00	38.79	27.33	22.82	18.74	35.51	56.23	42.04	33.78			
M36089	XRCC1	19.53	6.36	6.93	9.04	4.45	2.04	8.06	5.57	4.68	12.32	12.60	8.90	4.70	4.32	7.58	10.13	9.52	8.20	4.38	13.85	11.21	9.55			
Other DNA Synthesis, Recombination & Repair Proteins																										
X90392	DNASE1L1	4.92	4.37	3.20	2.60	1.44	1.80	3.05	2.46	2.38	6.80	5.40	1.96	2.42	2.60	3.43	2.83	3.70	4.10	7.23	9.46	7.53	5.81	1.90		
M60974	GADD45A	7.25	11.41	7.80	9.28	8.65	19.73	10.68	6.61	9.90	35.59	14.14	9.07	40.49	10.48	18.04	11.39	11.14	5.36	28.27	27.10	30.06	18.88	1.69	1.77	
X74262	RBBP4	50.57	39.42	39.08	52.29	43.10	70.90	49.22	25.38	30.23	73.31	49.64	42.54	62.28	49.55	47.56	31.88	21.37	19.82	49.13	63.73	34.30	36.54			

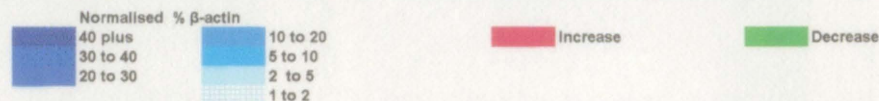


Increase Decrease

Appendix II The ratios of difference of each gene for cancer versus normal, polyp versus normal and cancer versus polyp subjects. Genes with a ratio of difference of 1.4 or above are highlighted. All genes are normalised to the housekeeping gene β -actin and expressed as % of β -actin.

For a full list of gene names see **Appendix III**

Accession Number	Gene Name	1	3	Normal Subjects				Ave	Syn05	Syn13	Syn15	Cancer Subjects				Syn58	Syn77	Ave	Syn40	Polyp Subjects				Syn49	Syn74	Ave	Canc vs Norm	Polyp vs Norm	Canc vs Polyp	
	Angiogenic factors			4	5	6	7					Syn30	Syn38							Syn65	Syn70	Syn42								
M32977	VEGF	8.51	25.23	11.21	23.77	16.64	12.06	16.23	11.63	15.72	42.10	13.83	23.92	30.06	29.32	23.80	16.66	14.64	7.96	23.17	32.78	38.70	22.31						1.47	
U43142	VEGFC	1.98	5.65	2.69	2.55	1.26	2.02	2.69	2.80	2.23	9.97	3.57	2.61	2.61	2.65	3.78	1.46	3.21	4.99	2.26	7.23	7.45	4.43						1.40	1.65
U48801	VEGFB	4.63	5.25	6.45	6.46	2.76	3.36	4.82	7.63	3.21	8.30	6.04	7.86	8.94	8.25	7.17	7.26	8.08	10.61	8.09	13.55	10.80	9.73						1.49	2.02
J05081	endothelin 3	3.23	7.44	4.49	5.04	1.25	1.02	3.74	3.10	1.39	7.09	5.50	2.63	3.31	2.26	3.61	2.91	3.05	2.05	3.07	3.49	10.78	4.22							
M65199	endothelin 2	5.07	6.16	3.24	2.42	1.45	0.84	3.19	3.31	1.96	10.53	6.53	3.09	3.60	3.02	4.58	1.71	2.73	4.50	4.21	7.05	11.13	5.22						1.43	1.63
U01134	FLT1	17.73	48.20	45.04	22.84	26.01	33.60	32.22	21.61	51.33	10.27	19.36	23.49	30.56	25.21	25.97	16.67	15.79	8.27	29.56	32.97	25.05	21.38							1.51
	Functionally Unclassified																													
U32907	LRRC17	2.89	5.24	5.89	2.64	3.45	3.08	3.86	1.74	2.75	7.14	5.48	2.85	8.04	2.95	4.42	3.29	3.04	2.29	7.48	9.23	14.19	6.59							1.71
U37688	RATS1	2.35	14.53	6.35	4.46	0.55	1.49	4.95	3.25	14.24	6.33	2.87	1.80	4.65	3.98	5.16	1.54	2.82	4.87	3.82	6.57	8.91	4.75							
D25216	LRRC14	6.39	6.53	3.38	6.59	3.69	5.12	5.28	2.66	3.59	7.85	8.47	4.11	5.94	3.36	5.14	3.38	3.00	5.68	3.80	8.57	9.93	5.72							



Appendix II The ratios of difference of each gene for cancer versus normal, polyp versus normal and cancer versus polyp subjects. Genes with a ratio of difference of 1.4 or above are highlighted. All genes are normalised to the housekeeping gene β -actin and expressed as % of β -actin.

For a full list of gene names see **Appendix III**

Appendix III

Accession Number	Gene Symbol	Gene Name
	Transcription Activators & Repressors	
U76638	BARD1	BRCA1 associated RING domain 1
M96577	E2F1	E2F transcription factor 1
Y10479	E2F3	E2F transcription factor 3
U15642	E2F5	E2F transcription factor 5, p130-binding
M62829	EGR1	early growth response 1
M91196	homeo box D3	homeo box D3
D11117	ICSBP1	interferon consensus sequence binding protein 1
X14454	IRF1	interferon regulatory factor 1
X17620	NME1	non-metastatic cells 1, protein (NM23A) expressed in
L16785	NME2	non-metastatic cells 2, protein (NM23B) expressed in
M96684	PURA	purine-rich element binding protein A
X06538	RARA	retinoic acid receptor, alpha
X07282	RARB	retinoic acid receptor, beta
M24857	RARG	retinoic acid receptor, gamma
M97935	STAT1	signal transducer and activator of transcription 1, 91kD
M97934	STAT2	signal transducer and activator of transcription 2, 113kD
U47686	STAT5B	signal transducer and activator of transcription 5B
U59863	TANK	TRAF family member-associated NFkB activator
L23959	TFDP1	transcription factor Dp-1
U18422	TFDP2	transcription factor Dp-2 (E2F dimerization partner 2)
	Cell Cycle	
U33286	CSE1L	CSE1 chromosome segregation 1-like (yeast)
L34075	FRAP1	FK506 binding protein 12-rapamycin associated protein 1
AF007111	Mdm2	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)
Z12020	Mdm4	Mdm4, transformed 3T3 cell double minute 4, p53 binding protein (mouse)
U63295	SIAM1	seven in absentia homolog 1 (Drosophila)
Y11416	tumor protein p73	tumor protein p73
	Cyclins	
X51688	cyclin A2	cyclin A2
M25753	cyclin B1	cyclin B1
M74091	cyclin C	cyclin C
X59798	cyclin D1	cyclin D1 (PRAD1: parathyroid adenomatosis 1)
D13639	cyclin D2	cyclin D2
M92287	cyclin D3	cyclin D3
M73812	cyclin E1	cyclin E1
U47413	cyclin G1	cyclin G1
U47414	cyclin G2	cyclin G2
U11791	cyclin H	cyclin H
	Cell cycle-regulating kinases	
L29222	CDC-like kinase 1	CDC-like kinase 1
L29216	CDC-like kinase 2	CDC-like kinase 2
L29220	CDC-like kinase 3	CDC-like kinase 3
M68520	CDK 2	cyclin-dependent kinase 2
X66357	CDK 3	cyclin-dependent kinase 3
M14505	CDK 4	cyclin-dependent kinase 4
X66364	CDK 5	cyclin-dependent kinase 5

Appendix III : Full list of gene symbols and corresponding gene names

Accession Number	Gene Symbol	Gene Name
	<i>Cell cycle-regulating kinases</i>	
X66365	CDK 6	cyclin-dependent kinase 6
L20320	CDK 7	cyclin-dependent kinase 7 (MO15 homolog, <i>Xenopus laevis</i> , cdk-activating kinase)
L25576	CDK 9	cyclin-dependent kinase 9 (CDC2-related kinase)
L33264	CDK 10	cyclin-dependent kinase (CDC2-like) 10
X66358	CDKL1	cyclin-dependent kinase-like 1 (CDC2-related kinase)
X05360	CDC2	cell division cycle 2, G1 to S and G2 to M
M8629	CDC2L5	cell division cycle 2-like 5 (cholinesterase-related cell division controller)
X60188	MAPK3	extracellular signal-regulated kinase 1 (ERK1); insulin-stimulated MAP2 kinase; MAP kinase 1 (MAPK 1); p44-MAPK; microtubule-as
U02687	FLT3	fms-related tyrosine kinase 3
X59727	MAPK4	mitogen-activated protein kinase 4
X80692	MAPK6	mitogen-activated protein kinase 6
U25278	MAPK7	mitogen-activated protein kinase 7
X79483	MAPK12	mitogen-activated protein kinase 12
L35253	MAPK14	mitogen-activated protein kinase 14
L05624	MAP2K1	mitogen-activated protein kinase kinase 1
U25265	MAP2K5	mitogen-activated protein kinase kinase 5
U39657	MAP2K6	mitogen-activated protein kinase kinase 6
U78876	MAP3K3	mitogen-activated protein kinase kinase kinase 3
U01038	PLK1	poio-like kinase (<i>Drosophila</i>)
	<i>CDK inhibitors</i>	
U09579	CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
U22398	CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)
L27211	CDKN2A	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
U17075	CDKN2B	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)
U40343	CDKN2D	cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)
U10564	WEE1	WEE1+ homolog (<i>S. pombe</i>)
	<i>Other Cell Cycle Controls</i>	
U77949	CDC6	CDC6 cell division cycle 6 homolog (<i>S. cerevisiae</i>)
S72008	CDC10	CDC10 cell division cycle 10 homolog (<i>S. cerevisiae</i>)
U18291	CDC16	CDC16 cell division cycle 16 homolog (<i>S. cerevisiae</i>)
U63131	CDC37	CDC37 cell division cycle 37 homolog (<i>S. cerevisiae</i>)
	<i>Other Cell Cycle Controls</i>	
S57153	ARID4A	retinoblastoma binding protein 1
M81933	CDC25A	cell division cycle 25A
M81934	CDC25B	cell division cycle 25B
M34065	CDC25C	cell division cycle 25C
U00301	CDC27	cell division cycle 27
X80343	CDK5R1	cyclin-dependent kinase 5, regulatory subunit 1 (p35)
U34051	CDK5R2	cyclin-dependent kinase 5, regulatory subunit 2 (p39)
U66469	CGRRF1	cell growth regulatory with ring finger domain
L13398	GAS1	growth arrest-specific 1
S66431	JARID1A	retinoblastoma binding protein 2
D13365	MT3	metallothionein 3 (growth inhibitory factor (neurotrophic))
D63878	NEDD5	neural precursor cell expressed, developmentally down-regulated 5
U49070	PIN1	protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1
X85134	RBBP5	retinoblastoma binding protein 5

Appendix III : Full list of gene symbols and corresponding gene names

Accession Number	Gene Symbol	Gene Name
	<i>Other Cell Cycle Controls</i>	
X85133	RBBP6	retinoblastoma binding protein 6
U58334	TP53BP2	tumor protein p53 binding protein, 2
	Cell Adhesion Receptors/Proteins	
M81104	CD34	CD34 antigen
Z24680	GARP	glycoprotein A repetitions predominant
	<i>Matrix Adhesion Receptor</i>	<i>matrix adhesion receptors</i>
M24795	CD36	CD36 antigen (collagen type I receptor, thrombospondin receptor)
M14219	decorin	decorin
X56654	desmoglein 1	desmoglein 1
Z26317	desmoglein 2	desmoglein 2
U43901	LAMR1	laminin receptor 1 (67kD, ribosomal protein SA)
U14588	paxillin	paxillin
L12350	thrombospondin 2	thrombospondin 2
	<i>Cell-Cell Adhesion Receptors</i>	
M34064	cadherin 2	cadherin 2, type 1, N-cadherin (neuronal)
X63629	cadherin 3	cadherin 3, type 1, P-cadherin (placental)
L34059	cadherin 4	cadherin 4, type 1, R-cadherin (retinal)
X79981	cadherin 5	cadherin 5, type 2, VE-cadherin (vascular epithelium)
D31784	cadherin 6	cadherin 6, type 2, K-cadherin (fetal kidney)
L34060	cadherin 8	cadherin 8, type 2
L34056	cadherin 11	cadherin 11, type 2, OB-cadherin (osteoblast)
L34057	cadherin 12	cadherin 12, type 2 (N-cadherin 2)
L34058	cadherin 13	cadherin 13, H-cadherin (heart)
D63542	cadherin 15	cadherin 15, M-cadherin (myotubule)
D13866	catenin alpha 1	catenin (cadherin-associated protein), alpha 1 (102kD)
M94151	catenin alpha 2	catenin (cadherin-associated protein), alpha 2
X67838	catenin beta 1	catenin (cadherin-associated protein), beta 1 (88kD)
X72925	desmocollin 1	desmocollin 1
X56807	desmocollin 2	desmocollin 2
X63929	desmocollin 3	desmocollin 3
M77830	desmoplakin	desmoplakin (DPI, DPII)
U53786	envoplakin	envoplakin
M74387	L1CAM	L1 cell adhesion molecule (hydrocephalus, stenosis of aqueduct of Sylvius 1, MASA (mental retardation, aphasia, shuffling gait
X16841	NCAM1	neural cell adhesion molecule 1
U72661	ninjunn 1	ninjunn 1
L34774	OPCML	opoid binding protein/cell adhesion molecule-like
U52111	plexin B3	plexin B3
AAA36418	protocadherin 1	protocadherin 1 (cadherin-like 1)
L11373	PCDHGC3	protocadherin gamma subfamily C, 3
U60800	SEMA4	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D
X65978	TAX1	TAX1, axonin-1/TAQ1
	Integrins	
X68742	integrin, alpha 1	integrin, alpha 1
M26249	integrin, alpha 2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)
M55911	integrin, alpha 3	integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)
L12002	integrin, alpha 4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)

Appendix III : Full list of gene symbols and corresponding gene names

Accession Number	Gene Symbol	Gene Name
	Integrins	
X06256	integrin, alpha 5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
X53586	integrin, alpha 6	integrin, alpha 6
X74295	integrin, alpha 7	integrin, alpha 7
L36531	integrin, alpha 8	integrin, alpha 8
D25303	integrin, alpha 9	integrin, alpha 9
L25351	integrin, alpha E	integrin, alpha E (antigen CD103, human mucosal lymphocyte antigen 1, alpha polypeptide)
M34189	integrin, beta 1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
J02703	integrin, beta 3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
U37139	integrin beta 3 (BP)	integrin beta 3 binding protein (beta3-endonexin)
X53587	integrin, beta 4	integrin, beta 4
J05633	integrin, beta 5	integrin, beta 5
M35198	integrin, beta 6	integrin, beta 6
M62880	integrin, beta 7	integrin, beta 7
M73780	integrin, beta 8	integrin, beta 8
	Immune System Proteins	
M34671	CD59	CD59 antigen p18-20 (antigen identified by monoclonal antibodies 16.3A5, EJ16, EJ30, EL32 and G344)
M11886	HLA-C	major histocompatibility complex, class I, C
M21626	TRAV14DV4	T cell receptor alpha variable 14/delta variable 4
	Oncogenes & Tumor Suppressors	
U23435	abl-interactor 2	abl-interactor 2
M74088	APC	adenomatosis polyposis coli
U43746	BRCA2	breast cancer 2, early onset
X03663	CSF1R	colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog
X76132	DCC	deleted in colorectal carcinoma
U49089	DLG3	discs large (Drosophila) homolog 3 (neuroendocrine-dlg)
AF010314	ENC1	ectodermal-neural cortex (with BTB-like domain)
X00588	EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)
M99487	FOLH1	folate hydrolase (prostate-specific membrane antigen) 1
M19722	FGR	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog
L29511	GRB2	growth factor receptor-bound protein 2
AF001954	ING1	inhibitor of growth family, member 1
M29039	JUNB	jun B proto-oncogene
U43527	KISS1	KISS-1 metastasis-suppressor
X06182	KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
X13967	LIF	leukemia inhibitory factor (cholinergic differentiation factor)
AF010312	LITAF	LPS-induced TNF-alpha factor
J02958	MET	met proto-oncogene (hepatocyte growth factor receptor)
U35113	MTA1	metastasis associated 1
U68162	MPL	myeloproliferative leukemia virus oncogene
AF010315	p53-induced protein	p53-induced protein
X02811	PDGFB	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)
D89667	prefoldin 5	prefoldin 5
AF010310	PRODH	proline dehydrogenase (oxidase) 1
AF010316	PTGES	prostaglandin E synthase
AF010309	TP5313	quinone oxidoreductase homolog
L25080	RHOA	ras homolog gene family, member A

Appendix III : Full list of gene symbols and corresponding gene names

Accession Number	Gene Symbol	Gene Name
Oncogenes & Tumor Suppressors		
L25081	RHOC	ras homolog gene family member C
D85815	RHOD	ras homolog gene family member D
X95282	RHOE	ras homolog gene family member E
X61587	RHOG	ras homolog gene family member G (rho G)
Z35227	RHOH	ras homolog gene family member H
M31213	oncogene RET	ret proto-oncogene (multiple endocrine neoplasia and medullary thyroid carcinoma 1, Hirschsprung disease)
M15400	RB1	retinoblastoma 1 (including osteosarcoma)
X74594	RBL2	retinoblastoma-like 2 (p130)
U16296	TIAM1	T-cell lymphoma invasion and metastasis 1
Z29383	TPBG	trophoblast glycoprotein
M14694	tumor protein p53	tumor protein p53 (Li-Fraumeni syndrome)
M35296	ABL2	v-abl Abelson murine leukemia viral oncogene homolog 2 (arg, Abelson-related gene)
M63167	AKT1	v-akt murine thymoma viral oncogene homolog 1
M77198	AKT2	v-akt murine thymoma viral oncogene homolog 2
M11730	ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
M29366	ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
L07368	ERBB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)
K00650	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog
M54968	KRAS2	v-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog
M13228	MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)
M95712	BRAF	v-raf murine sarcoma viral oncogene homolog B1
X03484	RAF1	v-raf-1 murine leukemia viral oncogene homolog 1
NM_005417	SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
M15990	YES1	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1
Extracellular Matrix Proteins		
M55172	aggrecan 1	aggrecan 1 (chondroitin sulfate proteoglycan 1, large aggregating proteoglycan, antigen identified by monoclonal antibody A0122)
J04599	biglycan	biglycan
U16306	CSPG2	chondroitin sulfate proteoglycan 2 (versican)
X02761	fibronectin 1	fibronectin 1
M85289	HSPG2	heparan sulfate proteoglycan 2 (perlecan)
X78565	tenascin C	hexabrachion (tenascin C, cytactin)
U86759	netrin 2-like	netrin 2-like (chicken)
M30269	nidogen	nidogen (enactin)
J03040	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)
X98085	tenascin R	tenascin R (restonectin, janusin)
X14787	thrombospondin 1	thrombospondin 1
X03168	vitronectin	vitronectin (serum spreading factor, somatomedin B, complement S-protein)
Trafficking/Targeting Proteins		
X55525	COL1A2	collagen, type I, alpha 2
X16468	COL2A1	collagen, type II, alpha 1 (primary osteoarthritis, spondyloepiphyseal dysplasia, congenital)
X14420	COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)
X05610	COL4A2	collagen, type IV, alpha 2
M92993	COL4A3	collagen, type IV, alpha 3 (Goodpasture antigen)
D21337	COL4A6	collagen, type IV, alpha 6
X15879	COL6A1	collagen, type VI, alpha 1
M34570	COL6A2	collagen, type VI, alpha 2

Appendix III : Full list of gene symbols and corresponding gene names

Accession Number	Gene Symbol	Gene Name
	Trafficking/Targeting Proteins	
X52022	COL6A3	collagen, type VI, alpha 3
X57527	COL8A1	collagen, type VIII, alpha 1
J04177	COL11A1	collagen, type XI, alpha 1
U32169	Col11A2	collagen, type XI, alpha 2
M92642	COL16A1	collagen, type XVI, alpha 1
L22548	COL18A1	collagen, type XVIII, alpha 1
M61916	laminin, beta 1	laminin, beta 1
S77512	laminin, beta 2	laminin, beta 2 (laminin S)
X70904	laminin, alpha 4	laminin, alpha 4
J03202	laminin, gamma 1	laminin, gamma 1 (formerly LAMB2)
X97442	TMP21	transmembrane trafficking protein
	Metabolism	
X01677	GAPD	glyceraldehyde-3-phosphate dehydrogenase
U90313	GSTO1	glutathione-S-transferase like, glutathione transferase omega
V00530	HPRT1	hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)
Y07604	NME4	non-metastatic cells 4, protein expressed in
M13267	SOD1	superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult))
	Ribosomal Proteins	
X65923	FAU	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed (fox derived); ribosomal protein S30
X56932	ribosomal protein L13a	ribosomal protein L13a
U14971	ribosomal protein S9	ribosomal protein S9
	Apoptosis Associated Proteins	
	<i>Death Receptor-Associated Proteins & Adaptors</i>	
U84388	CRADD	CASP2 and RIPK1 domain containing adaptor with death domain
U82938	SIVA	C/27-binding (Siva) protein
AF015956	DAXX	death-associated protein 6
L41390	TRADD	TNFRSF1A-associated via death domain
U78798	TRAF6	TNF receptor-associated factor 6
U90875	TNFRSF10A	tumor necrosis factor receptor superfamily, member 10a
	<i>Caspases</i>	
U13699	caspase 1	caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)
U13021	caspase 2	caspase 2, apoptosis-related cysteine protease (neural precursor cell expressed, developmentally down-regulated 2)
U13737	caspase 3	caspase 3, apoptosis-related cysteine protease
U28014	caspase 4	caspase 4, apoptosis-related cysteine protease
U20537	caspase 6	caspase 6, apoptosis-related cysteine protease
U37448	caspase 7	caspase 7, apoptosis-related cysteine protease
X98176	caspase 8	caspase 8, apoptosis-related cysteine protease
U56390	caspase 9	caspase 9, apoptosis-related cysteine protease
U60519	caspase 10	caspase 10, apoptosis-related cysteine protease
	<i>BCL2 Family Proteins</i>	
U23765	BAK	BCL2-antagonist/killer 1
U66879	BAD	BCL2-antagonist of cell death
S83171	BAG1	BCL2-associated athanogene
L22474	BAX	BCL2-associated X protein
M14745	BCL2	B-cell CLL/lymphoma 2
U29680	BCL2A1	BCL2-related protein A1

Appendix III : Full list of gene symbols and corresponding gene names

Accession Number	Gene Symbol	Gene Name
	<i>BCL2 Family Proteins</i>	
U59747	BCL2L2	BCL2-like 2
U34584	BIK	BCL2-interacting killer (apoptosis-inducing)
S62185	BRAG-1	brain-related apoptosis gene (BRAG-1) Bcl-2 homolog
L08246	MCL1	myeloid cell leukemia sequence 1 (BCL2-related)
	<i>Death Kinases</i>	
X76104	DAPK1	death-associated protein kinase 1
Y10256	MAP3K14	mitogen-activated protein kinase kinase kinase 14
X66363	PCTK1	PCTAIRE protein kinase 1
X66360	PCTK2	PCTAIRE protein kinase 2
X66362	PCTK3	PCTAIRE protein kinase 3
	<i>Other Apoptosis-Associated Proteins</i>	
U45879	BIRC2	baculoviral IAP repeat-containing 2
U45878	BIRC3	baculoviral IAP repeat-containing 3
U45880	BIRC4	baculoviral IAP repeat-containing 4
U75285	BIRC5	baculoviral IAP repeat-containing 5 (survivin)
AF010127	CFLAR	CASP8 and FADD-like apoptosis regulator
U18321	DAP3	death associated protein 3
U91985	DFFA	DNA fragmentation factor, 45 kD, alpha polypeptide
S40706	DDIT3	DNA-damage-inducible transcript 3
S78385	PDCD2	programmed cell death 2
AF022385	PDCD10	programmed cell death 10
U25994	RIPK1	receptor (TNFRSF)-interacting serine-threonine kinase 1
	RNA processing, turnover, and transport	
U12431	ELAVL2	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)
	Cell Receptors	
	<i>Growth Factor & Chemokine Receptors</i>	
L17075	ACVRL1	activin A receptor type II-like 1
M6C459	EPOR	erythropoietin receptor
X68203	FLT4	fms-related tyrosine kinase 4
L37882	FZD2	frizzled homolog 2 (Drosophila)
U28811	GLG1	golgi apparatus protein 1
XC4434	IGF1R	insulin-like growth factor 1 receptor
YC0285	IGF2R	insulin-like growth factor 2 receptor
M3E410	IGFBP2	insulin-like growth factor binding protein 2 (36kD)
U12140	NTRK2	neurotrophic tyrosine kinase, receptor, type 2
U05012	NTRK3	neurotrophic tyrosine kinase, receptor, type 3
M9C437	Notch homolog 2	Notch homolog 2 (Neurogenic locus notch homolog protein 2 precursor)
U77493	Notch homolog 2	Notch homolog 2 (Drosophila)
U95299	Notch homolog 4	Notch homolog 4 (Drosophila)
U43148	PATCH	patched homolog (Drosophila)
M21574	PDGFRA	platelet-derived growth factor receptor, alpha polypeptide
M21616	PDGFRB	platelet-derived growth factor receptor, beta polypeptide
LC7594	TGFB3	transforming growth factor, beta receptor III (betaglycan, 300kD)
X85960	TRK-fused gene	TRK-fused gene
AF016268	TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b
YC9392	TNFRSF25	tumor necrosis factor receptor superfamily, member 12 (translocating chain-association membrane protein)

Appendix III : Full list of gene symbols and corresponding gene names

Accession Number	Gene Symbol	Gene Name
	<i>Growth Factor & Chemokine Receptors</i>	
M33294	TNFRSF1A	tumor necrosis factor receptor superfamily, member 1A
M32315	TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B
X60592	TNFRSF5	tumor necrosis factor receptor superfamily, member 5
Z70519	TNFRSF6	tumor necrosis factor receptor superfamily, member 6
	<i>FGF receptors</i>	
M37722	FGFR1	fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)
U11814	FGFR2	fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1)
M58051	FGFR3	fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)
	<i>Ephrin receptors</i>	
M59371	EphA2	EphA2
L40636	EphB1	EphB1
L41939	EphB2	EphB2
U07695	EphB4	EphB4
	<i>Interleukin Receptors</i>	
M63099	interleukin 1 receptor	interleukin 1 receptor antagonist
M20566	interleukin 6 receptor	interleukin 6 receptor
	<i>Interferon receptors</i>	
J03171	IFNAR1	interferon (alpha, beta and omega) receptor 1
X77722	IFNAR2	interferon (alpha, beta and omega) receptor 2
A09781	IFNGR	interferon-gamma receptor (IFNGR)
J03143	IFNGR1	interferon gamma receptor 1
U05875	IFNGR2	interferon gamma receptor 2 (interferon gamma transducer 1)
	<i>Hormone Receptors</i>	
X52773	RXRA	retinoid X receptor, alpha
M84820	RXRB	retinoid X receptor, beta
	<i>Protein Kinases</i>	
X61656	KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)
U33635	PTK7	PTK7 protein tyrosine kinase 7
YC9328	NTRK1	neurotrophic tyrosine kinase, receptor, type 1
D17517	TYRO3	TYRO3 protein tyrosine kinase
	<i>Other Receptors</i>	
X95425	EPHA5	ephrin type-A receptor 5 precursor; tyrosine-protein kinase receptor EHK1, EPH homology kinase-1, HEK7
U43318	FZD5	frizzled homolog 5 (Drosophila)
U82169	FZD9	frizzled homolog 9 (Drosophila)
X02492	GIP3	interferon alpha-inducible protein (clone IFI-6-16)
X13916	LRP1	low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor)
U04441	LRP2	low density lipoprotein-related protein 2
M14764	NGFR	nerve growth factor receptor (TNFR superfamily, member 16)
U08839	PLAUR	plasminogen activator, urokinase receptor
X67852	SEX gene	SEX gene
X60957	TIE	tyrosine kinase with immunoglobulin and epidermal growth factor homology domains
	Cell Signaling, Extracellular Communication Proteins	
	<i>Neuropeptides</i>	
L19063	GDNF	glial cell derived neurotrophic factor
X52599	NGFB	nerve growth factor, beta polypeptide
M13980	Notch homolog 1	Notch homolog 1, translocation-associated (Drosophila)
S41541	NTF6G	neurotrophin 6 gamma pseudogene
X53655	neurotrophin 3	neurotrophin 3

Appendix III : Full list of gene symbols and corresponding gene names

Accession Number	Gene Symbol	Gene Name
	<i>Growth Factors, Cytokines & Chemokines</i>	
M30704	amphiregulin	amphiregulin (schwannoma-derived growth factor)
M15530	B-cell growth factor 1	B-cell growth factor 1 (12xD)
M61176	BDNF	brain-derived neurotrophic factor
M73238	CNTFR	ciliary neurotrophic factor
M92934	CTGF	connective tissue growth factor
U15979	DLK1	delta-like 1 homolog (Drosophila)
M66278	DTR	diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor)
U49262	DVL3	dishevelled, dsr homolog 3 (Drosophila)
X04571	EGF	epidermal growth factor (beta-urogastrone)
U04806	FLT3LG	fms-related tyrosine kinase 3 ligand
M86492	GMFB	glia maturation factor, beta
D49493	GDF10	growth differentiation factor 10
U46010	HGF	hepatocyte growth factor (hepatoietin A, scatter factor)
D16431	HDGF	hepatoma-derived growth factor (high-mobility group protein 1-like)
D14012	HGF activator	HGF activator
A25270	IK cytokine	IK cytokine, down-regulator of HLA II
M59964	KITLG	KIT ligand
M74178	MST1	macrophage stimulating 1 (hepatocyte growth factor-like)
X72755	CXCL9	monokine induced by gamma interferon
L12260	neuregulin 1	neuregulin 1
X54936	PGF	placental growth factor, vascular endothelial growth factor-related protein
X06374	PDGFA associated prote	platelet-derived growth factor alpha polypeptide
M57399	pleiotrophin	pleiotrophin (heparin binding growth factor 8, neurite growth-promoting factor 1)
M54995	PPBP	pro-platelet basic protein (includes platelet basic protein, beta-thromboglobulin, connective tissue-activating peptide III, ne
L42379	quiescin Q6	quiescin Q6
L26081	SEMA3A	sema domain, immunoglobulin domain (Ig), short basic domain secreted (semaphorin) 3A
ABC00220	SEMA3C	sema domain, immunoglobulin domain (Ig), short basic domain secreted (semaphorin) 3C
U33920	SEMA3F	sema domain, immunoglobulin domain (Ig), short basic domain secreted (semaphorin) 3F
X02530	CXCL10	small inducible cytokine subfamily B (Cys-X-Cys) member 10
L38518	SHH	sonic hedgehog homolog (Drosophila)
L36034	CXCL12	stromal cell-derived factor 1
M96956	TDGF3	teratocarcinoma-derived growth factor 3, pseudogene
L36052	thrombopoietin	thrombopoietin (myeloproliferative leukemia virus oncogene ligand, megakaryocyte growth and development factor)
K03222	TGFA	transforming growth factor, alpha
X02812	TGFB1	transforming growth factor, beta 1 (Camurati-Engelmann disease)
M19154	TGFB2	transforming growth factor, beta 2
J03241	TGFB3	transforming growth factor, beta 3
L07414	TNFSF5	tumor necrosis factor (ligand) superfamily, member 5 (hyper-IgM syndrome)
D38122	TNFSF6	tumor necrosis factor (ligand) superfamily, member 6
L08096	TNFSF7	tumor necrosis factor (ligand) superfamily, member 7
L09753	TNFSF8	tumor necrosis factor (ligand) superfamily, member 8
U57059	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10
	<i>Fibroblast Growth Factor</i>	
X51943	FGF1	fibroblast growth factor 1 (acidic)
M27968	FGF2	fibroblast growth factor 2 (basic)
X14445	FGF3	fibroblast growth factor 3 (murine mammary tumor virus integration site (v-int-2) oncogene homolog)

Appendix III : Full list of gene symbols and corresponding gene names

Accession Number	Gene Symbol	Gene Name
	<i>Fibroblast Growth Factor</i>	
M37825	FGF5	fibroblast growth factor 5
X63454	FGF6	fibroblast growth factor 6
M60828	FGF7	fibroblast growth factor 7 (keratinocyte growth factor)
U36223	FGF8	fibroblast growth factor 8 (androgen-induced)
D14838	FGF9	fibroblast growth factor 9 (glia-activating factor)
U66197	FGF12	fibroblast growth factor 12
L03840	FGFR4	fibroblast growth factor receptor 4
	<i>Bone Morphogenetic Protein</i>	
M22488	BMP1	bone morphogenetic protein 1
M22489	BMP2	bone morphogenetic protein 2
M22491	BMP3	bone morphogenetic protein 3 (osteogenic)
D30751	BMP4	bone morphogenetic protein 4
M60314	BMP5	bone morphogenetic protein 5
M60315	BMP6	bone morphogenetic protein 6
M60316	BMP7	bone morphogenetic protein 7 (osteogenic protein 1)
M97016	BMP8A	bone morphogenetic protein 8 (osteogenic protein 2)
	<i>Ephrins</i>	
M57730	ephrin-A1	ephrin-A1
U26403	ephrin-A5	ephrin-A5
U09304	ephrin-B1	ephrin-B1
L38734	ephrin-B2	ephrin-B2
U66406	ephrin-B3	ephrin-B3
	<i>Interleukins</i>	
X02851	interleukin 1, alpha	interleukin 1, alpha
K02770	interleukin 1, beta	interleukin 1, beta
A14844	interleukin 2	interleukin 2
M14743	interleukin 3	interleukin 3 (colony-stimulating factor, multiple)
M13982	interleukin 4	interleukin 4
X04688	interleukin 5	interleukin 5 (colony-stimulating factor, eosinophil)
X04602	interleukin 6	interleukin 6 (interferon, beta 2)
J04156	interleukin 7	interleukin 7
X17543	interleukin 9	interleukin 9
M57627	interleukin 10	interleukin 10
M57765	interleukin 11	interleukin 11
M65291	interleukin 12A	interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)
M65290	interleukin 12B	interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)
L06301	interleukin 13	interleukin 13
L15344	interleukin 14	interleukin 14
U14407	interleukin 15	interleukin 15
U32659	interleukin 17	interleukin 17 (cytotoxic T-lymphocyte-associated serine esterase 8)
	<i>Interferon</i>	
M28622	IFNB1	interferon, beta 1, fibroblast
J00209	interferon, alpha 10	interferon, alpha 10
X01992	interferon gamma	interferon, gamma

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Accession Number	Gene Symbol	Gene Name
	Insulin-like Growth Factor Binding Protein	
M27544	IGF1	insulin-like growth factor 1 (somatomedin C)
M31159	IGFBP3	insulin-like growth factor binding protein 3
M62403	IGFBP4	insulin-like growth factor binding protein 4
M65062	IGFBP5	insulin-like growth factor binding protein 5
M62402	IGFBP6	insulin-like growth factor binding protein 6
	Other Extracellular Communication Proteins	
U83508	angiopoietin 1	angiopoietin 1
M36690	CD9 antigen (p24)	CD9 antigen (p24)
U24163	FRZB	frizzled-related protein
AF028593	jagged 1	jagged 1 (Alagille syndrome)
AF003521	jagged 2	jagged 2
U94354	LFNG	luratic fringe homolog (Drosophila)
M25639	MIF	macrophage migration inhibitory factor (glycosylation-inhibiting factor)
U94352	MFNG	manic fringe homolog (Drosophila)
U41745	PDAP1	PDGFA associated protein 1
AF017986	SFRP2	secreted frizzled-related protein 2
AF017988	SFRP5	secreted frizzled-related protein 5
X07876	WNT2	wingless-type MMTV integration site family member 2
Z71621	WNT2B	wingless-type MMTV integration site family, member 2B
L20861	WNT5A	wingless-type MMTV integration site family, member 5A
X91940	WNT8B	wingless-type MMTV integration site family, member 8B
X97057	WNT10B	wingless-type MMTV integration site family, member 10B
	Intracellular Transducers/Effectors/Modulators	
M76125	AXL	AXL receptor tyrosine kinase
U24166	MAPRE1	microtubule-associated protein, RP/EB family, member 1
L11353	neurofibromin 2	neurofibromin 2 (bilateral acoustic neuroma)
Y00815	P*PRF	protein tyrosine phosphatase, receptor type, F
L06139	TEK	TEK tyrosine kinase, endothelial (venous malformations, multiple cutaneous and mucosal)
U77845	TRIP	TRAF interacting protein
	Adaptor & Receptor-Associated Proteins	
U07707	EPS15	epidermal growth factor receptor pathway substrate 15
U12535	EPS8	epidermal growth factor receptor pathway substrate 8
U69276	GRB10	growth factor receptor-bound protein 10
AKC26432	HCK	hemopoietic cell kinase
U40282	ILK	integrin-linked kinase
M63959	LRPAP1	low density lipoprotein-related protein-associated protein 1 (alpha-2-macroglobulin receptor-associated protein 1)
U84401	SMO	smoothened homolog (Drosophila)
U12597	TRAF2	TNF receptor-associated factor 2
U21092	TRAF3	TNF receptor-associated factor 3
U69108	TRAF5	TNF receptor-associated factor 5
L05148	ZAP70	zeta-chain (TCR) associated protein kinase (70 kD)
AF000974	TRIP6	zyxin related protein ZRP-1

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Accession Number	Gene Symbol	Gene Name
Intracellular Kinase Network Members (non-receptor protein kinases)		
X58957	BTk	Bruton agammaglobulinemia tyrosine kinase
X86779	FAST kinase	FAST kinase
L26318	MAPK8	mitogen-activated protein kinase 8
L31951	MAPK9	mitogen-activated protein kinase 9
U34819	MAPK10	mitogen-activated protein kinase 10
U24152	PAK1	p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)
U24153	PAK2	p21 (CDKN1A)-activated kinase 2
U43522	PAK2B	protein tyrosine kinase 2 beta
L13616	PTK2	PTK2 protein tyrosine kinase 2
U43195	ROCK1	Rho-associated, coiled-coil containing protein kinase 1
G-Proteins		
M35543	cell division cycle 42	cell division cycle 42 (GTP binding protein, 25kD)
YC7923	GTP-binding protein	GTP-binding protein
X06820	RHOB	ras homolog gene family member B
X95456	RHON	GTP-binding protein Rho7
M31470	RHOQ	likely ortholog of mouse TC10-alpha
M29870	RAC1	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)
M64595	RAC2	ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)
L24564	RRAD	Ras-related associated with diabetes
GTP/GDP Exchangers & G-Protein GTPase Activity Modulators		
Z18951	caveolin 1	caveolin 1, caveolae protein, 22kD
U32114	caveolin 2	caveolin 2
U11690	faciogenital dysplasia	faciogenital dysplasia (Aarskog-Scott syndrome)
U59752	PSCD2	pleckstrin homology, Sec7 and coiled/coil domains 2-like
U02570	ARHGAP1	Rho GTPase activating protein 1
X78817	ARHGAP4	Rho GTPase activating protein 4
X69550	ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha
L20388	ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta
U82532	ARHGDIG	Rho GDP dissociation inhibitor (GDI) gamma
Phospholipases & Phosphoinositol Kinases		
U38545	phospholipase D1	phospholipase D1, phosphatidylcholine-specific
M86400	YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide
Other Intracellular Transducers Effectors Modulators		
L22005	cell division cycle 34	cell division cycle 34
U46461	DVL1L1	dishevelled, dish homolog 1 (Drosophila)
U56976	PDE1B	phosphodiesterase 1B, calmodulin-dependent
Protein Turnover		
Serine Proteases		
D49742	HABP2	hyaluronan binding protein 2
X05199	plasminogen	plasminogen
M15518	PLAT	plasminogen activator, tissue
M15476	PLAU	plasminogen activator, urokinase
Metalloproteinases		
X05231	MMP1	matrix metalloproteinase 1 (interstitial collagenase)
J03210	MMP2	matrix metalloproteinase 2 (gelatinase A, 72kD gelatinase, 72kD type IV collagenase)
X05232	MMP3	matrix metalloproteinase 3 (stromelysin 1, progelatinase)

Appendix III : Full list of gene symbols and corresponding gene names

Accession Number	Gene Symbol	Gene Name
	Metalloproteinases	
X07819	MMP7	matrix metalloproteinase 7 (matrilysin, uterine)
J05556	MMP8	matrix metalloproteinase 8 (neutrophil collagenase)
J05070	MMP9	matrix metalloproteinase 9 (gelatinase B, 92kD gelatinase, 92kD type IV collagenase)
X07820	MMP10	matrix metalloproteinase 10 (stromelysin 2)
X57766	MMP11	matrix metalloproteinase 11 (stromelysin 3)
L23808	MMP12	matrix metalloproteinase 12 (macrophage elastase)
X75308	MMP13	matrix metalloproteinase 13 (collagenase 3)
D26512	MMP14	matrix metalloproteinase 14 (membrane-inserted)
Z48482	MMP15	matrix metalloproteinase 15 (membrane-inserted)
D65511	MMP16	matrix metalloproteinase 16 (membrane-inserted)
X69576	MMP17	matrix metalloproteinase 17 (membrane-inserted)
X92521	MMP19	matrix metalloproteinase 19
U41766	ADAM9	a disintegrin and metalloproteinase domain 9 (meltrin gamma)
U69611	ADAM17	a disintegrin and metalloproteinase domain 17 (tumor necrosis factor, alpha, converting enzyme)
	Protease Inhibitors	
M11313	A2M	alpha-2-macroglobulin
M68516	SERPINA5	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antitrypsin, antitrypsin), member 5
M18082	SERPINE2	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2
X04429	SERPINE1	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
X03124	TIMP1	tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)
J05593	TIMP2	tissue inhibitor of metalloproteinase 2
Z30183	TIMP3	tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)
U76456	TIMP4	tissue inhibitor of metalloproteinase 4
	Others Involved in Protein Turnover	
L20471	BSG	basigin (OK blood group)
M26880	ubiquitin C	ubiquitin C
	Cytoskeleton/Motility Proteins	
X00351	beta actin	actin, beta
U29343	RHAMM	hyaluronan-mediated motility receptor (RHAMM)
M23410	junction plakoglobin	junction plakoglobin
M77349	TGF- β induced	transforming growth factor, beta-induced, 68kD
K00558	alpha tubulin	tubulin, alpha, ubiquitous
X51521	villin 2	villin 2 (ezrin)
X94991	zyxin	zyxin
	Intermediate Filament Proteins	
M63618	DS1	bullous pemphigoid antigen 1 (230/240kD)
M95063	cytokeratin 2	cytokeratin 2
U59167	desmin	desmin
M98776	keratin 1	keratin 1 (epidermolytic hyperkeratosis)
M95061	keratin 2A	keratin 2A (epidermal ichthyosis bullosa of Siemens)
X67683	keratin 4	keratin 4
M21389	keratin 5	keratin 5 (epidermolysis bullosa simplex, Dowling-Meara/Kobner/Weber-Cockayne types)
J00269	keratin 6A	keratin 6A
L42592	keratin 6B	keratin 6B
X03212	keratin 7	keratin 7
M34225	keratin 8	keratin 8
Z29074	keratin 9	keratin 9 (epidermolytic palmoplantar keratoderma)

Appendix III: Full list of gene symbols and corresponding gene names

Accession Number	Gene Symbol	Gene Name
	Intermediate Filament Proteins	
M19156	keratin 10	keratin 10 (epidermolytic hyperkeratosis, keratosis palmaris et plantaris)
D78367	keratin 12	keratin 12 (Meesmann corneal dystrophy)
X52426	keratin 13	keratin 13
J00124	keratin 14	keratin 14 (epidermolysis bullosa simplex, Dowling-Meara, Koebner)
M26326	keratin 18	keratin 18
Y00503	keratin 19	keratin 19
M21772	K16 CK16	type I cytoskeletal 16 keratin, cytokeratin 16 (K16, CK 16), pseudo-keratin K16 type I
X56134	vimentin	vimentin
	DNA Synthesis, Recombination & Repair	
	DNA Replication: Polymerases, Replication Factors & Topoisomerases	
M15796	PCNA	proliferating cell nuclear antigen
X06745	POLA	polymerase (DNA directed), alpha
M63488	RPA1	replication protein A1 (70kD)
M87338	RFC2	replication factor C (activator 1) 2 (40kD)
L07541	RFC3	replication factor C (activator 1) 3 (38kD)
M87339	RFC4	replication factor C (activator 1) 4 (37kD)
L07540	RFC5	replication factor C (activator 1) 5 (36.5kD)
J03250	TOP1	topoisomerase (DNA) I
J04088	TOP2A	topoisomerase (DNA) II alpha (170kD)
	DNA Damage Signaling/Repair Proteins & DNA Ligases	
U33841	ATM	ataxia telangiectasia mutated (includes complementation groups A, C and D)
M13194	ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1 (includes overlapping antisense sequence)
X52221	ERCC2	excision repair cross-complementing rodent repair deficiency, complementation group 2 (xeroderma pigmentosum D)
M31899	ERCC3	excision repair cross-complementing rodent repair deficiency, complementation group 3 (xeroderma pigmentosum group B complement)
L20046	ERCC5	excision repair cross-complementing rodent repair deficiency, complementation group 5 (xeroderma pigmentosum, complementation group 5)
X64740	LIG3	ligase III, DNA, ATP-dependent
X63441	LIG4	ligase IV, DNA, ATP-dependent
U07418	MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)
U04045	MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)
U54777	MSH6	mutS homolog 6 (E. coli)
M29971	MGMT	O-6-methylguanine-DNA methyltransferase
U35835	PRKDC	protein kinase, DNA-activated, catalytic polypeptide
D21235	RAD23A	RAD23 homolog A (S. cerevisiae)
U63139	RAD50	RAD50 homolog (S. cerevisiae)
D13804	RAD51	RAD51 homolog (RecA homolog, E. coli) (S. cerevisiae)
U12134	RAD52	RAD52 homolog (S. cerevisiae)
M32865	G22P1	thyroid autoantigen 70kD (Ku antigen)
	DNA Damage Signaling/Repair Proteins & DNA Ligases	
M30938	XRCC5	X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining; Ku autoantigen, 80kD)
M74524	UBE2A	ubiquitin-conjugating enzyme E2A (RAD6 homolog)
M36089	XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1
	Other DNA Synthesis, Recombination & Repair Proteins	
X90392	DNASE1L1	deoxyribonuclease I-like 1
M60974	GADD45A	growth arrest and DNA-damage-inducible, alpha
X74262	RBBP4	retinoblastoma binding protein 4

Appendix III : Full list of gene symbols and corresponding gene names

Accession Number	Gene Symbol	Gene Name
	Angiogenic factors	
M32977	VEGF	vascular endothelial growth factor
U43142	VEGFC	vascular endothelial growth factor C
U48801	VEGFB	vascular endothelial growth factor B
J05081	endothelin 3	endothelin 3
M65199	endothelin 2	endothelin 2
U01134	FLT1	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)
	Functionally Unclassified	
U32907	LRRC17	37 kDa leucine-rich repeat (LRR) protein
U37688	RATS1	RATS1
D25216	LRRC14	KIAA0014 gene product

Appendix III : Full list of gene symbols and corresponding gene names