The effect of oxidative stress in lymphocytes from patients with inflammatory bowel disease and various cancer states compared with healthy control individuals.

By

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2010

Abstract

In the present investigation peripheral blood lymphocytes from patients with inflammatory bowel disease (IBD) and different cancer states were treated with various agents and compared with lymphocytes from healthy control individuals (HCI) treated in the same way and measured in the Comet assay. For inflammatory bowel disease, patient's responses in IBD patients treated with H_2O_2 were higher than in HCI and crohn's patients (CD) were found to have higher responses than Ulcerative colitis (UC) patients. The responses for all IBD and HCI were all reduced in the presence of chaga mushroom extract which behaved in an antioxidant manner. A second group of IBD patients were treated with the heterocyclic amine (food mutagen), IQ and H_2O_2 and responses were reduced in the presence of the flavonoids, quercetin and epicatechin and compared with HCI similarity treated. In all cells responses were reduced with flavonoids and again CD had higher responses than the UC patients and IBD patients higher than HCI. The responses with CD and UC were that confirmed in two independent studies with IBD, one with chaga mushroom extract and the other with flavonoids.

Peripheral lymphocytes from malignant melanoma and suspected melanoma patients and colon cancer and polyposis patients were compared to the lymphocytes from HCI and treated with UVA. There were differential sensitivities when measured in the micronucleus and Comet assays. The cancer patients had higher responses than those in the precancerous states and they in turn were higher than responses in HCI. In all the studies, untreated baseline DNA damage values were also higher in IBD and cancer patients and pre-cancerous patients than HCIs. This would suggest that baseline frequencies of different diseases compared to controls could be an important biomarker in the diagnosis of pre-cancers and early stage cancers. Also peripheral lymphocytes are a useful surrogate for cancers and pre-cancerous disease states since, blood is present in all organs and tissues and DNA is basically the same in all cells.

Key words: Oxidative stress, cancers, malignant melanoma, colorectal cancer, Suspected melanoma, polyposis, comet and micronucleus assays, Inflammatory bowel disease, chaga mushroom, flavonoids, IQ, H_2O_2, UVA

ACKNOWLEDGMENTS

I would like to thank Professor Diana Anderson, Established Chair of Biomedical Sciences, University of Bradford, who has been far more than a supervisor for me. In the past four years, her generous support and wise advice has always helped me to overcome the obstacles and find my way through this research.

I am also very grateful to Mr Justin B. Davies, Dr Andrew Wright and Dr P. Dominic Reynolds, the consultants and to the staff of outpatient clinics in St Luke's Hospital, for spending precious time to help recruit patients for which I took the samples for this study. It was an extra burden on their ever-increasing workload.

I owe a special thanks to Dr Adolf Baumgartner and my other colleagues in Biomedical Sciences Department, University of Bradford for sharing their experience with me and helping me whenever I needed.

I will never forget the support I had from my family, my beloved children and my caring and compassionate husband. Finally my parents have a special place here, those who gave me everything with love and without any expectation.

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ABBREVIATIONS

AAPC Attenuated adenomatous polyposis coli

AAs African Americans

APC Adenomatous polyposis coli

APC antigen-presenting cell

AMPK Adenosine monophosphate-activated protein kinase

ATG16L1 autophagy related 16-like 1

ATP synthesis adenosine triphosphate synthesis

BiBuds binucleated buds

BiNPBs binucleated nucleoplasmic bridges

BRAF B-Raf proto-oncogene serine/threonine-protein kinase

BRI Bradford Royal Infirmary

CBPI cytokinesis-block proliferation index

CCD-camera charge-coupled device camera

CD Crohn's disease

CD4+ cluster of differentiation 4

CD8+ cluster of differentiation 8

CDKs cyclin-dependent protein kinases

CIN chromosomal instability

C-Myc myelocytomatosis cods

CMNs Congenital melanocytic Nevis

COX cycloxigenase

CPDs cyclobutanate-pyrimidine dimers

CPG C (cytosine), G (guanine) CpG" is shorthand for "—C—phosphate—G—", that is, cytosine and guanine separated by a phosphate

CRC colorectal cancer

CRD15 caspase activating recruitment domain 15

CTLs cytolytic T lymphocytes

DBL dihydroxybenzalacetone

DCC deleted in colon cancer

DiMeIQx 2-amino-3,4,8-dimethylimidazo[4,5-*f*]quinoxaline

DMSO dimethyl sulphoxide

DN Dysplastic naevi

DNA deoxyribonucleic acid

DPPH diphenyl-2-picrylhydrazyl

EBV-EA Epstein-Barr virus early antigen

ECG epicatechine-3 gallate

EDTA ethylenediaminetetraacetic acid

EGC epigallocatechin

EGCG epigallocatechin-3 gallate

EF Fisher's Exact

8-OhdG 8-hydroxy-deoxy-guanosine

FAP Familial adenomatous polyposis

FBS foetal bovine serum

Gadd45 Growth Arrest and DNA Damage

HCA heterocyclic amines

HCI healthy control individual

HIF1α hypoxia inducible 1**α**

HIF2α hypoxia inducible 2**α**

HLA Human Leukocyte Antigen

H_2O_2 hydrogen proxide

hMSH2 DNA mismatch repair gene

H-MSI high frequency microsatellite instability

HNPCC Hereditary nonpolyposis colorectal cancer

HOIQ 2-amino-3*H*-imidazo [4,5-*f*] quinoline-7-one

HPV human papillomavirus

IFN interferon

IGE Infectious gastroenteritis

IGFBP7 Insulin-like growth factor-binding protein 7

IQ 2-amino-3-methylimidazo[4,5-*f*]quinoline

IL interleukin

IBD Inflammatory Bowel Disease

KCl Potassium chloride

K-W Kruskal-Wallis

KRAS Kirsten rat sarcoma viral oncogene homolog

LFA-3 lymphocyte functional antigen-3

LMP low melting point

LOH loss of heterozygosity

LV Leucovorin

MeIQ 2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline

MeIQx 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline

MDP muramyl depeptide

MHC major histocompatibility complex

MC1R melanocortin-1 receptor

MCs melanocytes

MDM2 murinedouble minute 2

MLH1 MutL homolog 1

MM malignant melanoma

MMC mitomycin C

MMR enzyme DNA mismatch repair

MN micronucleus

MNBN Micronuclei in binucleated cells

MonoMN mononucleated micronuclei

MSH2 mutS homolog 2

MTOR mammalian target of rapamycin

MUTYH mutY Homolog (E. coli)

NaCl sodium chloride

NER Nucleotide excision repair

NF-kappaB nuclear factor-kappaB

NIH National Cancer Institute

NO$^{\bullet}$ nitric oxide

Nod2 nucleotide oligomerisation domain 2

OCT optical coherence tomography

ODD oxidative DNA damage

PC Polyposis Coli

phIP 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine

PMN polymorphonuclear

PMS2 Mismatch repair endonuclease

RPMI Rothwell Park Memorial Institute

ROS reactive oxygen species

RNA Ribonucleic acid

RNS reactive nitrogen species

SB small bowel

SCGE single cell gel electrophoresis

6-4 PPs 6-4 photoproducts

SM Suspected melanoma

SMAD4 Mothers against decapentaplegic homolog 4

SPSS solar-powered satellite system

SSBs single strand breaks

SSP Superficial spreading melanoma

SNP Single nucleotide polymorphisms

SODIS Summary Solar disinfection

SSB single strands break

TBARS thiobarbituric acid reactive substance

TCA cycle tricarboxylic acid cycle

TCF4 T cell factor 4

TEP total extracellular phenol

TIP total intercellular phenol

TP Thymidine phosphorylase

TNF Tumour necrotising factor

TNM Tumor, Node, Metastasis

TPA tetracanoylphrobol-13-acetate

UC Ulcerative colitis

UVA ultraviolet A

VEGF Vascular endothelial growth factor

WSR Wilcoxon Signed Rank

XP Xeroderma pigmentosum

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CHAPTER 1
INTRODUCTION

1.0. Introduction

1.1. Inflammatory Bowel disease

Inflammatory bowel disease (IBD) covers a group of disorders that cause inflammation of the intestine and consists of both Crohn's disease (CD) and Ulcerative colitis (UC) ((author,year)Rhodes, Thomas et al. 1997). These disorders have distinct pathologic and clinical characteristics but their pathogenesis remains poorly understood. Infectious gastroenteritis (IGE) is known to exacerbate previously diagnosed inflammatory bowel disease IBD. The initiation of IBD is a multifactorial process that might include the disruption of normal gut homeostatic mechanisms. More studies are warranted to evaluate the pathogen-specific risks, identify susceptible populations, and better understand the pathophysiologic relationship between IGE and IBD (Porter, 2008).

Environmental factors play an important role in the pathophysiology of IBD. There is a strong and consistent association between smoking and Crohn's disease, and between non-smoking and Ulcerative colitis. Despite extensive research, the exact pathophysiological mechanisms for these associations remain unclear. In spite of this, some clinical trials with nicotine-patches showed beneficial effects for the treatment of Ulcerative colitis. Associations of Crohn's disease and Ulcerative colitis with other environmental factors are weaker like the association with use of oral contraceptives or those less well investigated such as the association with childhood hygiene. Most studies suggesting a potential pathogenetic role of *Mycobacterium paratuberculosis* or an effect of tuberculostatic therapy in Crohn's disease could not be reproduced by others

(Andus, 2000). Perinatal or childhood infections by viruses like measles are heavily debated, but not proven to be causal for IBD. Coagulation disorders have been described as protecting from inflammatory bowel disease, suggesting hypercoagulability to be a pathogenetic factor (Andus, 2000). Some studies described that appendectomy may prevent the onset of Ulcerative colitis in man and mice. Other environmental factors such as hydrogen sulphide, tonsillectomy, diet, blood transfusions, and Listeria also require confirmation (Andus, 2000). There are, however, convincing data from genetic animal models and twin studies that environmental factors such as the intestinal bacterial flora interact with susceptible hosts to cause inflammatory bowel disease. Inflammatory bowel diseases have multifactorial aetiologies, which require a differentiated approach for treatment and prevention (Andus, 2000).

1.1.1.1. Signs and Symptoms of Crohn’s disease

In contrast to Ulcerative colitis, Crohn's disease is characterized by transmural rather than superficial mucosal inflammation and by skip lesions rather than continuous disease. The transmural inflammatory nature of Crohn's disease often leads to fibrosis and to obstructive clinical presentations which are not typically seen in Ulcerative colitis. The transmural inflammation can also result in sinus tracts that burrow through and penetrate the serosa, giving rise to microperforations and fistulae. Crohn's disease may involve the entire gastrointestinal tract from mouth to perianal area (Nigg, 2008).

1.1.1. 2. Distribution of Crohn's disease

The studies have shown that both Crohn's disease and Ulcerative colitis affect people in white collar occupations associated with higher income and higher social class more frequently than other groups in the population (Sonnenberg, 1990).

These associations were found in the complete data for 1982-8 as well as in the separate data for the two half periods 1982-5 and 1986-8. Highly significant correlations between the occupational distribution of Crohn's disease and Ulcerative colitis were found among both male and female employees. It seems that occupations involving work in the open air and physical exercise are protective, while being exposed to air conditioned artificial working conditions or extended and irregular shift working confer a risk of contracting inflammatory bowel disease (Sonnenberg, 1990).

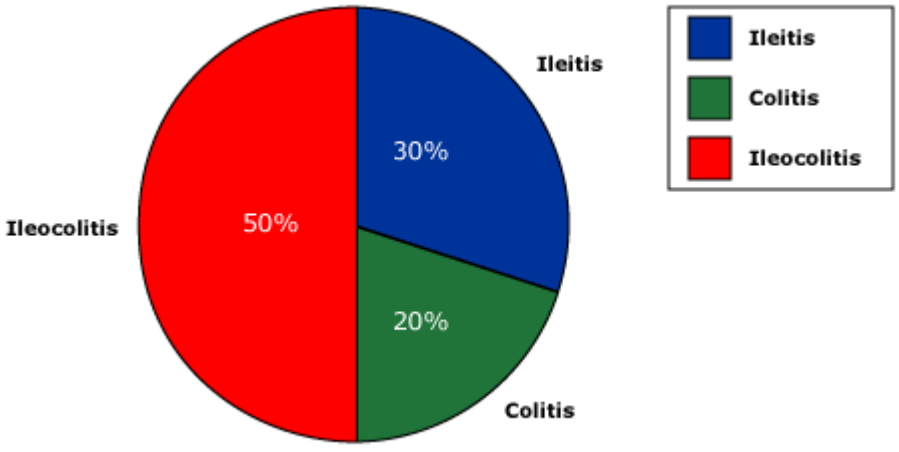

Figure 1.1. Approximate frequency of ileal and colonic involvement in Crohn's disease. Crohn's disease can involve the entire gastrointestinal tract from mouth to perianal area. Courtesy of the American Gastroenterological Association©.

1.1.1.3. Signs and Symptoms of Ulcerative colitis

Ulcerative colitis is characterized by recurring episodes of inflammation limited to the mucosal layer of the colon. It almost invariably involves the rectum and may extend in a proximal and continuous fashion to involve other portions of the colon. Different terms are used to describe the degree of involvement. Ulcerative proctitis refers to disease limited to the rectum (Farrell, 2002 ;Farrell, 2002).

Patients with Ulcerative colitis can have a variable presentation. For therapeutic and prognostic purposes, it has been useful to classify these presentations as mild, moderate, and severe. The severity of the symptomatology often correlates with the anatomic extent of the disease, another parameter that will guide therapy.

1.1.1.3.1. Mild disease

Patients whose disease is confined to the rectum (proctitis) or rectosigmoid (proctosigmoiditis or distal colitis), often present insidiously with intermittent rectal bleeding associated with the passage of mucus, and the development of mild diarrhoea with fewer than four small loose stools per day. Mild crampy pain, tenesmus, and periods of constipation are also common, but severe abdominal pain, profuse bleeding, fever, and weight loss are not part of the spectrum of mild disease.

1.1.1.3.2. Moderate disease

Moderate disease is usually characterized anatomically by involvement of more than the distal colon, with the inflammatory process extending to at least the splenic flexure (left-sided colitis). The clinical picture is characterized by frequent loose, bloody stools (up to 10 per day), mild anaemia not requiring blood transfusions, abdominal pain that is not severe, and low grade fever. Adequate nutrition is usually maintained. Approximately 80 percent of patients have small bowel involvement, usually in the distal ileum, with one-third of patients having exclusively ileitis (Gitnick, 1994).

Approximately 50 percent of patients have ileocolitis which refers to involvement of both the ileum and colon. Approximately 20 percent have disease limited to the colon (Lewis, 2004). Although this pattern is similar to that in Ulcerative colitis, roughly one-half of such patients have sparing of the rectum which is rare in Ulcerative colitis.

1.1.1.3.3. Severe disease

Severe Ulcerative colitis, the least common form of the disease, occurs in 15% of all patients with Ulcerative colitis. This form of the disease may be the initial

presentation or may represent a progression from a less severe attack. Diarrhoea is profuse and rectal bleeding is constant and severe. Fever is marked and sustained, and appetite and weight are both severely diminished. Abdominal cramps are severe and tenderness may be localized, indicating impending perforation. Leukocytes greater than 10,000, severe anaemia, and hypoalbuminemia resulting from low protein intake (anorexia) and increased chronic loss of albumin are hallmarks of this form of the disease

Medical therapy is often ineffective for this type of patient, and colonectomy is often required (Thompson, 1997).

A small percentage has a predominant involvement of the mouth or gastroduodenal area, while fewer patients have involvement of the oesophagus and proximal small bowel. Approximately one-third of patients have perianal disease. Its precise aetiology remains unknown, but it is a multifactorial disorder involving genetic, environmental, microbial and immune factors (Fiocchi, 2005). IBDs are thought to result from unchallenged immune responses to normal flora in genetically susceptible hosts. The condition known as IBD has only been identified for 70 years and seems to be on the increase in developed countries within North America and Europe and Scandinavia. Crohn's disease was first described in 1932 and may affect any part of the gastrointestinal tract.

Ulcerative colitis is a chronic relapsing inflammatory disorder affecting the colonic and rectal mucosa. For the different disease states, although quite distinct pathologically, the treatment including drug therapy is often very similar.

TABLE 1.1. 1. Clinical differentiation of Ulcerative colitis from Crohn's colitis

Feature	*Ulcerative colitis*	*Crohn's colitis*
Clinical features		
Rectal bleeding	Very common - 90%	Uncommon: may be occult
Diarrhoea	Early, frequent, small stools	Less prevalent or absent
Abdominal pain	Predefecatory, urgency	Colicky, postprandial
Fever	Uncommon if uncomplicated	Frequent
Palpable mass	Rare	Frequent, right lower quadrant
Recurrence after resection	Rare	Frequent
Clinical course	Relapses/remissions 65% Chronic/continuous 20-30% Acute/fulminating 5-8%	Usually slowly progressive; fulminant
Endoscopical features		
Proctosigmoidoscopy	Diffuse pinpoint ulcerations,continuous lesions	Discrete aphthoid ulcerations, patchy lesions
Radiological features		
Rectal involvement	Invariable	Infrequent
Distribution	Continuous	Segmental, discontinuous
Mucosa	Fine ulcerations	"Cobblestones"
Strictures	Rare	Frequent
Fistulas	Rare	Frequent
Histological features		
Distribution	Mucosal	Transmural
Cellular infiltrate	Polymorphs	Lymphocytes
Glands	Mucin depletion • Gland destruction • Crypt abscesses	Gland preservation
Special features	None	Granulomas, aphthoid ulcers, histiocyte-lined fissures

Adapted From: First Principles of Gastroenterology: The Basis of Disease and an Approach to Management, 1997.

The most likely age of onset of IBD is bimodal and lies between 15-30 years or 60-80 years. The male to female ratio for UC is 1/1 and for CD is 1.8/ 1 (Kirsner, 1991). Inflammatory bowel diseases are a public health problem in industrialized countries, where 1 in 1000 people is affected. Most patients are young adults. The incidence of IBD has increased considerably in western countries since the Second World War but is beginning to level off. On the other hand, the incidence is still rising in low-incidence areas such as Eastern Europe, Asia and developing countries (Gismera, 2001; Guariso, 2010). One of the important factors in the immunopathogenesis of IBD is diet and basically it acts as luminal antigens, but whether antibodies against dietary antigens play a primary role in IBD aetiology or are secondary to intestinal inflammation is yet to be established (Yamamoto, 2009). Differences in incidence rates across age, time, and geographic areas suggest that environmental factors are involved in IBD, but only cigarette smoking and appendectomy have consistently been identified as risk factors. An important role of genetic factors in IBD was first suggested by epidemiological studies showing familial aggregation of IBD and by twin studies. Numerous studies from Europe and North America have provided a wealth of information regarding the epidemiological and clinical characteristics of IBD in Caucasians (Gismera, 2001). Previous studies in mainland China have been limited by small patient numbers or by lack of detailed information about clinical subgroups of the disease. This limited study was carried out to assess the demographic and clinical characteristics of IBD in Chinese patients. In the Sir Run Shaw Hospital between 1994 and 2003, 379 patients were diagnosed with IBD. Demographic

and clinical data were collected and analysed. Results: Of the 379 patients examined , 317 had Ulcerative colitis UC (83.6%, 168 male, 149 female, male-female ratio, 1.13/1, age range at diagnosis 14-79 years, mean age 44 years) and 62 had Crohn's disease CD (16.4%, 39 male and 23 female, male-female ratio 1.70/1, age range at diagnosis 13-70 years, mean age 33 year (Cao, 2005 ;Ullman, 2010).

Seasonal variation in flares of IBD (with peaks in the spring) have been suggested in some reports, however the magnitude of the association, if any, is weak, and discordant data have also been published (Lewis, 2004).

TABLE 1.1.2. Endoscopic grading of activities in Ulcerative colitis

Activity	Appearance
Quiescent	Distorted or absent mucosal vascular pattern Granularity
Mildly active	Continuous or focal erythema Friability (touch bleeding)
Moderately active	Mucopurulent exudate (mucopus) Single or multiple ulcers (<5 mm); fewer than 10 per 10 cm segment
Severe	Large ulcers (>5 mm); more than 10 per 10 cm segment Spontaneous bleeding

Adapted from: First Principles of Gastroenterology: The Basis of Disease and an Approach to Management, 1997.

1.1.1.4. Epidemiology of inflammatory bowel disease

TABLE1.1.3. Epidemiology of inflammatory bowel disease

Incidence, per 100,000 (North America)	3-14 (CD) 2-14 (UC)
Incidence, per 100,000 (North America)	26-199 (CD) 27-246 (UC)
Geography	Northern Countries > Southern Countries
Age of onset	Peak: 15-30 Second peak: 50-80 (CD)
Sex	M=F
Race	White > Blacks
Ethnic	Jewish > Non-Jewish
Smoking	Associated with CD: protective in UC
Appendectomy	Maybe protective in UC
Possible – genetic associations' chromosome	Chromosome 16 (CD) Chromosome 3,5,7,12,19 (UC and CD), TNF-(CD); IL-1A (CD), IL-23 receptor

Adopted from: ***First Principles of Gastroenterology: The Basis of Disease and an Approach to Management***, 1997.

In Ulcerative colitis, 11.4% of patients had proctitis, 25.2% had proctosigmoiditis, 18.6% were diseased to the splenic flexure and 44.8% had extensive colitis. Nine patients with UC (2.8%) had arthritis; three patients (0.9%)

had iritis or conjunctivitis. Of the 62 CD patients, 16 (25.8%) had diseases restricted to the terminal ileum; 15 (24.2%) had colonic diseases; 20 (32.3%) had ileocolonic disease and 11 (17.7%) had disease involving the upper gastrointestinal tract. This study showed similar characteristics of IBD to that in the West but there are some differences with respect to severity and extraintestinal manifestations. The ethnic and geographic differences may give important clues to the aetiology of IBD (Gismera, 2001).

TABLE 1.1.4. Incidence and prevalence* of IBD from selected registries

Location	Year	Incidence	Prevalence*
Crohn's disease			
Rochester, New York	1943-1982	4.3	90.5
Malmo, Sweden	1958-1973	4.8	75.2
Copenhagen, Denmark	1958-1978	1.8	34.0
Tel Aviv, Israel	1970-1976	1.3	12.3
North Tees, England	1971-1977	5.3	35.0
Ulcerative colitis			
Rochester, New York	1960-1979	15.0	212.6
Malmo, Sweden	1958-1973	6.4	89.0
Copenhagen, Denmark	1962-1978	8.1	117.0
Tel Aviv, Israel	1961-1970	3.7	37.4
North Tees, England	1971-1977	15.1	99.0

*Per100,00population

IBD may cause a delay in puberty or growth problems because it can interfere with nutrition. Patients usually need extensive therapeutic treatment as a result of the chronic relapsing nature of the disease. Ten percent of patients have a first degree relative with the disease while siblings are affected 30 times more often

than the general population suggesting that genetic factors help susceptibility to IBD (Ueda, 1992).

1.1.1.5. Diagnosis

The diagnosis of inflammatory bowel disease can pose a significant challenge to gastroenterologists, mainly due to a wide array of clinical presentations that are related to the involvement of different portions of the gastrointestinal tract (Kirsner, 1991). Due to an absence of uniformity in clinical presentation and a lack of a single gold standard test, an accurate diagnosis and appropriate classification of IBD can often be challenging. Diagnosing small bowel (SB) involvement has been particularly challenging in the past due to a lack of adequate endoscopic and radiologic modalities to evaluate the entire SB. Endoscopy remains the cornerstone in the evaluation of IBD, with the diagnosis usually established by a combination of clinical symptoms, laboratory markers e.g. perinuclear antineutrophil cytoplasmic antibodies (pANCA), autoantibodies to intestinal goblet cells (GAB) and autoantibodies to exocrine pancreas (PAB) and endoscopic and radiologic features. One of the new methods to diagnose this disease is capsule endoscopy (Pasha, 2007; Homsak, 2010).

TABLE1.1.5. Inflammatory bowel disease: indications for colonoscopy

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Differentiating IBD from other diseases and differentiating Crohn's from Ulcerative colitis
Establishing the extent of the disease
Screening for malignancy and malignant precursors
Evaluation of abnormalities on radiographs

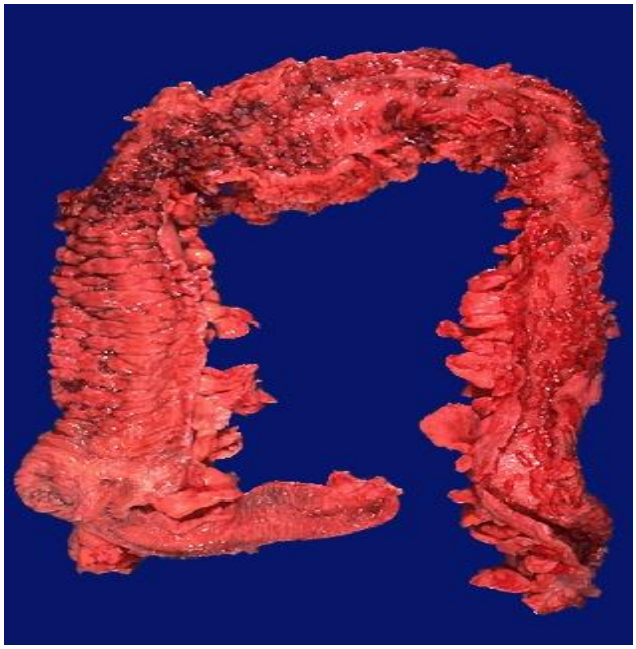
- Strictures
- Masses

Evaluation of patients not responsive to standard therapy or examination to explain recent flare; searching for complications
Examination prior to surgery

- Detection of intestinal involvement (active IBD) in fistulous disease
- Differentiation of Ulcerative colitis from Crohn's colitis

Adapted from: ***First Principles of Gastroenterology: The Basis of Disease and an Approach to Management***, 1997.

Photograph 1.1.1. Pseudomembranous colitis appears as a tan to yellow-green exudate over an erythematous bowel mucosa. *www.mda-sy.com/pathology/TUTORIAL/IBD/IBD011.HTM*



Photograph 1.1.2. Ulcerative colitis is seen here grossly in a total colectomy. There is diffuse involvement of the mucosa from the rectum proximally to the ascending colon. The caecum is not so

Severely involved, and the terminal ileum is not involved.

www.mda-sy.com/pathology/TUTORIAL/IBD/IBD011.HTM

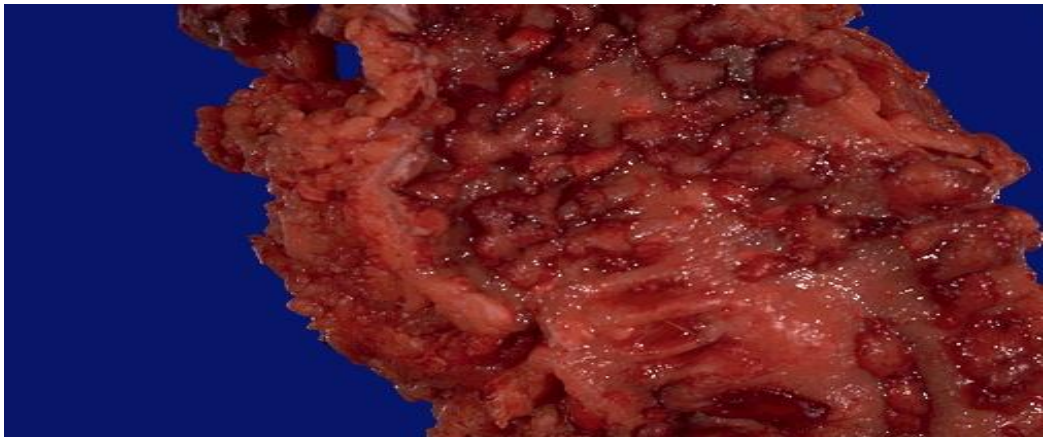
Photograph 1.1.3. Colonic adenocarcinoma is a long term risk with Ulcerative colitis. A large mass is seen extending into the lumen of the colon here. *www.mda-sy.com/pathology/TUTORIAL/IBD/IBD011.HTM*

Photograph 1.1.4. Crohn's disease is seen in this segmental resection of terminal ileum to involve the centre portion where the small intestinal wall is thickened and the mucosa is inflamed and ulcerated. *www.mda-sy.com/pathology/TUTORIAL/IBD/IBD011.HTM*

Crohn's disease is a chronic, transmural, granulomatous disorder that can involve any segment of gastrointestinal tract from the mouth to the anus. In the bowel it may affect multiple distinct segments, with normal intervening bowel. Crohn's disease also may be complicated by intestinal strictures, fistulas, and perianal fistulas. The clinical presentation of Crohn's disease depends on the area of

gastrointestinal tract involved and ranges from intestinal obstruction, to bloody or non-bloody diarrhoea, to malabsorption (Kirsner, 1991; Huprich, 2010).

Ulcerative colitis is a nongranulomatous inflammatory condition that always starts in the rectum and extends proximally throughout the colon in a continuous and confluent fashion, never involving the small bowel. The clinical presentation of Ulcerative colitis is more uniform than that of Crohn's disease and includes rectal bleeding or bloody diarrhoea. In addition to gastrointestinal symptoms, extraintestinal manifestations can occur and may involve the skin (e.g., erythema nodosum, pyoderma gangrenosum), joints (sacroiliitis, ankylosing spondylitis, and peripheral arthritis), eyes (iritis and uveitis), and liver (sclerosing cholangitis). Extraintestinal manifestations are more common with colonic than with small-bowel disease and are believed to be due to the release of bacterial antigens from the colonic lumen (Farrell, 2002 ;Mader, 2005).



Photograph 1.1.5. Ulcerative colitis is seen grossly in the colon at higher magnification with the typical pattern of "pseudopolyps" from severe inflammation and mucosal erosion. The pseudopolyps are remaining islands of mucosa after the bulk of the mucosa has ulcerated away. *www.mda-sy.com/pathology/TUTORIAL/IBD/IBD011.HTM*

The differential diagnostic considerations in IBD are wide and varied. In patients with new-onset bloody diarrhoea and abdominal cramps, an infectious cause must first be ruled out. In addition to routine cultures, cultures for *Clostridium difficile,* ova and parasites, and haemorrhagic *Escherichia coli* should be done. Colonic ischemia presents with symptoms similar to those of IBD--abdominal cramps, rectal bleeding, and diarrhoea--and should be a consideration in older patients. Nonsteroidal anti-inflammatory drugs can also cause colonic ulcerations that mimic colonic Crohn's disease (Farrell, 2002 ;Hussain, 2010).

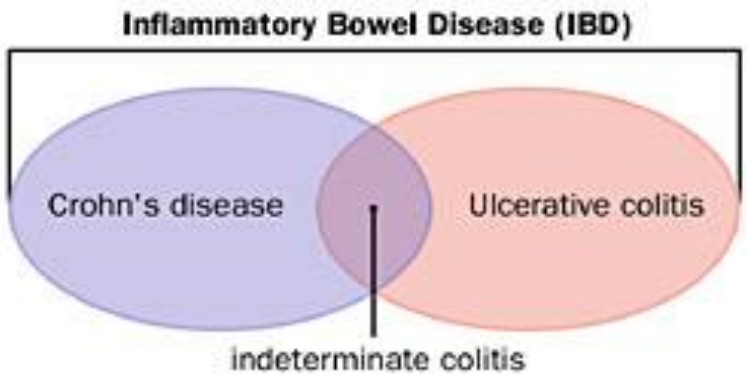


Figure 1.1.2. Inflammatory bowel disease subsets (Bayless, 2008).

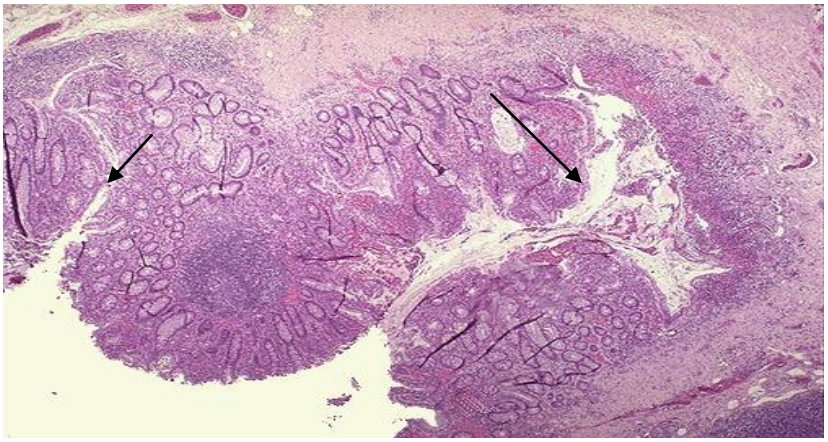
UC and CD have similar features to many other diseases. In the absence of a key diagnostic test, a combination of features is used. Once a diagnosis of IBD is made, distinguishing between UC and CD is impossible in up to 15% of cases. These are termed indeterminate colitis (Guindi, 2004; Tertychnyi, 2010).

Ulcerative colitis usually begins in the rectosigmoid and extends proximally; however, there is a subtype that is milder in degree which is limited to the rectum

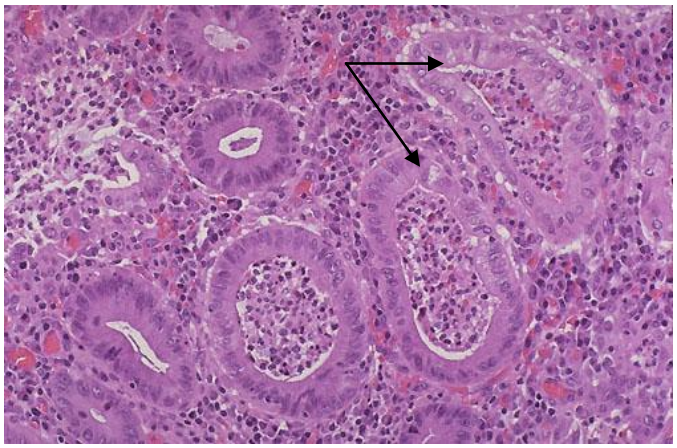
(Ulcerative proctitis). In young patients, the entire colon is more often involved than in adults (Farrell, 2002) (Photograph 1.1.5).

1.1.1.6. Pathology

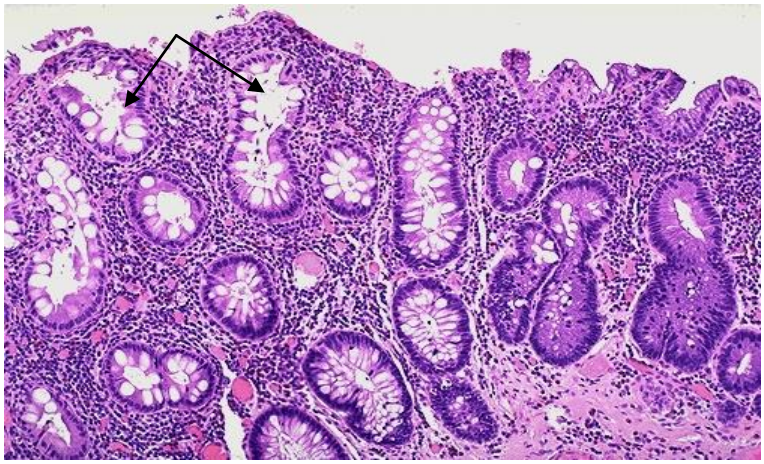
In Ulcerative colitis the characteristic histological findings are acute and chronic inflammation of the mucosa by polymorphonuclear leukocytes and mononuclear cells, crypt abscesses, distortion of the mucosal glands, and goblet cell depletion. Crohn's disease, in contrast to UC, can involve any part of the gastrointestinal tract from the oropharynx to the perianal area (see later Photographs 1.1.9, 1.1.10, 1.1.11). Diseased segments frequently are separated by intervening normal bowel, leading to the term "skip areas." Inflammation can be transmural, often extending through to the serosa, resulting in sinus tracts or fistula formation. Histological findings include small superficial ulcerations over a Peyer's patch (aphthoid ulcer) and focal chronic inflammation extending to the submucosa, sometimes accompanied by noncaseating granuloma formation. The most common location is the ileocecal region, followed by the terminal ileum alone, diffuse small bowel, or isolated colonic disease in decreasing order of frequency (Andus, 2000 ;Asquith, 2010).



Photograph 1.1.6. Ulcerative colitis is shown microscopically at low power magnification to reveal mucosal inflammation and erosion. *www.mda-sy.com/pathology/TUTORIAL/IBD/IBD011.HTM*

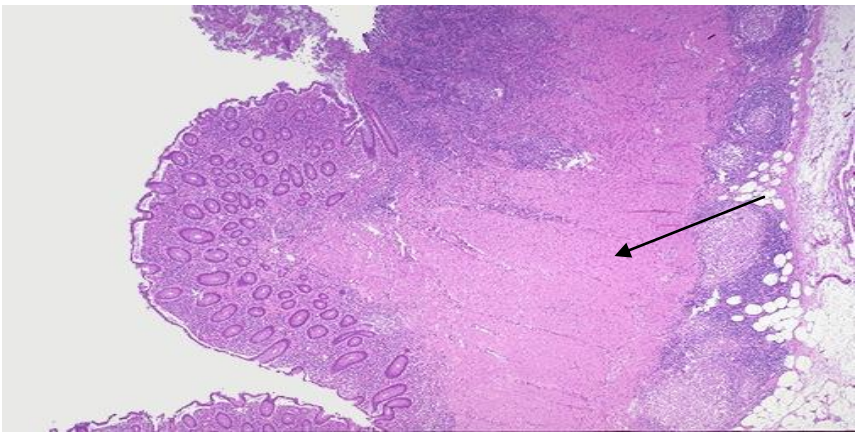


Photograph 1.1.7. Ulcerative colitis seen here microscopically at high power magnification demonstrates the presence of acute inflammatory cells within crypts, the so-called "crypt abscesses".*www.mda-sy.com/pathology/TUTORIAL/IBD/IBD011.HTM*

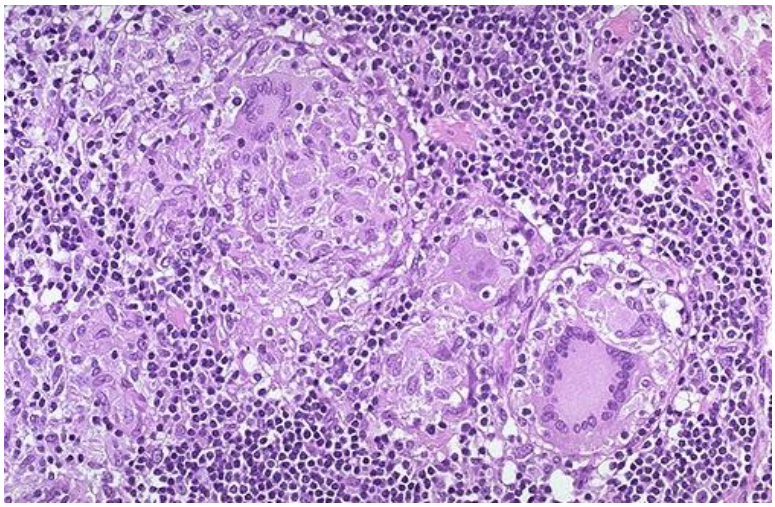


Photograph 1.1.8. Ulcerative colitis in other areas may demonstrate crypt distortion and dysplasia, as seen here on the right microscopically. *www.mda-sy.com/pathology/TUTORIAL/IBD/IBD011.HTM*

In Ulcerative colitis the pathological change begins with degeneration of the mucosal epithelium (specifically the reticulin fibres), occlusion of the subepithelial capillaries, and progressive infiltration of the lamina propria with white blood cells; all types invade but there is a predominance of PMN's (polymorphonuclear). Eventually, crypt abscesses, epithelial necrosis, and mucosal ulcerations develop (Bousvaros, 2007) (see later Photographs 1.1.6, 1.1.7, 1.1.8).

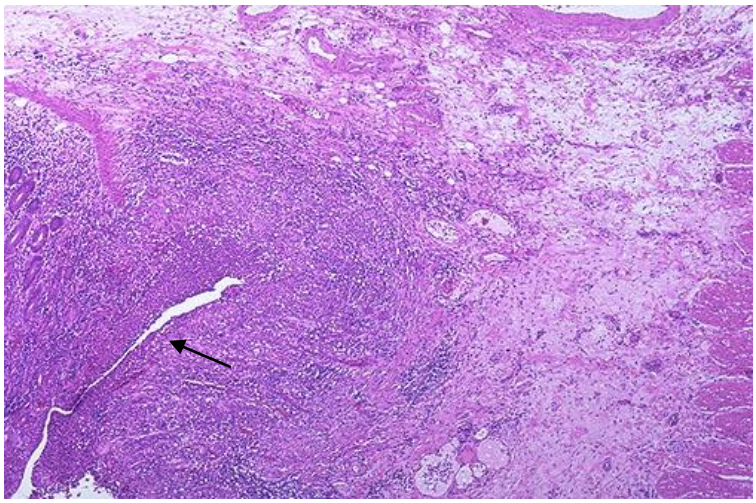


Photograph 1.1.9. Crohn's disease microscopically at low power magnification can be seen to involve the full thickness of the wall from the mucosa to the serosa. Granulomas are seen toward the serosal surface here. *www.mda-sy.com/pathology/TUTORIAL/IBD/IBD011.HTM*



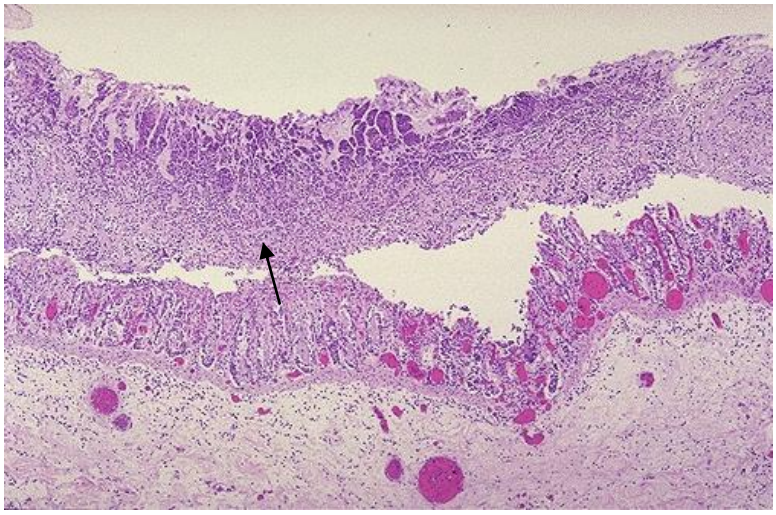
Photograph 1.1.10. Crohn's disease microscopically at high power magnification may demonstrate granuloma formation. Special stains for infectious organisms will be negative.

www.mda-sy.com/pathology/TUTORIAL/IBD/IBD011.HTM



Photograph 1.1.11. Crohn's disease microscopically at medium power magnification is shown with a deep fissure extending through mucosa to the submucosa. This can result in fistula and/or abscess formation.

www.mda-sy.com/pathology/TUTORIAL/IBD/IBD011.HTM



Photograph 1.1.12. Pseudomembranous colitis microscopically is seen as an adherent membrane of inflammatory cells and necrotic debris overlying the mucosa. *www.mda-sy.com/pathology/TUTORIAL/IBD/IBD011.HTM*

1.1.2. Oxidative stress and IBD

Overproduction of colonic oxidants contributes to mucosal injury in inflammatory bowel disease (IBD) but the mechanisms are unclear. The findings using monolayers of intestinal cells suggest that the mechanism could be oxidant induced-damage to cytoskeletal proteins. However, oxidants and oxidative damage have not been well characterised in IBD mucosa (Keshavarzian, 2003).

It has been shown in experimental animals as well as in humans that free radicals are released during inflammatory colitis (Loguercio, 1996; O'Connor, 2010). Hydrogen peroxide and reactive oxygen metabolites are generated in greater amounts in the inflamed bowel; the non-enzymatic pathway may be an additional route for nitric oxide radical ($NO^{\bullet}$) production in UC (Simmonds, 1993; Amini-Shirazi, 2009). Whilst IBD is associated with free radical damage, xanthine oxidase activity is not elevated in UC (Simmonds, 1993) but increased nitric

oxide synthesis is confirmed in UC (Reynolds, 1996;Hosseini, 2008 ; Colon, 2004). This pathway involves the reaction of hydrogen peroxide with both L- and D-arginine (Nagase, 1997). Conventionally, the activity of nitric oxide synthase is thought to be the main factor for $NO^{\bullet}$ generation in UC (Smith, 1993; Hosseini, 2008). When the large bowel wall becomes inflamed and hypoxic, other alternative sources become more important like the reduction of nitrite by xanthine oxidase and bacterial nitrate/nitrite reductases as well as the non-enzymatic reaction of hydrogen peroxide with arginine. Oxygen radicals, particularly superoxide and hydroxyl radicals are very reactive species believed to be involved in cell and tissue damage in a variety of diseases including inflammatory bowel disease (Allgayer, 1991; Colon, 2004). Both CD and UC patients showed increased free radical derived DNA damage within peripheral leukocytes, by examining the amount of 8-hydroxy-deoxy-guanosine (8-OHdG) present in blood and decreased plasma antioxidant defences (Hanauer, 2006; D'Odorico, 2001).

Oxidant levels increase in IBD along with oxidation of tissue and cytoskeletal proteins. Oxidative injury is correlated with disease severity but it is also present in substantial amounts in normal appearing mucosa of IBD patients, suggesting that oxidative injury does not necessarily lead to tissue injury and is not entirely a consequence of tissue injury. Marked actin oxidation (>50%) and NFkappaB which appear to result from cumulative oxidative damage was only seen in inflamed mucosa, suggesting that oxidant induced cytoskeletal disruption is required for tissue injury, mucosal disruption, and IBD flare-up (Keshavarzian, 2003; Sunil, 2010).

Neutrophil accumulation within epithelial crypts and in the intestinal mucosa directly correlates with clinical disease activity and epithelial injury in IBD. Advances have defined the mechanisms by which neutrophils are activated or migrate across endothelial and mucosal epithelial cells. A better understanding of this process will likely provide new insights into novel treatment strategies for IBD. Especially, activated neutrophils produce reactive oxygen and nitrogen species and myeloperoxidase within intestinal mucosa, which induce oxidative stress (Naito, 2007).

Emerging evidence demonstrates that prostaglandins play an important role in inflammation and cancer and recently the roles of the different prostaglandins in colorectal cancer and IBD have been studied (Sica, 2008).

The cyclooxygenase (COX) is a key enzyme in the conversion of arachidonic acid to prostaglandins (Park, 2007).

Intestinal inflammation is accompanied by excessive production of reactive oxygen and nitrogen metabolites. In order to counteract their harmful effects, the intestinal mucosa contains an extensive system of antioxidants. It has previously been shown that the levels of and the balance between the most important antioxidants are seriously impaired within the intestinal mucosa from inflammatory bowel disease patients compared with normal mucosa (Kruidenier, 2003; Sunil, 2010).

Oxidative stress is thought to play a significant role in the pathogenesis of inflammatory bowel disease, including CD. In an Italian study, subjects (37 CD and 46 UC patients) were compared with 386 healthy controls. Oxidative DNA damage was measured by examining the amount of 8-hydroxy-deoxy-guanosine (8-OhdG) present in blood. In addition, evaluation of plasma levels of vitamins A

and E and carotenoids demonstrated significant decreases in patients with either CD or UC when compared to controls. The specific carotene found to be most significantly reduced was beta-carotene, with plasma levels at only 50 percent of that of the controls. No significant differences were detected between CD and UC patients. Levels of 8-OhdG were considerably higher in IBD patients than in the controls, indicating increased oxidative DNA damage (Tanaka, 2001). Reduced plasma antioxidant concentrations and increased oxidative DNA damage were found in IBD (D'Odorico, 2001; Romier, 2009).

Endogenous antioxidants such as superoxide dismutase (SOD), glutathione, and catalase are normally able to counteract oxidative stress in the intestinal mucosa. However, inflammation increases the demand for these important antioxidants and results in an imbalance between pro-oxidants and antioxidants, with subsequent mucosal damage (Jahanshahi, 2004).

Depletion of antioxidants is likely to be important in the pathophysiology of IBD. Ulcerative colitis and CD patients showed increased free radical peripheral leukocyte DNA damage and decreased plasma antioxidant defences, by examining the amount of 8-hydroxy-deoxy-guanosine (8-OHdG) present in blood and decreased plasma antioxidant defences (Hanauer, 2006). This pathway involves the reaction of hydrogen peroxide with both L- and D-arginine (Nagase, 1997; Klink, 2001). Conventionally, the activity of nitric oxide synthase is thought to be the main factor for $NO^{\bullet}$ generation in UC (Smith, 1993). When the large bowel wall becomes inflamed and hypoxic, other alternative sources become more important like the reduction of nitrite by xanthine oxidase and bacterial nitrate/nitrite reductases as well as the non-enzymatic reaction of hydrogen peroxide with arginine. Oxygen radicals, particularly superoxide and

hydroxyl radicals are very reactive species believed to be involved in cell and tissue damage in a variety of diseases including inflammatory bowel disease (Allgayer, 1991; Keshavarzian, 2003).

Hypoxia inducible factors 1α and 2α (HIF1α and HIF2α) are hypoxia regulated transcriptional factors, which control the expression of a variety of genes responsible for angiogenesis, glycolysis, and the inhibition of apoptosis. Because angiogenesis and tissue regeneration are integral components of the inflammatory process, a study was designed to investigate the role of HIFα molecules in inflammatory bowel disease (Giatromanolaki, 2003).

HIF1α was expressed focally (epithelial cells, stromal fibroblasts, and myocytes) in both UC and CD, whereas HIF2α was expressed focally in UC and diffusely in CD. Thymidine phosphorylase (TP) is the key enzyme for capecitabine activation in tumour cells. Thymidine phosphorylase expression was uniformly positive in both diseases. VEGF expression vascular endothelial growth factor in healing wounds provokes dermal angiogenesis through complex mechanisms involving promotion of endothelial cell proliferation (Ferrara and Henzel 1989; Zanetti, Barozzi et al. 2010). VEGF was absent in CD, and weakly positive in UC. The VEGF–KDR reactivity of the submucosal vasculature was only slightly increased in UC and CD compared with normal tissue. The inflammatory cells stained with HIF2α and TP in all cases, but the reactivity was generalised in CD and focal in UC. In both diseases, vascular density was significantly higher than that seen in normal tissue (Giatromanolaki, 2003).

1.1.3. Genetic disorders

Mutations in the *Nod*2 gene may be associated with distinct, phenotypic expressions of Crohn's disease. Indeed, genotype/phenotype associations have already been identified which suggest that patients with Crohn's disease and *Nod*2 mutations are more likely to have "phenotypic expressions" of ileal disease that develop fibrostenosing complications (Abreu, 2002; Huebner, 2010). The scientists have shown that the wild-type Nod2 protein is a general sensor of peptidoglycan through the recognition of muramyl dipeptide (MDP), the minimal bioactive peptidoglycan motif common to all bacteria, and that the 3020insC (a cytosine insertion) frameshift mutation variant of Nod2, most frequently associated with Crohn's disease, fully abrogates Nod2-dependent detection of peptidoglycan and MDP (Abreu, 2002;Marrakchi, 2009).These observations have prompted the necessity of further elucidation of genetic patterns and, in particular, the likelihood of immune interactions with common enteric bacteria, inducing vulnerability to the development of the IBDs, and Crohn's disease in particular (Stephen, 2003 ; Haller, 2010).

The first CD susceptibility gene, NOD2/CARD15 on chromosome 16, was characterized (Gao, 2005; Torok, 2009). Other susceptibility genes have since been located (Gao, 2005). Their identification should help to understand the complex interaction between the environment and the intestinal immune system (Colombel, 2007). IBD may cause a delay in puberty or growth problems because it can interfere with nutrition. Patients usually need extensive therapeutic treatment as a result of the chronic relapsing nature of the disease. Ten percent of patients have a first degree relative with the disease while siblings are affected 30

times more often than the general population suggesting that genetic factors help susceptibility to IBD (Ueda, 1992).

The principal antigenic barrier to transplantation is a series of molecules which are polypeptide products of a closely linked cluster of genes known as the Major Histocompatibility Complex (MHC) or HLA (for Human Leukocyte Antigens) in humans (Jahanshahi, 2004). The MHC is highly polymorphic from individual to individual, and segregates in families in a Mendelian codominant fashion.

1.1.4. MHC classes

The abnormalities of T cells or macrophages and their interaction still remains the most feasible hypothesis (Bross, 1996; Guri, 2010). The genes of the HLA locus are located on the short arm of chromosome 6 (Suthanthiran, 1994; Chen, 2010). They encode two distinct classes of cell surface molecules, I and II (Duquesnoy, 1988; Ferguson, 2007). Class I molecules are expressed on the surfaces of virtually all nucleated cells at varying densities, while class II molecules are more restricted to cells of the immune system, primarily B lymphocytes and monocytes. However, cytokines secreted by lymphocytes and monocytes during immune activation can cause dramatic increases in class II HLA antigen expression, even on cell types which normally have little or no surface expression (Halloran, 1986; Ebert, 2005).

There are three different class I (HLA-A, -B, -C) and class II (HLA-DQ, -DR, -DP) antigens. Studies in renal transplantation indicate that mismatches at the A, B, and DR loci are associated with worse allograft survival (Doxiadis, 1996).

The normal function of the MHC is now better understood. The principal task of the immune system is to distinguish self from non-self.

HLA molecules provide the crucial surface upon which the antigen receptors on T lymphocytes recognize foreign (non-self) antigens. On antigen presenting cells such as macrophages, class II molecules present antigenic fragments to the CD4+ inducer (or helper) T cells, while class I molecules function at the effector phase of immunity by presenting antigens to CD8+ T cells, which generally have cytotoxic/suppressor function (Suthanthiran, 1994; Yap, 2004). This process of antigen presentation consists of the binding of a single T cell receptor to a complex on the surface of an antigen presenting cell consisting of the MHC molecule and a peptide fragment derived from the foreign antigen (Figures 1.1.3 and 1.1.4).

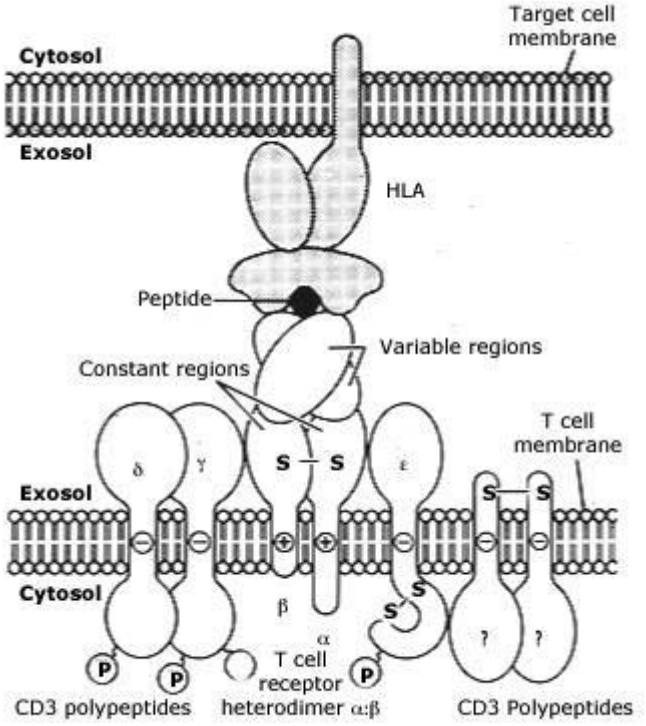


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Figure 1.1.3. Interaction of HLA and the T cell antigen receptor complex.The T cell antigen receptor is composed of an alpha and a beta chain which are linked by disulfide bonds forming a heterodimer. The TCR recognizes and interacts with processed peptide antigen {Krensky, 1990 #7;Krensky, 2005 #35}

The T cell antigen receptor is composed of an alpha and a beta chain which are linked by disulfide bonds forming a heterodimer. The TCR recognizes and interacts with processed peptide antigen presented in the context of the major histocompatibility complex. Adapted from (Krensky, 2005).

The first CD susceptibility gene, NOD2/CARD15 on chromosome 16, was characterized (Gao , 2005). Other susceptibility genes have since been located (Gao, 2005). Their identification should help to understand the complex interaction between the environment and the intestinal immune system (Colombel , 2007).

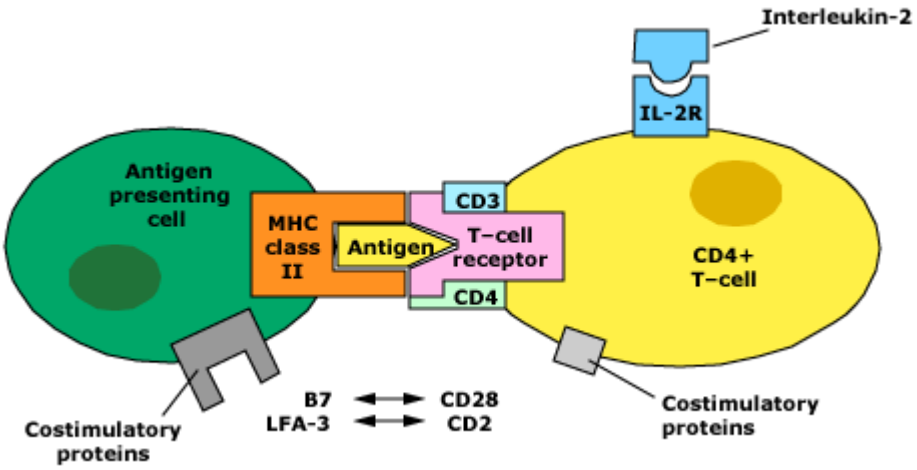
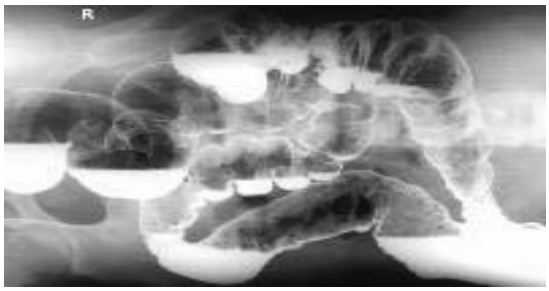


Figure1.1.4. Representation of T-cell activation, *Nature Immunology (2001)*

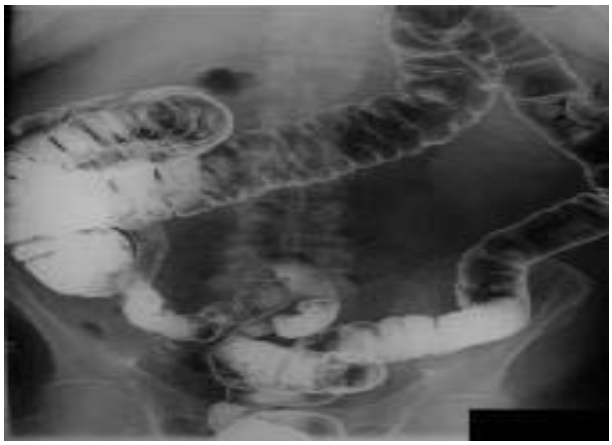
This is a schematic representation of initiation and immunologic response to an antigen. The antigen binds to a groove in MHC class II molecules on antigen-presenting cells (APCs, such as macrophages). This binding allows the antigen to be presented to antigen receptors on autoreactive CD4 inducer or helper T cells which, in type 1 diabetes mellitus, initiate autoimmune injury to the pancreatic beta-cells. In addition, the respective binding of B7 proteins and LFA-3 (lymphocyte functional antigen-3) on APCs to CD28 and CD2 on T cells are important costimulatory pathways that further increase T-cell activation. Other molecules also can participate in the immune response, such as the binding of interleukin-2 to its receptor (IL-2R) (Krensky, 2005) (Photograph 1.1.4).

1.2. Polyposis Coli

A GI polyp is defined as a mass of the mucosal surface protruding into the lumen of the bowel. Polyps can be neoplastic, nonneoplastic, or submucosal. GI polyposis is characterized by multiple polyps within the GI tract. The adenoma and hyperplastic polyp are two major types of colorectal epithelial polyps (Aust and Baretton 2010). The adenomas in the colon and rectum are developed due to west countries widespread of familial adenomatous polyposis. The number of polyps can range from no detectable polyps at colonoscopy to more than 7,000 seen on resected specimens of bowel. The polyposis predominantly affects the left colon (80-90%) (Debinski, Love et al. 1996). A variety of polyposis syndromes can affect the GI tract. These polyposis syndromes may be classified as familial inherited (autosomal dominant) or nonfamilial. The inherited polyposis syndromes can be further subdivided into 2 groups depending on whether the polyps are adenomas or hamartomas. The adenomatous polyposis syndromes include the classic familial adenomatous polyposis (FAP) (Photographs 1.2.1, 1.2.2) Gardner syndrome, and Turcot syndrome. Hamartomatous familial polyposis syndromes include Peutz-Jeghers syndrome, juvenile polyposis syndrome, Cowden disease, and Ruval-Caba-Myhre-Smith syndrome (Chan and Haghighi 2006; Aust and Baretton 2010).

. 

Photograph 1.2.1.Colon, polyposis syndromes. Polyposis coli. Left lateral decubitus image obtained as part of a barium enema study shows numerous small polyps in the transverse and descending colon. *emedicine.medscape.com*



Photograph 1.2.2.Colon, polyposis syndromes. Polyposis coli. Double-contrast enema study in a man with a family history of familial colonic polyposis shows a solitary polyp with malignant change.

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1.2.1. Distribution of polyposis coli

FAP is inherited in an autosomal dominant manner with 80% penetrance, but sporadic occurrence has been recorded in one third of patients. The gene for FAP is located on chromosome 5. The prevalence is quoted as 1 in 7,000-24,000 live births. From fly to man, APC is strongly expressed in the developing and adult nervous system (Koester, Muller et al. 2007).

Gardner syndrome probably has the same genotype as FAP coli. It is transmitted as an autosomal dominant trait with complete penetrance and variable expressivity. The incidence of familial polyposis of the colon is between 1 in 8,300 and 1 in 14,025 live births (Nandakumar, Morgan et al. 2004). The estimated prevalence is 1 in 14,000 populations (Nannery, Barone et al. 1990).

Peutz-Jeghers syndrome has a prevalence of 1 in 7,000 live births with a half familial and half sporadic distribution. Cronkhite-Canada syndrome has no hereditary factors; 100 cases had been published as of 1994. Turcot syndrome is inherited in an autosomal recessive manner. Approximately 100 cases of Cowden's syndrome have been reported in the literature. Ruval-Caba-Myhre-Smith syndrome is rare, and the exact incidence is not known (Galiatsatos and Foulkes 2006). There is no racial or sex predominance predilection for colonic polyps and also it is reported in FAP coli, Gardner syndrome, or Peutz-Jeghers syndrome. However, the papillary thyroid carcinoma associated with Gardner syndrome has a female preponderance (Kupfer, McCaffrey et al. 2006).

1.2.2. Pathophysiology

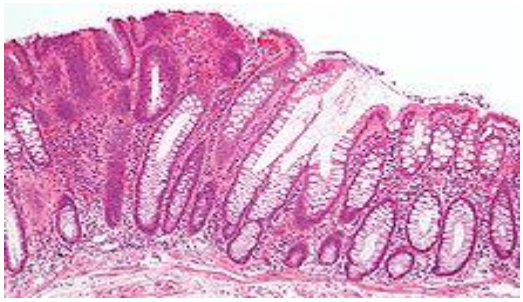
Familial adenomatous polyposis (FAP) is the most common adenomatous polyposis syndrome. It is an autosomal dominant inherited disorder characterized by the early onset of hundreds to thousands of adenomatous polyps throughout the colon. If left untreated, all patients with this syndrome develop colon cancer by age 35-40 years. In addition, an increased risk exists for the development of other malignancies (Lefevre J.H.Colas 2010). MYH associated polyposis is a hereditary syndrome, autosomal recessive responsible for early colorectal cancer

with a distinct genetic pathway from the Familial Adenomatous Polyposis or the Hereditary Non Polyposis Colorectal Cancer syndrome (Lefevre J.H.Colas 2010). The genetic defect in FAP is a germline mutation in the adenomatous polyposis coli (*APC*) gene. Syndromes once thought to be distinct from FAP are now recognized to be, in reality, part of the phenotypic spectrum of FAP (Castellsague, Gonzalez et al. 2010).

Syndromes with a germline mutation in the *APC* gene include FAP, Gardner syndrome, some families with Turcot syndrome, and attenuated adenomatous polyposis coli (AAPC) (Edlich, Cross et al. 2009). Gardner syndrome is characterized by colonic polyposis typical of FAP, along with osteomas (bony growth most commonly on the skull and the mandible), dental abnormalities, and soft tissue tumours. Turcot syndrome is characterized by colonic polyposis typical of FAP, along with central nervous system tumours (medulloblastoma). The APC is characterized by fewer colonic polyps (average number of polyps, 30-35) as compared to classic FAP. The polyps also tend to develop at a later age (average age, 36 y), and they tend to involve the proximal colonic area (Edlich, Cross et al. 2009).

1.2.3. Anatomy and clinical presentation

The groups of symptoms that are shared by all polyposis syndromes include vague abdominal pain, rectal bleeding and mild diarrhea, passage of mucus, intussusception, rectal prolapse, and bowel obstruction. GI symptoms of rectal bleeding and abdominal pain usually develop in the third decade in FAP (Cappell 2007).



Photograph 1.2.3. 4,272 × 2,848 pixels, file size: 4.47 MB, MIME type: image/jpeg. Micrograph of a tubular adenoma (left of image), a type of colonic polyp and a precursor of colorectal cancer. Normal colorectal mucosa is seen on the right. H&E stain. *emedicine.medscape.com*

The process of developing colon cancer from adenomas polyp is a multistep, relating to mutations of the DCC, k-ras, and p53 genes; loss of heterozygosity in which cells loose one allele of some genes from chromosomal loss; and DNA methylation which can silence DNA expression. While colon cancer in an advanced and incurable stage often produces clinical findings, premalignant adenomatous polyps (Photograph 1.2.3) and early, highly curable, colon cancer are often asymptomatic (Cappell 2007).

1.2.4. Treatment

The colorectal lesions in early stage prone to cancer can be detected and removed by colonoscopic screening test. The strong controversy is to decide when endoscopic treatment is enough or when surgical resection is necessary (Ruiz-Tovar, Jimenez-Miramon et al. 2010). In addition, metformin suppresses colonic epithelial proliferation via the inhibition of the mTOR (mammalian target of rapamycin) pathway through the activation of AMPK (adenosine monophosphate-activated protein kinase). As metformin is already used daily as

an antidiabetic drug, it might be a safe and promising candidate for the chemoprevention of colorectal cancer (Hosono, Endo et al. 2010).

1.3. Colorectal cancer

Colorectal cancer, also called colon cancer large intestine cancer or large bowel cancer, includes cancerous growths in the colon, in the last six inches of the colon (the rectum) and appendix it is the fourth most common form of cancer in the United States and the third leading cause of cancer-related death in the Western world (WHO 2006). The large bowel cancer (also known as colorectal cancer) is a common form of malignancy in developed countries and Colon and rectal cancer incidence was negligible before 1900. The incidence of colorectal cancer has been rising dramatically following economic development and industrialization but occurs much less frequently in the developing world. Around 106 new cases of colorectal cancer are diagnosed each day in the UK and it is the third most common cancer after breast and lung. In 2007 there were 38,608 new cases of large bowel cancer registered in the UK: around two-thirds (24,274) in the colon and one-third (14,334) in the rectum (Registry 2010; Statistics 2010) (Figure 1.3.1).

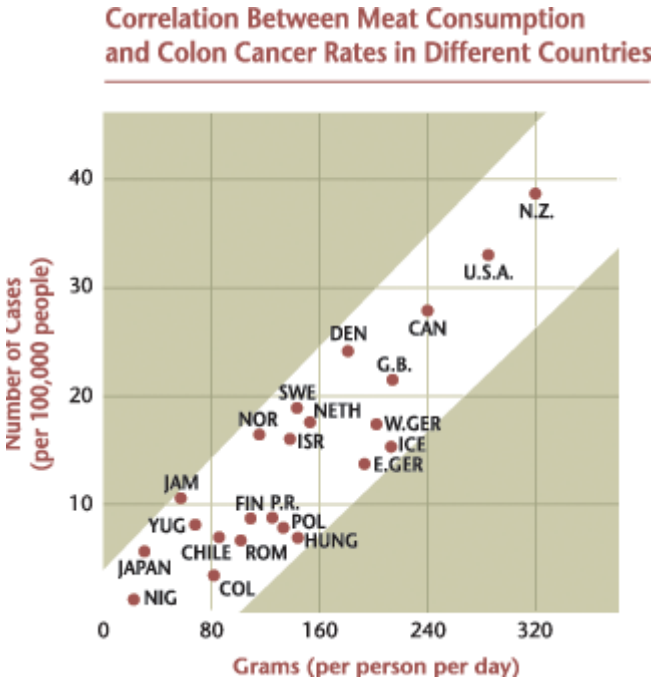


Figure1.3.1. Correlation between meat consumption and colon cancer rates in different countries.

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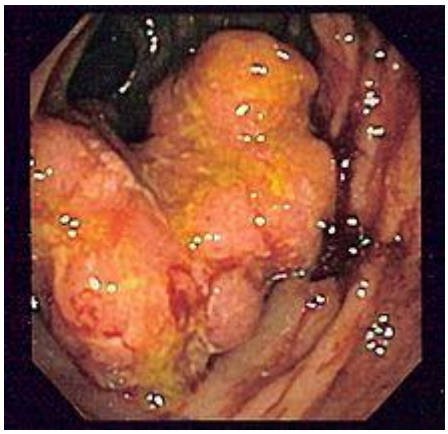
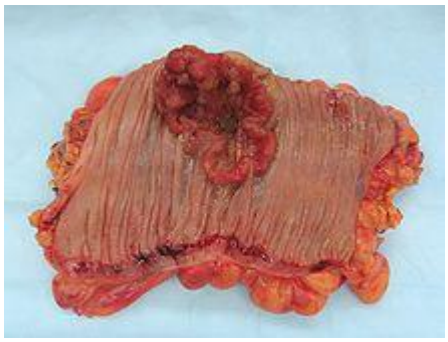


Photograph 1.3.1. Colorectal cancer endoscopy: 473 × 456 pixels, file size: 141 KB, MIME type: image/jpeg. *en.wikipedia.org/wiki/Colorectal_cancer*



Photograph 1.3.2.Gross appearance of a colectomy specimen containing one invasive colorectal carcinoma (the crater-like, reddish, irregularly shaped tumour). *en.wikipedia.org/wiki/Colorectal_cancer*

Colorectal cancers arise from adenomatous polyps in the colon. These mushroom-shaped growths are usually benign, but some develop into cancer over time. Localized colon cancer is usually diagnosed through colonoscopy (Photograph 1.3.1). Invasive cancers that are confined within the wall of the colon (TNM stages I and II) are curable with surgery. If untreated, they spread to regional lymph nodes (stage III), where up to 73% are curable by surgery and chemotherapy (Photograph 1.3.2). Cancer that metastasizes to distant sites (stage IV) is usually not curable, although chemotherapy can extend survival, and in rare cases, surgery and chemotherapy together have seen patients through to a cure (Markowitz and Bertagnolli 2009). Radiation is used with rectal cancer. At the cellular and molecular level, colorectal cancer starts with a mutation to the Wnt signaling pathway. Therefore, the Wnt binds to a receptor on the cell that sets in motion a chain of molecular events that ends with β-catenin moving into the nucleus and activating a gene on DNA. In colorectal cancer, genes along this chain are damaged. Usually, a gene called APC, which is a "brake" on the Wnt pathway, is damaged. Without a working APC brake, the Wnt pathway is stuck in the "on" position (Markowitz and Bertagnolli 2009).

1.3.1. Signs and symptoms

Unfortunately, most people with colorectal cancer do not experience any symptoms in the early stage of the disease. That's why screening tests, such as a colonoscopy, are so important (Le Marchand, Wilkens et al. 1999).The symptoms of colorectal cancer depend on the location of the tumour in the bowel, and whether it has spread elsewhere in the body. Most of the symptoms may occur in other diseases as well, and hence none of the symptoms mentioned here is diagnostic of colorectal cancer. Changes in bowel habits for example, blood in the stool (can have other causes, too), problems related to blood loss (anemia, weakness, fatigue, shortness of breath, pounding or racing heart, chest pain, and intolerance to exercise), abdominal discomfort (frequent gas, bloating, fullness, cramps, and pain), unexplained weight loss, pain with bowel movement and feeling that the bowel doesn't empty completely (Fauci , 2008).

There are two different types of signs and symptoms local and constitutional (Carrion, Marin et al. 2010).

1.3.1. 1. Local

Local symptoms are more likely if the tumour is located closer to the anus. There may be a change in bowel habit (new-onset constipation or diarrhea in the absence of another cause), and a feeling of incomplete defecation (rectal tenesmus) and reduction in diameter of stool; tenesmus and change in stool shape are both characteristic of rectal cancer. Lower gastrointestinal bleeding,

including the passage of bright red blood in the stool, may indicate colorectal cancer, as may the increased presence of mucus. Melena, black stool with a tarry appearance, normally occurs in upper gastrointestinal bleeding (such as from a duodenal ulcer), but is sometimes encountered in colorectal cancer when the disease is located at the beginning of the large bowel.

A tumour that is large enough to fill the entire lumen of the bowel may cause bowel obstruction. This situation is characterized by constipation, abdominal pain, and abdominal distension and vomiting. This occasionally leads to the obstructed and distended bowel perforating and causing peritonitis.

Certain local effects of colorectal cancer occur when the disease has become more advanced. A large tumor is more likely to be noticed on feeling the abdomen, and it may be noticed by a doctor on physical examination. The disease may invade other organs, and may cause blood or air in the urine (invasion of the bladder) or vaginal discharge (invasion of the female reproductive tract).

1.3.1.2. Constitutional

If a tumour has caused chronic occult bleeding, iron deficiency anemia may occur; this may be experienced as fatigue, palpitations and noticed as pallor (pale appearance of the skin). Colorectal cancer may also lead to weight loss, generally due to a decreased appetite.

More unusual constitutional symptoms are an unexplained fever and one of several paraneoplastic syndromes. The most common paraneoplastic syndrome is thrombosis, usually deep vein thrombosis.

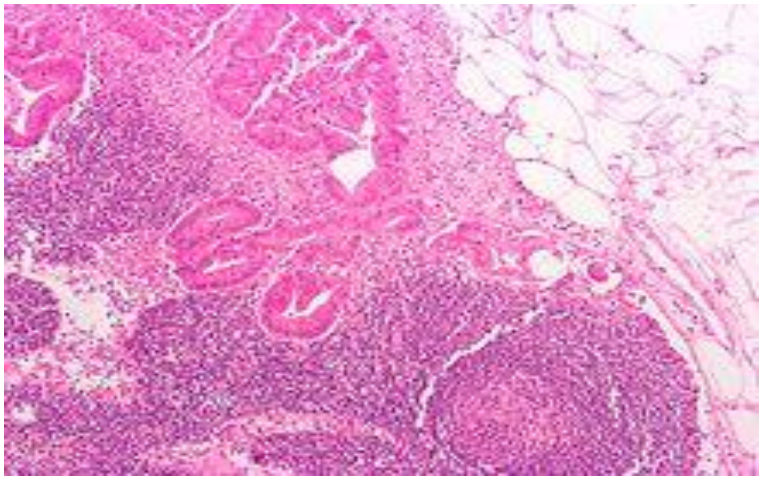
1.3.1.3. Metastatic

Colorectal cancer most commonly spreads to the liver. This may go unnoticed, but large deposits in the liver may cause jaundice and abdominal pain (due to stretching of the capsule). If the tumour deposit obstructs the bile duct, the jaundice may be accompanied by other features of biliary obstruction, such as pale stools (Partelli, Mukherjee et al. 2010).

1.3.2. Risk factors

Risk factors for colorectal cancer include, age (being over 50) (Prevention, 2010) having colorectal cancer previously and a history of adenomatous polyps or family history of colorectal cancer (Nieuwenhuis, Douma et al. 2010; Slattery, Herrick et al. 2010), eating a high-fat diet, smoking, being overweight (Wernli, Newcomb et al. 2010), heavy use of alcohol, inflammatory bowel disease, diabetes (Pais, Silaghi et al. 2009; Campbell, Deka et al. 2010). The exposure to some viruses (such as particular strains of human papilloma virus) may be associated with colorectal cancer (Bean, Chhieng et al. 2010). The style of infection and following that transformation resembles very much the pathogenesis of cervical and other HPV-associated cancers (Bean, Chhieng et al. 2010; von Knebel Doeberitz and Reuschenbach 2010). Recent trends in some of the western countries suggest a disproportionally higher incidence and death from colon cancer in African Americans (AAs) than in whites. Hispanic persons have the lowest incidence and mortality from colorectal cancer and an increased

prevalence of polyps has also been noted in men regardless of race (Penn, Garrow et al. 2010).



Photograph 1.3.3. Micrograph of a colorectal adenocarcinoma metastasis to a lymph node. The cancerous cells are at the top center-left of the image, in glands (circular/ovoid structures) and eosinophilic (bright pink). H&E stain. *en.wikipedia.org/wiki/Colorectal_cancer*

1.3.3. Diagnosis

The doctor will take a complete medical history, and will perform a physical examination to order one or more tests to diagnose colorectal cancer. Standard tests used to diagnose colorectal cancer include sigmoidoscopy, colonoscopy, and barium enema (Taouqi, Ingrand et al. 2010).

During a sigmoidoscopy or a colonoscopy, a biopsy (sample of tissue) is removed from the colon or rectum and examined under a microscope in order to detect abnormal growths (Photograph 1.3.3)

Stages of colorectal cancer include:

Stage 0: The earliest stage; cancer is found only in the innermost lining of the colon and/or rectum.

Stage 1: Cancer has grown through the innermost lining but hasn't spread beyond the colon wall or rectum.

Stage 2: Cancer has spread to deeper layers of the wall of the colon or rectum, but not the lymph nodes.

Stage 3: Cancer has spread to nearby lymph nodes but not to other parts of the body.

Stage 4: Cancer has spread to other parts of the body, such as the liver and lungs (Fauci , 2008). Dukes system (Table 1.3.1)

AJCC stage	TNM stage	
Stage 0	Tis N0 M0	Tis: Tumour confined to mucosa; cancer-*in-situ*
Stage I	T1 N0 M0	T1: Tumour invades submucosa
Stage I	T2 N0 M0	T2: Tumour invades muscularis propria
Stage II-A	T3 N0 M0	T3: Tumour invades subserosa or beyond (without other organs involved)
Stage II-B	T4 N0 M0	T4: Tumour invades adjacent organs or perforates the visceral peritoneum
Stage III-A	T1-2 N1 M0	N1: Metastasis to 1 to 3 regional lymph nodes. T1 or T2.
Stage III-B	T3-4 N1 M0	N1: Metastasis to 1 to 3 regional lymph nodes. T3 or T4.
Stage III-C	any T, N2 M0	N2: Metastasis to 4 or more regional lymph nodes. Any T.
Stage IV	any T, any N, M1	M1: Distant metastases present. Any T, any N.

Table 1.3.1.TNM stage criteria for colorectal cancerAJCC *Cancer Staging Manual* (Sixth ed.). Springer-Verlag New York, Inc.. 2002

1.3.5. Preventive Care

Regular screening for colorectal cancer can prevent, even cure, when it is in a very early stage. Detecting polyps before they become cancerous allows the patients to survive with in situ colorectal cancer. Regular screening is operated at age 50 for people who have an average risk of for colorectal cancer (Lieberman, 2008). Eating plenty of fruits and vegetables as well as foods rich in omega-3 fatty acids (such as salmon and halibut) and calcium (such as sea vegetables and kale) can help reduce the risk of colorectal cancer. Limiting alcohol consumption, quitting smoking, and reducing the intake of high-fat and fried foods, particularly red meats, may also protect developing colorectal cancer (Barros, Xavier et al. 2010; Limsui, Vierkant et al. 2010).

1.3.6. Pathogenesis

The most common colon cancer cell type is adenocarcinoma which accounts for 95% of cases. Other, rarer types include lymphoma and squamous cell carcinoma. Genetically, colorectal cancer represents a complex disease, and genetic alterations are often associated with progression from premalignant lesion

(adenoma) to invasive adenocarcinoma. Sequence of molecular and genetic events leading to transformation from adenomatous polyps to overt malignancy has been characterized in other studies (Goel and Boland 2010). The early event is a mutation of *APC* (adenomatous polyposis gene), which was first discovered in individuals with familial adenomatous polyposis (FAP). The protein encoded by *APC* is important in activation of oncogene c-*myc* and cyclin D1, which drives the progression to malignant phenotype. Although FAP is a rare hereditary syndrome accounting for only about 1% of cases of colon cancer, *APC* mutations are very frequent in sporadic colorectal cancers (Velmurugan, Gangar et al. 2010). Genome-wide association studies have recently linked CRC to 10 common genetic variants or single-nucleotide polymorphisms that map to chromosomes 8q23, 8q24, 10p14, 11q23, 14q22, 15q13, 16q22, 18q21, 19q13 and 20p1. Recent studies indicate that the single-nucleotide polymorphism rs6983267, which maps to 8q24, serves as an enhancer of MYC expression by binding T cell factor 4 (TCF4) and influencing Wnt signalling (Goel, 2010).

Other important genes in colon carcinogenesis include *KRAS* oncogene, chromosome 18 loss of heterozygosity (LOH) leading to inactivation of *SMAD4* (*DPC4),* and *DCC* (deleted in colon cancer) tumour suppression genes (Koyama, Ito et al. 1999). Chromosome arm 17p deletion and mutations affecting *p53* tumour suppressor gene confer resistance to programmed cell death (apoptosis) and are thought to be late events in colorectal cancer (Risio, Casorzo et al. 2003). This phenotype has been linked to mutations of genes such as *MSH2*, *MLH1*, and *PMS2*. These mutations result in so-called high frequency microsatellite instability (H-MSI), which can be detected with an immunocytochemistry assay. H-MSI is a hallmark of hereditary nonpolyposis colon cancer syndrome

(HNPCC, Lynch syndrome), which accounts for about 6% of all colon cancers. H-MSI is also found in about 20% of sporadic colon cancers (Hall, Clarkson et al. 2010; Sjursen, Haukanes et al. 2010).

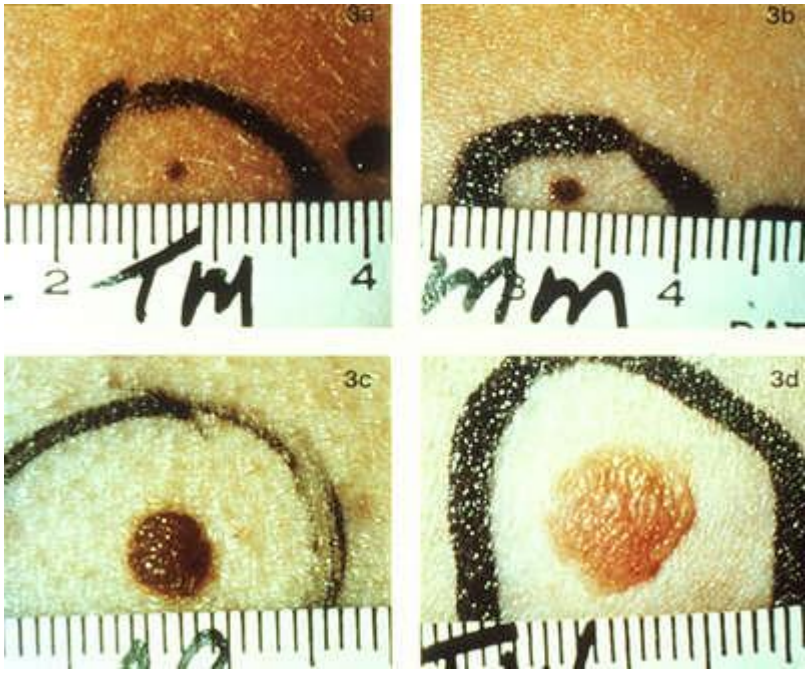
1.3.7. Treatment

Surgery to remove the part of the colon containing tumour is the primary treatment. Depending on the stage of the cancer, surgery is generally followed with chemotherapy (Jenab-Wolcott and Giantonio 2010). If the tumour is particularly large, radiation may be needed before or after surgery. An unhealthy lifestyle can increase the risk of developing the colorectal cancer by as much as 70% for some people (Young and Le Leu 2002). Many studies support that doing exercise by preventing colon tumorigenesis, at least partly via the suppression of iNOS expression associated with anti-inflammation (Aoi, Naito et al. 2010). Antioxidant foods, should be eaten including fruits (such as blueberries, cherries, and tomatoes), and vegetables (such as squash and bell peppers) and all high doses of hydrolyzed soybean treatments, except the green genotype significantly reduce colorectal cancer cell numbers compared to control reduction (Slavin, Kenworthy et al. 2009). 5-Fluorouracil remains the backbone of chemotherapy regimens for colon cancer, both in the adjuvant and metastatic setting (Boni, Bitarte et al. 2010). In addition to 5-fluorouracil, oral fluoropyrimidines such as capecitabine (Xeloda) and tegafur are increasingly used as monotherapy or in combination with oxaliplatin (Eloxatin) and irinotecan (Camptosar) and Leucovorin (LV, folic acid). Some of the standard combination regimens employ

prolonged continuous infusion of fluorouracil (FOLFIRI, FOLFOX) or capecitabine (CAPOX, XELOX, XELIRI) (Tournigand, Andre et al. 2004).

1.3. Melanocytic nevus, suspected melanoma

A nevus is a benign (non-cancerous) melanocytic tumour, more commonly called a mole. The American Academy of Dermatology announced that the majority of moles occure during the first two decades of a person's life, while about one in every 100 babies is born with moles (AAD. 2008). Nevi are not usually present at birth but begin to appear in children and teenagers. Most moles will never cause any problems, but a person who has more than 50 normal moles (or more than 5 atypical or "dysplastic" moles) has a higher risk of developing melanoma, the most aggressive form of skin cancer (Photograph 1.4.1). The number of melanocytic nevi is the strongest risk factor for cutaneous melanoma. As pigmented skin lesions are visible to everybody (Buettner and Garbe 2000) and cautiously detecting the suspicious lesions can expand the microscopic pathological data in the determination of prognosis also, these skin lesions, suspected to be melanoma should be photographed. The photograph and pathological report if it is followed up after surgical removal of the lesion could be a strong clue in the patient's permanent medical record (Hosono, Endo et al. 2010).



Photograph 1. 4.1. A normal mole, *Photo © National Cancer Institute*

1.4.1. Sign and symptoms

1.4.1.1. Location

There are different types of nevi based on the location of the melanocyte cells. The junctional nevus is which the nevus cells are located along the junction of the epithelium and the underlying dermis. A junctional nevus is flat and brown to black.

The other type is a compound nevus which is a mixture of junctional and intradermal proliferation. Compound nevi are slightly raised and brown to black. Beauty marks are usually compound nevi of either the acquired variety or congenital variety.

The last one is the intradermal nevus with cells located in the dermis only. Intradermal nevi are raised; most are flesh-colored (not pigmented) (Gutierrez, Barengo et al. 2009).

1.4.1.2. Dysplastic nevus

Dysplastic naevi (DN) are a known risk factor for malignant melanoma which is categorised to suspected melanoma. Their occurrence is closely connected with the degree of skin pigmentation though people with a light complexion are more likely to develop DN than dark-skinned individuals. DN cells contain higher concentrations of radical oxygen species than normal skin melanocytes from the same individuals also the increase of phaeomelanogenesis in DN cells is connected with oxidative imbalance, which is reflected by increased intracellular concentrations of reactive oxygen species and raised calcium and iron concentrations (Pavel, 2004).

The compound nevus with cellular and architectural dysplasia is a dysplastic nevus. Like typical moles, dysplastic nevi can be flat or raised. While they vary in size, dysplastic nevi are typically larger than normal moles and tend to have irregular borders and irregular coloration (Shrestha, Bishop et al. 2010). Hence, they resemble melanoma, appear worrisome, and are often removed to clarify the diagnosis. Dysplastic nevi are markers of risk when they are numerous (atypical mole syndrome). According to the National Cancer Institute (NIH), doctors believe that dysplastic nevi are more likely than ordinary moles to develop into the most virulent type of skin cancer called melanoma.

Oncogenic BRAF as an early and fundamental feature of melanocytic neoplasia has been confirmed with its identification in both melanoma and nevi. Oncogenic BRAF induces senescence/apoptosis by up-regulating the tumour suppressor IGFBP7, which acts through autocrine/paracrine pathways to inhibit BRAF-MEK-ERK signalling (Decarlo, Yang et al. 2010).

1.4.2.3. Blue nevus

It is blue in color as its melanocytes are very deep in the skin. The nevus cells are spindle shaped and scattered in deep layers of the dermis. The covering epidermis is normal (Lee, Na et al. 2010).

1.4.2.4. Spitz nevus

The distinct variant of intradermal nevus is found usually in children. They are raised and reddish (non-pigmented). A pigmented variant, called the nevus of Reed, typically appears on the leg of young women (Blum 2010; LeAnder, Chindam et al. 2010).

1.4.2.5. Acquired nevus

Acquired nevus is any melanocytic nevus that is not a congenital nevus or not present at birth or near birth. This includes junctional, compound and intradermal nevus. Damaged keratinocytes and inflammatory cells can release growth factors inducing nevus cell proliferation, and immunosuppression could end cellular surveillance keeping pre-existing nevus cell nests in check (Navarini, Kolm et al. 2010) (Photograph1.4.1).

1.4.2.6. Congenital nevus

Small to large nevus present at or near the time of birth. Small ones have low potential for forming melanomas; however the risk increases with size, as in the giant pigmented nevus. Congenital melanocytic nevi (CMNs) are diagnosed in 1% of newborn infants (Turkmen, Isik et al. 2010).

1.4.2.7. Giant pigmented nevus

These large, pigmented, often hairy congenital nevi are rare, with an estimated incidence of approximately 1 in 20,000 live births. They are important because melanoma may occasionally (10 to 15%) appear in them (Chien, Niu et al. 2010) (Photograph 1.4.1).

1.4.2.8. Intramucosal nevus

It is a Junctional nevus of the mucosa of the mouth or genital areas. In the mouth, they are found most frequently on the hard palate with pseudoepitheliomatous hyperplasia of the gingival (Suzuki, Kumamoto et al. 2002).

The other types of nevus are nevus of Ito and Nevus of Ota, Mongolian spot and recurrent nevus .

1.4.3. Genetics

Dysplastic nevi and atypical mole syndrome are hereditary conditions which causes a person to have a large quantity of moles (often 100 or more) with some

larger than normal or atypical. This often leads to a higher risk of melanoma, a serious skin cancer (Burkhart 2003). Developing a cancer more frequently happens in dysplastic nevi than ordinary moles. Dysplastic nevi are common and having more than 50 ordinary moles increases the risk of developing melanoma (Institute 2008). The Single nucleotide polymorphisms (SNP) on chromosomes 9 and 22 are associated with increased numbers of nevi. The importance of nevi in melanoma is the pathogenesis and increases understanding of their genetic determinants (Newton-Bishop, Chang et al. 2010).

1.4.4. Sunlight

The UV exposure from sun causes premature aging and skin damage that can lead to melanoma. The excessive exposure to sunlight, may play a role in the formation of acquired moles (van Schanke, van Venrooij et al. 2006).

1.5. Malignant Melanoma

Malignant melanoma is the most lethal form of skin cancer, which has become an important public health issue because in the U.K. over 8,000 cases are currently diagnosed each year and the numbers are steadily rising. Worldwide, the number of new cases of melanoma is increasing faster than for any other cancer type and mortality due to melanoma is also rising (Vasquez-Moctezuma, 2010). Such cells are found predominantly in skin, but are also found in the bowel and the eye (see uveal melanoma). Melanoma is one of the less common types of skin cancer, but causes the majority (75%) of skin cancer related deaths. These figures make melanoma an important cancer for study (Physician 2000). The majority of melanomas arise sporadically but in about 5% of cases there is a history of melanoma in two or more close relatives (Psaty, Scope et al. 2010).

1.5.1. Classification

1.5.1.1. Lentigo maligna

Lentigo maligna is known as lentiginous melanoma on sun-damaged skin. It is a melanoma *in situ*. It consists of malignant cells but does not show invasive growth (Erickson and Miller 2010). It is normally found in the elderly (peak

incidence in the 9th decade), on skin areas with high levels of sun exposure like the face and forearms. Some authors do not consider lentigo maligna to be a melanoma. The incidence of evolution to lentigo maligna melanoma is very low, about 2.2% to 5% in elderly patients (Erickson and Miller 2010).

1.5.1.2. Superficial spreading melanoma

Superficial spreading melanoma is usually characterized as the most common form of cutaneous melanoma in Caucasians. The average age at diagnosis is in the fifth decade, and it tends to occur on sun-exposed skin, especially on the backs of males and lower limbs of females (Amerio, Manzoli et al. 2009). The other types of MM are acral lentiginous melanoma, mucosal melanoma, nodular melanoma, polypoid melanoma , desmoplastic melanoma, amelanotic melanoma and soft-tissue melanoma.

1.5.2. Signs and symptoms

Early signs of melanoma are changes to the shape or color of existing moles. At later stages, the mole may itch, ulcerate or bleed. Early signs of melanoma are summarized as, asymmetry of the borders (irregular), color (variegated), and diameter (greater than 6 mm (0.24 in) (picture 1.5.5), about the size of a pencil eraser). Metastatic melanoma may cause non-specific paraneoplastic symptoms including loss of appetite, nausea, vomiting and fatigue. Metastasis of early melanoma is possible. However, fortunately less than a fifth of melanomas diagnosed early become metastatic (de la Fuente-Garcia and Ocampo-Candiani 2010).

1.5.3. Aetiology

There is the possibility of involving a multistep process of progressive genetic mutations that could be the alter cell proliferation, differentiation, and death and impact susceptibility to the carcinogenic effects of ultraviolet radiation (Demierre and Nathanson 2003). Recent data suggest multiple pathways of melanoma pathogenesis, with melanomas in sun-protected skin (trunk) developing in association with a high nevus count and intermittent ultraviolet radiation as opposed to those developing on sun-exposed skin in patients with low nevus counts and chronic sun exposure (Maldonado, Fridlyand et al. 2003; Whiteman, Watt et al. 2003). Primary cutaneous melanoma may develop in precursor melanocytic nevi (i.e., common, congenital, and atypical/dysplastic types), although more than 60% of cases are believed to arise de novo (i.e., not from a pre-existing pigmented lesion). Although the melanoma's development is multifactorial and related to multiple risk factors, for example fair complexion, excessive childhood sun exposure and blistering childhood sunburns, an increased number of common and dysplastic moles, a family history of melanoma, the presence of a changing mole or evolving lesion on the skin, and, importantly, older age (Califano and Nance 2009).

1.5.3.1. Genetics

Familial melanoma is genetically heterogeneous and loci for familial melanoma have been identified on the chromosome arms 1p, 9p and 12q. Multiple genetic

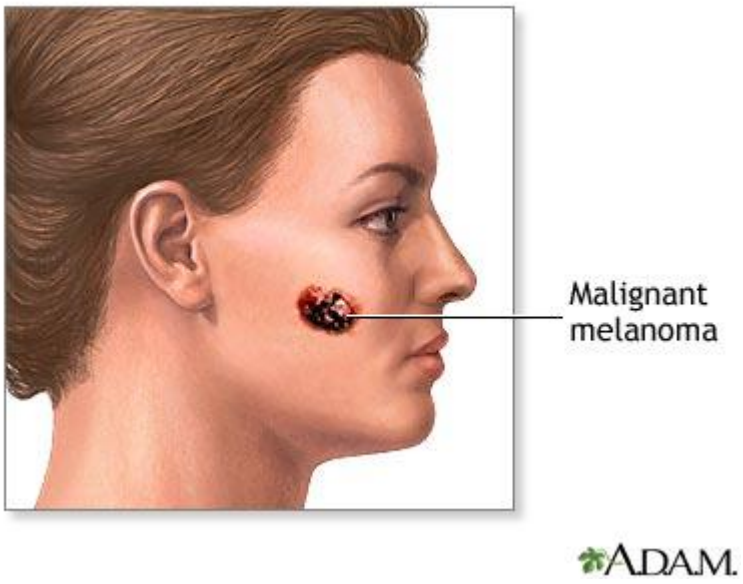
events have been related to the pathogenesis of melanoma (Yang, Liang et al. 2010). The multiple tumour suppressor 1 (CDKN2A/MTS1) gene encodes p16INK4a - a low-molecular weight protein inhibitor of cyclin-dependent protein kinases (CDKs) - which has been localized to the p21 region of human chromosome 9. Today, melanomas are diagnosed only after they become visible on the skin. In the future, however, physicians will hopefully be able detect melanomas based on a patient's genotype, not just his or her phenotype (Ibarrola-Villava, 2010). Recent genetic advances promise to help doctors to identify people with high-risk genotypes and to determine which of a person's lesions have the greatest chance of becoming cancerous. A number of rare mutations, which often run in families, are known to greatly increase one's susceptibility to melanoma. One class of mutations affects the gene CDKN2A (Ibarrola-Villava, 2010). An alternative reading frame mutation in this gene leads to the destabilization of p53, a transcription factor involved in apoptosis and in fifty percent of human cancers. Another mutation in the same gene results in a non-functional inhibitor of CDK4, a [cyclin-dependent kinase] that promotes cell division. Mutations that cause the skin condition Xeroderma Pigmentosum (XP) also seriously predispose one to melanoma. Scattered throughout the genome, these mutations reduce a cell's ability to repair DNA. Both CDKN2A and XP mutations are highly penetrant. Other mutations confer lower risk but are more prevalent in the population. People with mutations in the MC1R gene, for example, are two to four times more likely to develop melanoma than those with two wild-type copies of the gene. MC1R mutations are very common; in fact, all people with red hair have a mutated copy of the gene. Two-gene models of melanoma risk have already been created, and in the future, researchers hope to

create genome-scale models that will allow them to predict a patient's risk of developing melanoma based on his or her genotype (Ibarrola-Villava, Fernandez et al. 2010). In addition to identifying high-risk patients, researchers also want to identify high-risk lesions within a given patient. Many new technologies, such as optical coherence tomography (OCT), are being developed to accomplish this. OCT allows pathologists to view 3-D reconstructions of the skin and offers more resolution than past techniques could provide. In vivo confocal microscopy and fluorescently tagged antibodies are also proving to be valuable diagnostic tools. Mutation of the MDM2 SNP309 gene is associated with increased risk of melanoma in younger women (Capasso, Ayala et al. 2010) (Photograph 1.5.1).

1.5.3.2. UV exposure and malignant melanoma

The incidence of skin cancer has increased due to the increase in the amount of ultraviolet-B (UV-B) exposure as a result of ozone reduction across the world (Chang, Feng et al. 2010). The S100B-p53 protein complex was discovered in C8146A malignant melanoma (MM), therefore reducing S100B expression with siRNA and it was sufficient to activate p53 (Lin, Yang et al. 2010). Xeroderma pigmentosum (XP) patients have a 1,000-fold higher incidence of melanoma due to sunlight-induced "bulky" photoproducts to make melanomagenesis (Wang, Choi et al. 2010). Sunlight induces a high level of reactive oxygen species in melanocytes (MCs) then oxidative DNA damage (ODD) may also contribute to melanomagenesis, and XP gene products may participate in the repair of ODD. The melanin sensitizes UVA in the induction of ODD but not bulky UV photoproducts and the high susceptibility to UVA-induced ODD and the reduced DNA repair capacity in MCs contribute to carcinogenesis also the reduced repair

capacity for ODD contributes to the high melanoma incidence in XP patients (Wang, 2010). The skin aging process, implying oxidative stress, is associated with specific gene expression. Ultraviolet A (UVA) and hydrogen peroxide H_2O_2 both generate reactive oxygen species (ROS) making them relevant in the study of skin cell responses to oxidative stresses (Hazane-Puch, 2010). Melanocytes are producing melanin after UV irradiation as a defence mechanism. However, UV-induced damage is involved in melanoma initiation, depending on skin photo type. Melanocytes seem to be extremely susceptible to free radicals. Their main enzymatic antioxidants are superoxide dismutase and catalase (Baldea, 2009).



Photograph 1.5.1. A malignant melanoma lesion

1.5.4. Diagnosis

To detect melanomas it is important to check for changes (shape, size, color, itching or bleeding) in the moles and to show any suspicious moles to a doctor with an interest and skills in skin malignancy (Friedman, 1985 ; Torrens, 2009).

A popular method for remembering the signs and symptoms of melanoma is the mnemonic "ABCDE":

- Asymmetrical skin lesion.
- Border of the lesion is irregular.
- Color: melanomas usually have multiple colors.
- Diameter: moles greater than 6 mm are more likely to be melanomas than smaller moles.
- Enlarging: enlarging or evolving (Photograph 1.5.7).

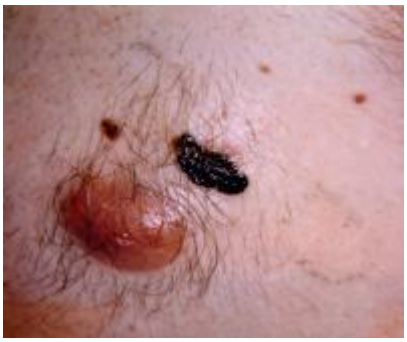
Seborrhea keratosis also in the ABCD criteria can lead to false positive signs sometimes even for physicians. An experienced doctor can generally distinguish seborrhea keratosis from melanoma upon examination, or with dermatoscopy (Califano, 2009 ; Torrens, 2009).

There is another E which stands for elevation. Elevation can help identify a melanoma, but lack of elevation does not mean that the lesion is not a melanoma. Most melanomas are detected in the very early stage, or in the in-situ stage, before they become elevated. By the time elevation is visible, they may have

progressed to the more dangerous invasive stage (Torrens, 2009; Muller, 2009). The other well-known sign novel method of melanoma detection is the "Ugly Duckling Sign" It is simple, easy to teach, and highly effective in detecting melanoma. Simply, correlation of common characteristics of a person's skin lesion is made. Lesions which greatly deviate from the common characteristics are labelled as an "Ugly Duckling", and further professional examination is required (Grob and Bonerandi 1998). However, molecular analysis has demonstrated different patterns of cell death, oncogene expression, gene amplification, and *BRAF* mutation frequency among the 4 main histogenetic types (Miracco, Santopietro et al. 1998; Bastian, Kashani-Sabet et al. 2000; Sasaki, Niu et al. 2004).

1.5.4.1. Superficial spreading melanoma

Superficial spreading melanoma (SSP) is characterized as the most common on the trunk in men and women and on the legs in women; this subtype is most commonly seen in individuals aged 30-50 years (photograph 1.5.2).



Photograph 1.5.2.Superficial spreading melanoma, left breast, 1.3-mm Breslow depth.

In addition, SSP manifests as a flat or slightly elevated brown lesion with variegate pigmentation (i.e., black, blue, pink, or white discoloration) (Demierre, Chung et al. 2005). It is generally greater than 6 mm in diameter, however, there are a lot of moles as lesions smaller than 6 mm in diameter with melanoma (Abbasi, Shaw et al. 2004). Histologically, it is characterized by buckshot (pagetoid) scatter of atypical melanocytes within the epidermis (Richard, 1999).

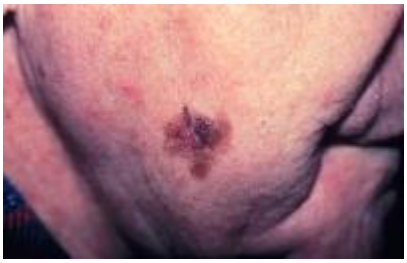
1.5.4.2. Nodular melanoma

Nodular melanoma is the subtype found in 15-30% of patients and it is seen most commonly on the legs and trunk. Additionally, it occurs with rapid growth over weeks to months and this subtype is responsible for most thick melanomas (Demierre, Chung et al. 2005). The colour of the lesions is dark brown-to-black papule or dome-shaped nodule, which may ulcerate and bleed with minor trauma; it may be clinically amelanotic (i.e., not pigmented) (Torrens and Swan 2009).

It tends to lack the typical ABCDE melanoma warning signs and, thus, may elude early detection. More commonly, it exhibits elevation, ulceration with bleeding, or both at presentation and also in histology (Photograph 1.5.8); it lacks a radial growth phase.

1.5.4.3. Lentigo maligna melanoma

One of the lentigo maligna melanoma characteristics is the incidence of lentigo maligna subtypes (*in situ* and invasive) which appears to be rising in the United States (Swetter, Boldrick et al. 2005). It is typically located on the head, neck, and arms (chronically sun-damaged skin) of fair-skinned older individuals (average age 65 y). It grows slowly over 5-20 years (Photograph 1.5.3).



Photograph 1.5.3.Lentigo maligna melanoma, right lower cheek. Centrally located erythematous papule represents invasive melanoma with surrounding macular Lentigo maligna (melanoma in situ)

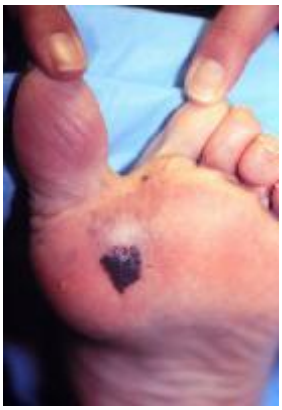
The *in situ* precursor lesion is usually large (>1-3 cm in diameter), present for a minimum of 10-15 years, and demonstrates macular pigmentation ranging from dark brown to black, although hypo pigmented (white) areas are common within lentigo maligna. Dermal invasion (progression to lentigo maligna melanoma) is characterized by the development of raised blue-black nodules within the in situ lesion.

From a histological viewpoint, it is characterized by a predominantly junctional confluent proliferation of melanocytes and extension along adnexal structures. Solar elastosis is typically prominent (Swetter, Boldrick et al. 2005).

1.5.4.4. Acral lentiginous melanoma

This type of malignant melanoma is the least common subtype of melanoma (2-8% of melanoma cases in white persons) and it accounts for 29-72% of melanoma cases in dark-skinned individuals (i.e., African American, Asian, and Hispanic persons) and, because of delays in diagnosis, may be associated with a worse prognosis (Byrd, Wilson et al. 2004).

Acral lentiginous melanoma occurs on the palms, on the soles, or beneath the nail plate (Subungual variant) (Photograph 1.5.4).



Photograph 1.5.4. Acral lentiginous melanoma (1-mm Breslow depth), left sole. Diagnostic punch biopsy site is located superiorly

Another type of melanoma is subungual melanoma and may manifest as diffuse nail discoloration or a longitudinal pigmented band within the nail plate. It must be differentiated from a benign junctional melanocytic nevus of the nail bed, which has a similar appearance. Pigment spread to the proximal or lateral nail

folds is termed the Hutchinson sign, which is a hallmark for acral lentiginous melanoma.

Rare melanoma variants (<5% of melanomas) include (1) desmoplastic/neurotropic melanoma, (2) mucosal (lentiginous) melanoma, (Rogers and Gibson 1997) (3) malignant blue nevus, (4) melanoma arising in a giant congenital nevus, and (5) melanoma of soft parts (clear cell sarcoma).

1.5.5. Race

Melanoma is primarily a malignancy of white individuals. African American persons develop melanoma approximately one twentieth as frequently as white persons, and the prevalence in Hispanic persons is approximately one sixth of that in white persons. However, mortality rates are higher in African Americans and Hispanics, who are more likely to have acral melanoma and advanced disease at presentation (Cress and Holly 1997; Hu, Soza-Vento et al. 2006; Hu, Parmet et al. 2009; Kundu, Kamaria et al. 2010).

1.5.6. Sex

In the western countries especially USA, the invasive melanoma has a higher female predilection from birth to age 39 years (1 in 370 women compared with 1

in 645 men). However, from age 40 years and older, melanoma in men predominates, occurring in 1 in 39 men compared with 1 in 58 women over a lifetime. Worldwide, of the 160,000 new cases estimated to have occurred in 2002, women were affected slightly more than men (male-to-female ratio, 0.97:1). Conversely, of the estimated 41,000 worldwide deaths in 2002, more occurred in men than in women (male-to-female ratio 1.2:1) (Coelho and Hearing 2010; Coras, Landthaler et al. 2010; Rouhani, Pinheiro et al. 2010). The additional exposure of adolescents to unnaturally large amounts of UVA from UVA-rich sunlamps which produce visible tan sources is implicated in the increasing incidence of malignant melanomas disproportionately in young women (Coelho and Hearing 2010).

1.5.7. Age

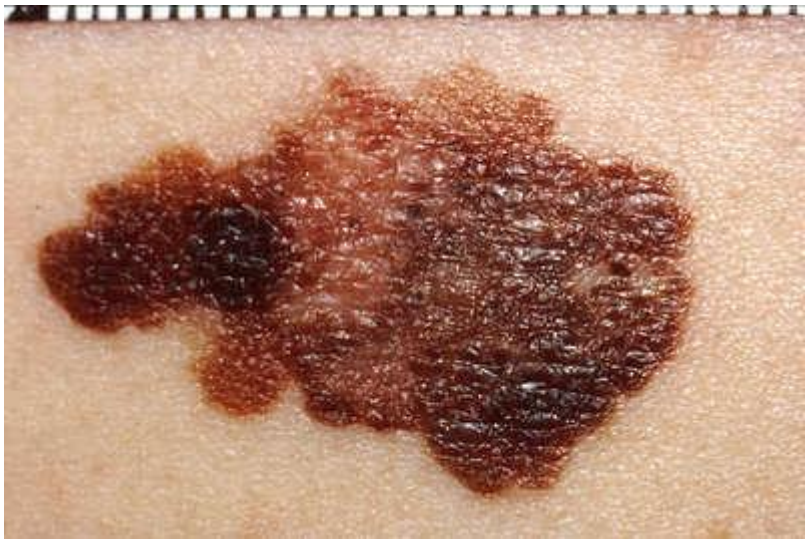
Overall the mean age at melanoma diagnosis is 53 years; however, it is the most common cancer in women aged 25-29 years and is second only to breast cancer in women aged 30-34 years. The most striking differences in melanoma incidence and mortality occur in individuals older than 65 years, although modest differences in age-specific incidence and mortality are notable in persons older than 50 years (Geller, Miller et al. 2002).

Older individuals are both more likely to acquire and to die from melanoma; thus, elderly persons should be a primary target for secondary melanoma prevention, including early detection and screening (van der Meijden, van Bruchem-Visser et al. 2010). Treatment options in elderly persons may also be limited because of comorbid medical conditions, an inability to tolerate adverse

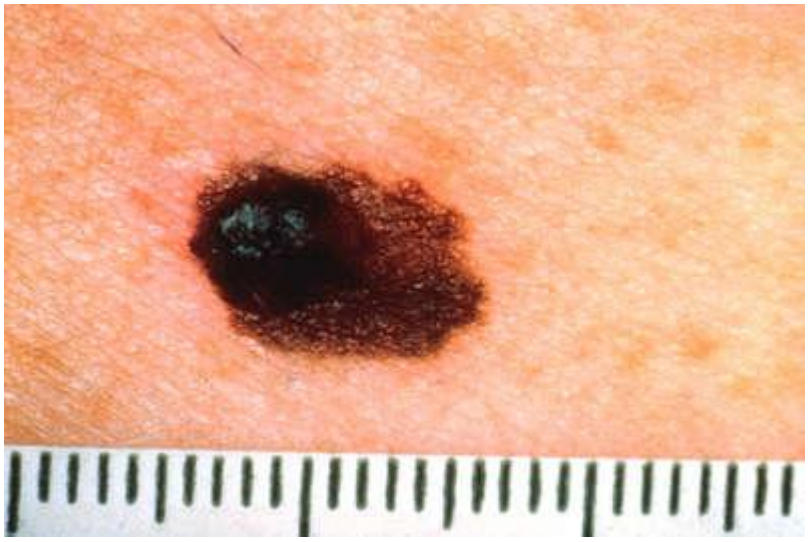
medication effects or toxicity, the increased likelihood of drug interactions, and potential exclusion from clinical trials based on age criteria.

1.5.5. Treatment

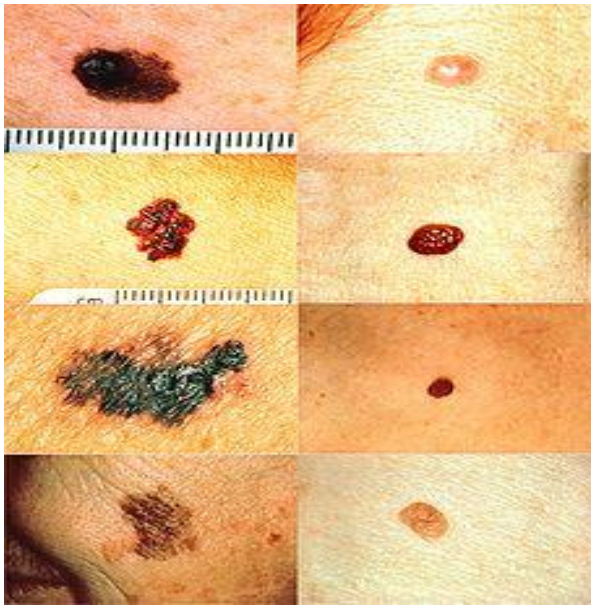
The only effective and urgent treatment is complete surgical removal. No survival benefit has been demonstrated for adjuvant chemotherapy, nonspecific (passive) immunotherapy, radiation therapy, retinoid therapy, vitamin therapy, or biologic therapy (Veronesi, Adamus et al. 1982). Adjuvant interferon (IFN) alfa-2b is the only adjuvant therapy approved by the US Food and Drug Administration for high-risk melanoma (currently defined as stages IIB, IIC, and III), which is associated with a 40-80% chance of relapse and death. Various experimental melanoma vaccines also show promise in the adjuvant setting (Kirkwood, et al. 1996; Kirkwood, et al. 2000).



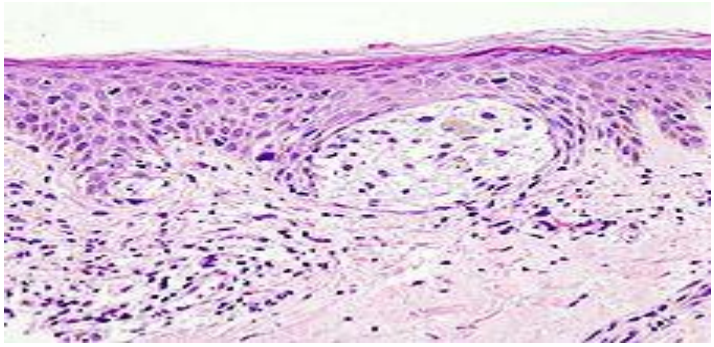
Photography 1.5.5. Picture of Melanoma Skin Cancer, *Photo © Skin Cancer Foundation*



Photograph 1.5.6. Melanoma Skin Cancer, *Photo © National Cancer Institute*



Photograph 1.5.7. ABCD rule illustration. On the left side from top to bottom: melanomas showing (A) Asymmetry, (B) a border that is uneven, ragged, or notched, (C) coloring of different shades of brown, black, or tan and (D) diameter that had changed in size. The normal moles on the right side do not have abnormal characteristics (no asymmetry, even border, even color, no change in diameter). *en.wikipedia.org*



Photograph 1.5.8. Malignant melanoma in skin biopsy with H&E stain. This case may represent superficial spreading melanoma. *en.wikipedia.org*

1.6. Oxidative stress and cancers

Oxygen radicals are continuously generated within mammalian cells, this being a consequence of the use of oxygen in aerobic respiration. Superoxide is generated within the mitochondria and is sequentially reduced to hydrogen peroxide and hydroxyl radicals (Wiseman, 1996). The development of malignancy can be the result of increasing in the ROS formation, In explaining this, most attention has been paid to direct oxidative damage to DNA by certain ROS, such as hydroxyl radical ($OH^{\bullet}$). However, increased levels of DNA base oxidation products such as the 8-OHdg (8-hydroxy-2´-deoxyguanosine) do not always lead to malignancy, although malignant tumours often show increased levels of DNA base oxidation. Oxidative stress constantly affects carcinoma cells *in vivo* and *in vitro* (Brown, 2001). Therefore the effects on P53 and cell proliferation, invasiveness and metastasis are the other results from of ROS. In chronic inflammation and developing cancers the function of ROS is very complicated and sometimes can

act as anti-inflammatory agents (Halliwell, 2007). One is oxidative damage to DNA that could lead to the mutational events of initiation and progression. The other is interruption of reactive oxygen-dependent cell signalling pathways controlling gene expression that contribute to all stages of cancer, in particular tumour promotion (Upham, 2001).

Interestingly, malignant cells are more sensitive to damage by ROS generating agents due to the production of O(2)(-) and subsequent apoptosis, compared to normal cells and they are under intrinsic stress. The intrinsic oxidative stress in cancer cells was associated with the upregulation of SOD and catalase protein expression, as a likely mechanism to tolerate increased ROS stress. The increase in SOD and catalase expression is observed both in primary human leukaemia cells and in primary ovarian cancer cells. One of the certain agents generating reactive oxygen species (ROS) is 2-methoxyestradiol (2-ME). O (2) (-) is an important mediator of 2-ME-induced apoptosis. The increased oxidative stress in cancer cells forces these cells to rely more on antioxidant enzymes such as SOD for O(2)(-) elimination, thus making the malignant cells more vulnerable to SOD inhibition than normal cells (Hileman, 2004). Ultraviolet light induces free radical formation in skin, causing photoaging and cancer (Jurkiewicz, 1994). Oxygen radicals can induce a number of disruptive cellular processes, including lipid peroxidation, DNA cleavage, altered enzyme activity, polysaccharide polymerization, and cell death (Reynolds, 2007).

An imbalance of prooxidants and antioxidants in the organism, is rapidly gaining recognition as a key phenomenon in chronic diseases (Ginter, 1996). Decreases

in antioxidant intake and high oxidative stress are linked with breast cancer risk (Sharhar, 2008).

The higher UVA radiation is a well-recognized generator of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which play a key role in mediating its biological effects (Hoerter, 2008). Unbalancing of antioxidants percentage and oxidative products, induces oxidative damage the accumulation of which is considered as a risk factor in photoaging, photoimmunosuppression and photocarcinogenesis (Wood, 2006). That UVA contributes to inflammation and to the deleterious effects observed in photoaging, immunosuppression and skin cancer has been demonstrated (Mouret, 2006; Berneburg, 1999; Okada, 2003). The production of ROS such as singlet oxygen ($^1O_2$) plays a key role in this pathway and direct evidence of its production in the skin has been obtained following UVA irradiation (Baier, 2007). In human keratinocytes, the high redox potential of O_2 allows it to react with all major classes of biomolecules, the most important of them in protein oxidation (Pattison, 2006), lipid peroxidation (Minami, 2008) and DNA damage (Ravanat, 2001), and it may trigger the generation of other ROS.

1.7. UVA radiation

UV radiation is a toxic agent with genotoxic effects. It has been found to be associated with chromosome aberrations caused by breaks in the DNA strands. Cellular growth rate, cell viability, mitotic index, chromosomal instability and biomarkers of oxidative metabolism were analysed in lymphocyte cells from healthy adults with different Ala16Val MnSOD polymorphisms that produced

three genotypes: AA, VV and AV. They found a differential response to UV exposure in cultures of lymphocyte cells from Ala16Val genotype donors. In general, AA cell cultures presented higher viability and mitotic index and lower TBARS (antioxidant) levels than VV and AV cells for both the control and UV exposure groups. However, when they compared the DNA damage among the three genotypes, AA lymphocyte cells presented the highest damage from UV exposure. These data suggest that the Ala16Val polymorphism affects the response of cellular oxidative metabolism in different ways (Montagner, 2010). Intracellular and extracellular oxidative stress initiated by reactive oxygen species (ROS) advance skin aging, which is characterized by wrinkles and atypical pigmentation. Because UV enhances ROS generation in cells, skin aging is usually discussed in relation to UV exposure (Masaki, 2010).

UV-induced damage is involved in the initiation of MM as melanocytes seem to be extremely susceptible to free radicals (Baldea, 2009; Wang, 2010). UVB (290–320 nm) exposure was thought to be responsible for non-melanoma skin cancer as it generates CPDs in skin cells which in turn can develop into cutaneous squamous cell or basal cell carcinomas (Mouret, 2006). By the increasing usage of UVA tanning beds and the parallel rise in the development of melanomas, the adverse effects of UVA exposure (320-400 nm) was also suggested (Mouret, 2006). Like UVB, UVA can cause non-melanoma skin cancers, but tumours take longer to develop and require much higher doses. UVA-induced skin cancers have been thought to derive from indirect damage to DNA caused primarily by the generation of reactive oxygen intermediates (Mouret, 2006).

1.8. Comet assay

One of the genetic endpoints to be presented in this dissertation is in the single cell gel electrophoresis or Comet Assay. The SCGE assay is a rapid and sensitive florescent microscopic method to examine DNA damage and repair at the individual cell level.

Rydberg and Johanson described the technique for the first time (Ryberg, 1978). Östling and Johanson 1984 developed a microgel electrophoresis technique, commonly known as the Comet assay.

The assay is an attractive test for measuring genotoxicity and investigating DNA repair, or monitoring humans/animals for exposure against environmental mutagenesis and chemical carcinogenesis.

The comet assay has widespread applications in toxicology testing/human biomounitoring *in vitro* and *in vivo* (Anderson *et al*, 1998).

Since the introduction of the alkaline comet assay in 1988 by Singh *et al*, a number of advances have greatly increased the flexibility and utility of this technique for detecting various forms of DNA damage, and DNA-DNA/DNA-protein/DNA-drug cross linking) and DNA repair in virtually any eukaryotic cell (Anderson *et al,* 1998).

Two versions of the comet assay are currently in use, one introduced by Singh *et al* 1998, who used alkaline electrophoresis (pH > 13) in order to increase the sensitivity for analysing DNA damage (after treatment with H_2O_2) which is capable of detecting DNA single strand breaks (SSBs), and alkali labile sites in

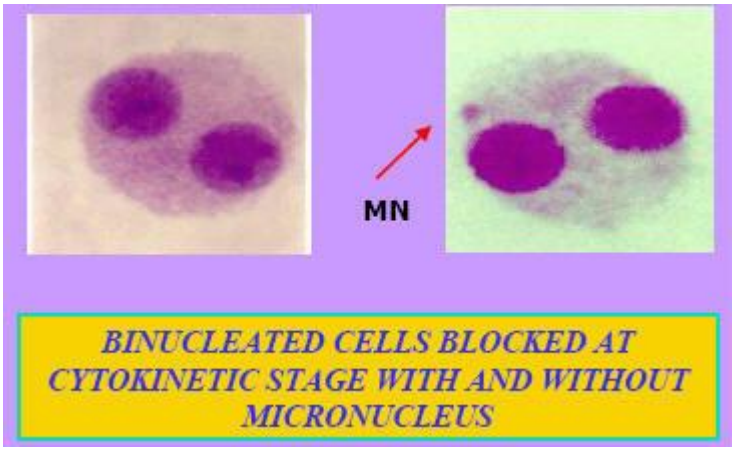
individual cells. This version is known as the “Single Cell Gel Electrophoresis (SCGE) technique”, although for historical reasons many investigators refer to this method as the “Comet assay” due to shape of the comet tail.

Also Olive and co-workers developed versions of the neutral technique of Östling and Johanson, which also involved lysis in less alkali treatment (Olive, 1989).

1.9. Micronucleus assay

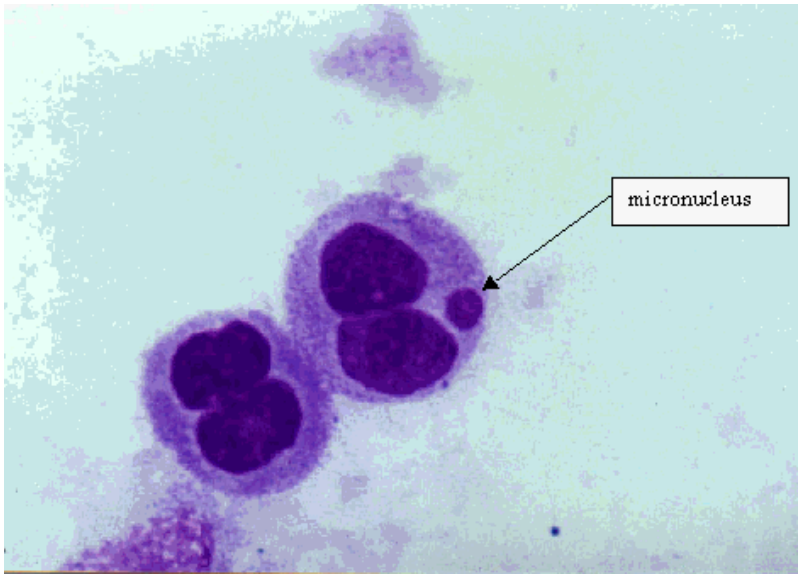
The other endpoint is the micronucleus (MN) is an acentric chromosome fragment or whole chromosome that is left behind during mitotic cellular division and appears in the cytoplasm of interphase cells as a small additional nucleus (Kirsch-Volders, 2003). The formation of MN in dividing cells is the result of chromosome breakage due to unrepaired or disrepaired DNA lesions, or chromosome malsegregation due to mitotic malfunction (Photograph 1.9.1, 1.9.2). These events may be induced by oxidative stress, exposure to clastogens or aneugens, genetic defects in the cell cycle checkpoint and/or DNA repair genes, as well as deficiencies in nutrients required as co-factors in DNA metabolism and chromosome segregation machinery (Fenech, 1999;Fenech, 2005). All these events can cause the formation of MN through chromosomal rearrangements, altered gene expression or aneuploidy, effects associated with the chromosome instability phenotype often seen in cancer (Fenech, 2002;Fenech, 2003) (Photographs 1.9.1, 1.9.2). The presence of an association between MN induction and cancer development is supported by a number of observations. The most substantiated include: the high frequency of MN in untreated cancer

patients and in subjects affected by cancer-prone congenital diseases, e.g. Bloom syndrome or ataxia telangiectasia (Fenech, 2002;Fenech, 1999), the presence of elevated MN frequencies in oral mucosa, used as a surrogate biomarker of cancer in clinical chemo-prevention trials(Van Schooten, 2002), the correlation between genotoxic MN-inducing agents and carcinogenicity, e.g. ionizing and ultraviolet radiation (Chang, et al.,1997;(Bettega, Calzolari et al. 2003)



Photograph 1.9.1. Micronuclei detected in binucleated cell.

www.igcar.ernet.in/igc2004/sg/micronuc.htm



Photograph 1.9.2. Micronuclei detected in binucleated cell. *www.igcar.ernet.in/igc2004/sg/micronuc.htm*

1.10. Aim and objectives of the present studies

The primary aim was to investigate the effect of oxidative stress and antioxidants on lymphocytes from patients with inflammatory bowel disease. Also the effect of UVA on cancerous and pre-cancerous states (malignant melanoma, suspected melanoma and colorectal cancer, polyposis coli patients). Lymphocytes from all diseases states were compared with those from healthy control individuals. Two genetic endpoints that were used were the Comet and micronucleus assays.

CHAPTER TWO

MATERIALS AND METHODS

2. Materials and Methods

2.1. Ethical approval studies for patients and healthy control individuals

Whole blood was collected by myself with venepuncture from donors before each experiment. The donors were gastroenterology patients from two clinics, St. Luke's Hospital and Bradford Royal Infirmary (BRI) in Bradford, West Yorkshire, UK. Healthy volunteers were recruited within the Division of Biomedical Sciences at the University of Bradford, West Yorkshire, UK. Ethical permission has been obtained from both the BRI Local Ethics Committee and the University of Bradford's Sub-Committee for Ethics in Research involving Human Subjects. Controls were from the University of Bradford. Ethical permission was obtained from both the BRI Local Ethics Committee for the experiments (reference numbers, malignant melanoma; 04/QI202/132 and colorectal cancer; 04/Q1202/15) and the University of Bradford's sub-committee of Research Ethics involving Human Subjects (reference number 0405/8).

2.2. The Comet assay and micronucleus assay

2.2.1. Comet assay

The single cell gel electrophoresis (SCGE) or Comet assay is a rapid and very sensitive fluorescent microscopic method to assess DNA damage and repair at the individual cell level (Tice, 2000). Since the introduction of the alkaline (pH >13) Comet assay (Singh, 1988), the flexibility and efficacy of this technique for detecting various forms of DNA damage (e.g., single- and double-

strand breaks, oxidative DNA base damage, and DNA-DNA/protein/DNA-drug cross-linking) and DNA repair have increased its use in virtually all eukaryotic cells (Tice, 2000). In the Comet assay, DNA migration is found to be a function of both size and the number of broken DNA ends. The length of the Comet tail increases initially with damage and then reaches a maximum size that depends on the electrophoretic conditions. Tail moment, a measure of tail length and the fraction of DNA in the Comet tail, was used as the arbitrary unit of assessment (Kumaravel, 2006;Anderson, 2003). The Comet assay was carried out as described by Tice *et al.* (2000) (Tice, 2000). In brief, ethanol flamed glass slides from BDH (Superfrost^{TM}) were coated with 1% agarose. The cell suspension was mixed 1:1 with 1% low-melting point agarose (< 40°C) and applied to dry agarose-coated slides. After setting, this second layer was covered with a top layer of 0.5% low-melting point agarose. For each dose point two replicate slides were produced. The slides were immersed laterally in a container with cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100, pH 10) and incubated at 4ºC overnight. After lysis, the slides were placed horizontally on the tray of an electrophoresis tank, filled with cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH <13), and incubated for 30 minutes at 4ºC in the dark to allow the unwinding of DNA and expression of the alkali labile damage. Electrophoresis was conducted at the same temperature for 30 minutes at 25 volts and an adjusted current of 300 mA (by raising or lowering the buffer level). After electrophoresis, the slides were removed from the tank and soaked three times each with neutralising buffer (400 mM Tris, pH 7.5) for a period of five minutes each (Anderson, 2003; Tice, 2000). Cells were stained with 20 µg/ml ethidium bromide and examined using a

fluorescence microscope equipped with a monochrome CCD-camera. For each replicate slide, 25 cells were scored (50 cells in total, making 1000 observations per experimental point (20 patients, 20 controls) allowing a more than adequate statistical power to detect effects). A computerised image analysis system, Komet 4.0 (Kinetic Imaging, Liverpool, UK), was employed to measure the Comet parameters; the median Olive tail moment was then used for statistical analysis. All other chemical reagents were obtained from VWR International laboratories suppliers address in the UK.

2.2.2. Lymphocyte isolation from peripheral blood

Blood was collected into heparin-coated tubes and diluted with 0.9% saline in a 50:50 proportion. Then, this diluted blood was carefully loaded on top of Lymphoprep (Axis, Norway) in a ratio of 2:1 without mixing the two layers.

The tubes were placed in a Mistral 3000 centrifuge at 800 g for 20 minutes at room temperature. After centrifugation, the cloudy, thin lymphocyte layer on top of the transparent Lymphoprep layer was transferred with a Pasteur pipette into universal tubes containing 0.9% saline. The tubes were centrifuged at 770 g for 15 minutes at room temperature. The supernatant was removed and the lymphocytes were used for the experiments after resuspension within RPMI. Some of the lymphocytes were stored in liquid nitrogen for long-term experimentation. The pellet was resuspended in 1 ml of foetal bovine serum (FBS) with 10% DMSO in a 1.5-ml cryovial and lodged in liquid nitrogen vapour overnight, before final storage after complete insertion in the storage Dewar.

2.2.3. Questionnaire for patients and controls (as shown in appendix 1)

A questionnaire was administered to each donor immediately after taking the blood sample. The completed questionnaire for the patient and control groups provided essential information about lifestyle, confounding factors and medical treatment or any other drug intake.

2.2.4. Treatment

The isolated lymphocytes were treated in two series of experiments. In the first one lymphocytes treated with different doses of Chaga extract, H_2O_2. Chaga mushroom extract was obtained from Fungi Perfecti's Mycomedicinals, Chicago, USA. According to the manufacturer's description, 30 drops of this alcohol-based extract are equivalent to 1g of Chaga mushroom mycelium fruit bodies. In the second experiment the lymphocytes from patients and healthy individual controls treated with different doses of IQ and flavonoids (quercetin, epicatechin) and for third experiment with UVA (PUVA).

2.2.5. Cell viability check

Cell viability at the concentrations chosen for each experiment was checked after treatment before performing the Comet assay. Viability was determined by trypan blue exclusion (10 µl of 0.05% trypan blue was added to 10 µl of cell suspension) and the percentage of cells excluding the dye was estimated using a haemocytometer under the microscope. Only concentrations with viability over

80% were accepted to avoid artefactual results from cytotoxicity (Henderson, 1998).

2.2.6. Statistical analysis for the Comet assay

Gaussian normality was violated for many of the scale variables as endorsed by the Kolmogorov-Smirnov test. Therefore, non-parametric test procedures were adopted wherever necessary, such as the Kruskal-Wallis (K-W) and the Mann-Whitney (M-W) tests for independent samples. The unit of assessment was end individual sample when testing intra-subject differences in DNA damage; the Wilcoxon Signed Rank (WSR) Test was applied. For the binary response variables, Fisher's Exact (FE) Test was applied. Throughout the analyses, a significance level of 5% was used and unilateral alternative hypotheses preferred to bidirectional tests (wherever appropriate). Graphical evidence in support of findings was described in terms of medians and quartiles, and illustrated using Box Plots. Outliers and extreme values were defined in the conventional manner by the statistical package SPSS (Version 13 and 16).

2.3. The cytokinesis block micronucleus (CBMN) assay

After collection, whole blood (500 µl) from each of the patients and healthy individuals was cultured on the same day as the collection in 25 cm^2 culture flasks in 4.5 ml RPMI 1640 medium with L-glutamine (Gibco, Paisley, UK) supplemented with 15% heat inactivated foetal calf serum (Gibco, Paisley, UK), 100 U/ml penicillin and 100 U/ml streptomycin (both from PAA Laboratory GmbH, Pasching, Austria) and 2% phytohaemaglutinin A 16 (PHA; Murex Biotech Ltd., Dartford, UK) to stimulate lymphocyte proliferation. Cultures were incubated for seventy two hours in a humidified incubator at 37°C and 5% CO2. From each sample (patients and controls) three culture flasks were prepared. Twenty four hours after starting the cultures one of the flasks from each set was exposed to UVA for 30 minutes at a mean sample surface intensity of 1.53 ± 0.01 $mWcm^{-2}$. While the second flask of each set remained untreated, the culture in the third flask was treated with mitomycin C (MMC) 0.4 µM as a positive control. Cytochalasin B (Sigma-Aldrich, ST. Louise. MO) was added 44 h after starting the cultures at 6 µg/ml per culture. After 72 h, the cultures were centrifuged at 190x g for 8 minutes at room temperature and cells were treated with cold hypotonic KCl solution (110 mM) at 4°C for 15 min. After a centrifugation at 190x g for 8 min at room temperature, cells were fixed with fresh Carnoy's solution (3:1 methanol: acetic acid) followed by three drops of 37% formaldehyde to each preparation. Fixation was repeated twice without adding formaldehyde. The fixed cells were incubated over night in the

refrigerator and then spread onto dry glass slides. Two slides per culture were prepared, dried overnight and stained with filtered 5% Giemsa (Merck, Darmstadt, Germany) in pH 6.5 Soerensen buffer. All chemical reagents not specified otherwise were obtained from VWR, UK.

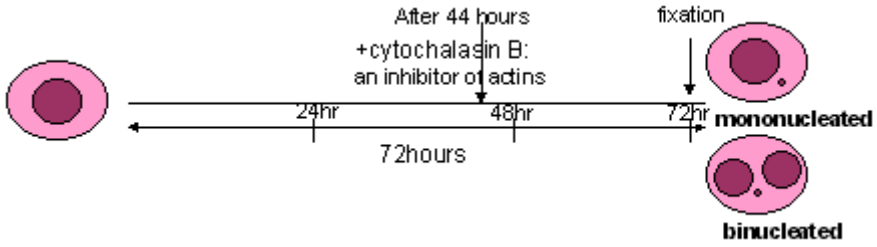


Figure 2.2.1. A schematic figure of CBPI. *Mutat Res.*, 2003

The slides were evaluated for quality, then mounted with cover-slips and coded. The slides were scored according to the criteria defined by Fenech and colleagues (Fenech, 2003). Micronuclei (MN) were evaluated in 1000 binucleated lymphocytes BiMN per treatment. Additionally, nuclear bridges (BiNPBs) and nuclear buds (BiBuds) were assessed in binucleated cells as well as micronuclei in mononucleated cells (MonoMN). The cytokinesis-block proliferation index (CBPI) was calculated from a total of 1000 mononucleated, binucleated and multinucleated cells per treatment. The CBPI is defined as: CBPI = [(number mononucleated cells + (2 * number binucleated cells) + (3 * number multinucleated cells) / total number of cells] (Kirsch-Volders, 2003).

2.3.1. Statistical analysis for the CBMI

For the CBMN assay, the numbers of MonoMN and BiMN, nuclear buds, nucleoplasmic bridges, CBPI and percentage of binucleated cells were used as

dependent variables. The different donors were considered as independent variables. The post-hoc analysis Fisher LSD test was used to determine the significant differences between group means. The level of statistical significance was set at 5% ($p < 0.05$). The statistical package STATISTICA 6.0 (IL, USA) was employed.

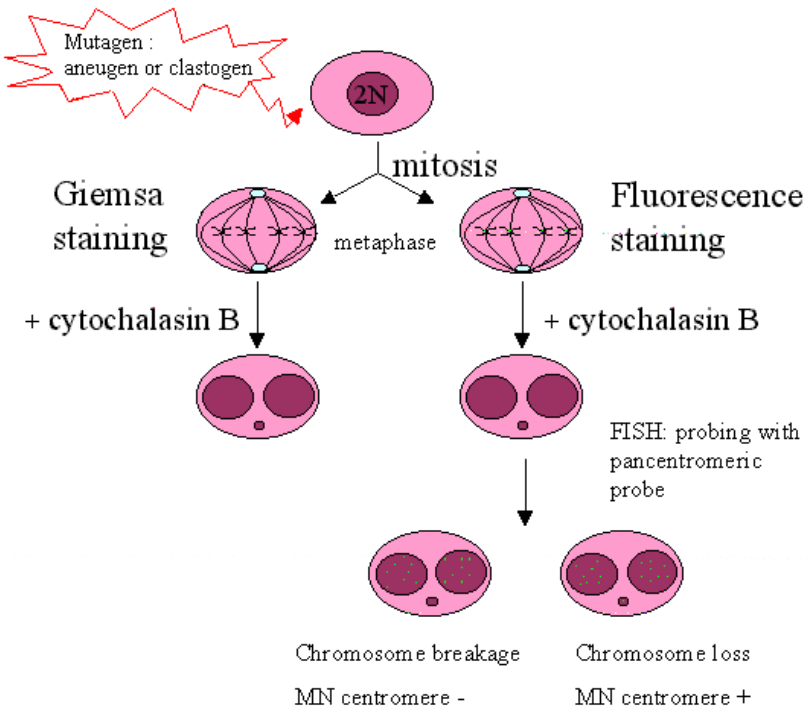


Figure 2.2.2. CBPI & Fish. The cytokinesis-block micronucleus (CBMN) assay has emerged as one of the preferred methods for assessing chromosome damage. Micronuclei (MN) are small, extranuclear bodies that are formed in mitosis from acentric chromosomal fragments or chromosomes that are not included in each daughter nucleus. Thus, MN contain either chromosomal fragments or whole chromosomes. The CBMN assay, together with a fluorescence in situ hybridization (FISH) technique *Mutat Res.*, 2003.

CHAPTER THREE

INFLAMMATORY BOWEL DISEASE

AND

CHAGA MUSHROOM

(*INNONOTOS OBLIQUUS*)

3. Introduction

In this chapter lymphocytes from patients with IBD were exposed to H_2O_2 to induce oxidative stress and treated with chaga mushroom extract. They were compared to similarly exposed lymphocytes from healthy control individuals.

3.1. Chaga mushroom

Medicinal mushrooms, such as Chaga "birch mushroom" which grows in northern regions on birch trees, have been found to inhibit the effect of carcinogens by exhibiting antioxidant properties, especially towards cancers of the stomach, oesophagus and lungs (Park *et al.*, 2005). Chaga *(Inonotus obliquus)* is a polypore that is a fungus with pores instead of gills. It is a fungal parasite and is medicine herbal supplement and is claimed to have beneficial properties for human health. The antioxidant effects of the mushroom protect cell components against free radicals. It has been shown in human lymphocytes in the Comet assay that Chaga mushroom extract treatment affords cellular protection against endogenous DNA damage produced by H_2O_2 (Park, 2005).

The Chaga mushroom is a fungal parasite, which is claimed to have natural beneficial properties for human health such as anti-bacterial, anti-allergic, anti-inflammatory and

antioxidant activities protecting cell components against free radicals (Naba, 1994; Chen, 2010). The Chaga mushroom extract has also been shown to prevent the inhibition of GJIC, a model system of gap junctional intercellular communication (GJIC) in WB-F344 normal rat liver epithelial cells, through the inactivation of ERK1/2 and P38 MAP kinases (Park, 2006). In addition to the three known lanostane-type triterpenoids, inotodiol, trametenolic acid and lanosterol, two new compounds, called inonotsuoxide A and B, were isolated from the sclerotia of *Inonotus obliquus* (Nakata, 2007). It has been shown that the former three compounds have inhibitory effects on the Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-O-tetracanoylphorbol-13-acetate (TPA) and could therefore be a potential natural source of cancer preventive medicine (Nakata, 2007). The anti-cancer effect of Chaga endo-polysaccharides is not directly tumorcidal but rather immuno-stimulating (Kim, 2006). An endo-polysaccharide acting as a specific activator of B-cells and macrophages was extracted from the mycelia of *Inonotus obliquus* (Chaga mushroom). Although, the *in vivo* anti-cancer effects and the chemical structure of this particular endo-polysaccharide were unknown for quite some time, the purified endo-polysaccharide, however, showed in a more recent study immuno-stimulating rather than tumorcidal activities *in vitro* as well as *in vivo* for both aqueous and ethanol extracts against various types of tumour cells (Youn, 2008; Youn, 2009 ;Mazurkiewicz, 2010). 3,4-dihydroxybenzalacetone (DBL) and sclerotia are the polyphenol derived from the medicinal fungi Chaga (Zheng, Zhang et al. 2009). Because most genes involved in inflammation, antiapoptosis, and cell proliferation are regulated by the transcription factor nuclear factor-kappaB (NF-kappaB), it was postulated that DBL activity is mediated via

modulation of the NF-kappaB activation pathway. The effects of DBL were investigated on NF-kappaB activation by the electrophoretic mobility shift assay and on NF-kappaB-regulated gene expression by Western blot analysis. The results indicated that DBL inhibits NF-kappaB activation and NF-kappaB-regulated gene expression, which may explain the ability of DBL to enhance apoptosis and inhibit invasion (Sung, 2008).

Four extracts from the fungus were evaluated for antioxidant activity against the 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide, and peroxyl radicals. The polyphenolic extract had a strong antioxidant activity, and the extract containing triterpenoids and steroids presented a relatively strong antioxidant effect. The polysaccharide extract, however, was inactive. The protective effects of these four extracts were assessed against hydrogen peroxide-induced oxidative stress using a human keratinocyte cell line, HaCaT. The results showed that the polyphenolic extract protected these cells against hydrogen peroxide-induced oxidative stress. The results indicated that *Inonotus obliquus* has the capacity to scavenge free radicals at concentrations higher than 5μg/ml and that the polyphenolic extract can protect cells against oxidative stress (Cui, 2005; Nakajima, 2009).

Phenolic compounds produced by *I. obliquus* in the control medium consisted of melanins, flavonoids, polyphenols and small phenolics. Their accumulation was affected by adding H_2O_2 to the medium, where increased levels of total intracellular phenols (TIP) and melanins, but less total extracellular phenol (TEP) occurred. Simultaneous exposure to H_2O_2 and arbutin resulted in a further increase in TIP production and reduced accumulation of TEP. Both TIP and TEP obtained at different culture ages and media were active in scavenging

superoxide anion and DPPH radicals. Therefore, production of phenolic compounds by *I. obliquus* is enhanced by imposing oxidative stress, which might allow it to be exploited as a reliable source of pharmaceutically important phenolic compounds (Zheng, 2008).

In the present study, we have investigated DNA damage using the Comet assay in human lymphocytes from patients with IBD compared with those from healthy control individuals. Cells have been treated *in vitro* with H_2O_2 in order to induce oxidative stress and also treated simultaneously with Chaga extract to determine if this can reduce the damage through its anti-oxidative properties.

3.2. Material and methods

The materials and methods used in this study have been described in section 2.1.

3.2.1. Treatment

Chaga mushroom extract was obtained from Fungi Perfecti's Mycomedicinals, Chicago, USA. According to the manufacturer's description, 30 drops of this alcohol-based extract are equivalent to 1g of Chaga mushroom mycelium fruit bodies. The purchased extract was further diluted in water to yield concentrations of 50, 100 and 500 µg of equivalent mycelium fruit body mass per millilitre. Isolated lymphocytes from 20 IBD patients were treated at first for 30 minutes at 37ºC with different doses of Chaga extract (0, 50, 100 and 500 µg/ml) and then for an additional 30 minutes with H_2O_2 (50 µg/ml) added to the cell suspension. Lymphocytes which were collected from 20 healthy individuals were used as

control samples, and treated exactly as those from patients under the same conditions. After incubation, the treated lymphocytes were centrifuged at 900 g for five minutes. For DNA damage studies, 900 µl of the supernatant were removed.

3.2.2. Statistical analysis

The statistical methods that have been applied, described in section 2.1.1. 50 cells were analyzed for individuals making a total of 1000 observations per experimental group and ensuring high statistical power throughout the study. However, data were analysed with the individuals as the unit of assessment.

3.3. Results

3.3.1. Patient versus control group

As shown in Figure 3.3.1, there was a significant difference in basic DNA damage within lymphocytes of IBD patients by comparing them with healthy control individuals controls (M-W, $p < 0.001$) before *in vitro* treatment The patient (study group) and the control groups showed increases in DNA damage from H_2O_2 alone ($p < 0.001$). When the dose levels of Chaga extract were increased in successive steps, the observed damage was reduced towards the initial base level in both the patient and the control groups. Chaga mushroom extract gave an overall 54.9% reduction ($p < 0.001$) of DNA damage within the patient group and a 34.9% reduction ($p < 0.001$) within the control group. The level of induced damage from H_2O_2 in the IBD group was statistically significantly greater than that of the control group ($p < 0.001$), even with reduction of the damage by Chaga. The minimum *in vitro* dose of Chaga extract, at which the effect of H_2O_2 was neutralised, was determined as 10µg/ml for the control group and 50 µg/ml for the patient group (Figure 3.3.1).

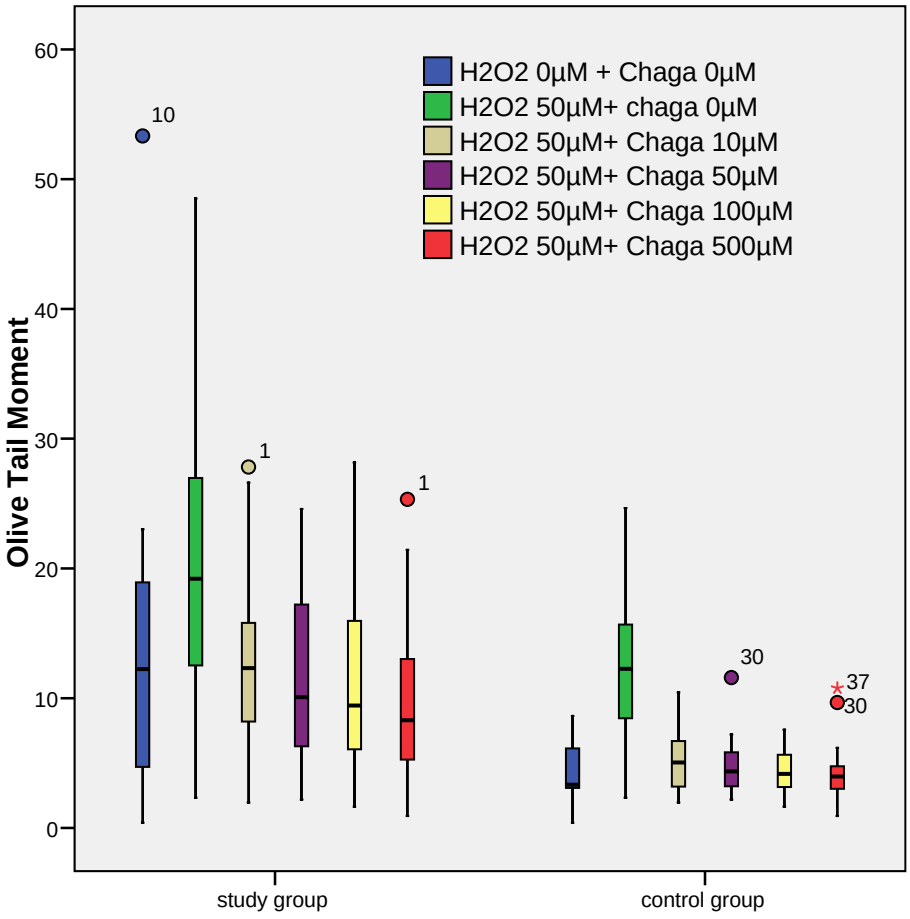


Figure 3.3.1. IBD patient group (n = 20) and control group (n = 20) after *in vitro* treatment with H_2O_2 (50 µg/ml) and supplementation with ethanolic Chaga extract at different dose levels, showing median levels of DNA damage and 75% quartiles. The ∗ and ° symbols followed by a number indicate individual outliers with the respective patient number (Kolmogorov-Smirnov test, $p < 0.001$). The number of individuals in study and control groups is 20 each.

3.3.2. Differences in IBD sub-groups

As shown in Figure 3.3.2, there was less damage in the UC (n = 8) patient group than in the CD (n = 8) group, which was significantly different ($p < 0.001$) when compared with the combined patient groups, which also included the indeterminate group where it was difficult to differentiate into UC or CD (n = 4) (Figure 3.3.2).

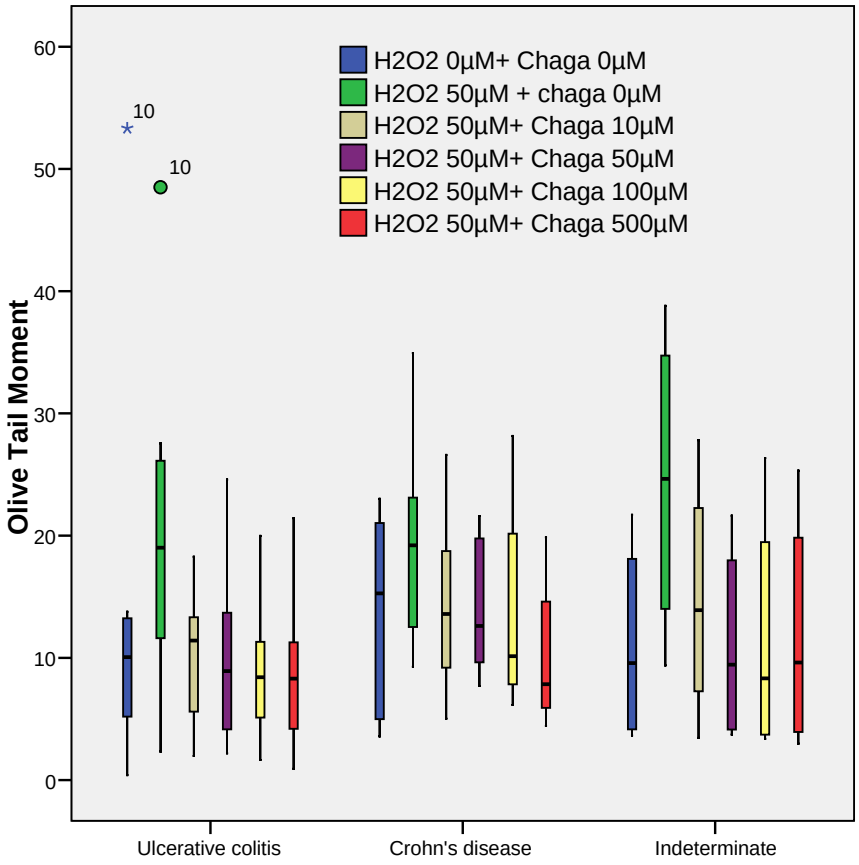


Figure 3.3.2. DNA damage within three IBD subgroups: the first two were diagnosed with Crohn's disease (n = 8) and Ulcerative colitis (n = 8). The third group was an indeterminate subgroup (n = 4). The ∗ and ° symbols followed by a number indicate individual outliers with the respective patient number (Kruskal-

Wallis (K-W) test, $p < 0.001$). The number of patients in the UC and CD is 8 each and the indeterminate is 4.

3.3.3. Confounding factors:

a) Ethnicity, age, gender, smoking and drinking habits

As shown in figures 3.3.3-3.3.6 there were small differences of median levels of DNA damage in Caucasians (n = 25) and Asians (n = 15) (Figure 3.3.6) as well as in males and females (Figure 3.3.3). However, these differences were not found to be statistically significant (M-W, $p = 0.118$); neither was there a statistically significant difference in the age distributions between patient (mean age = 40.20 years ± SD 11.62) and control (mean age = 32.35 years ± SD 9.00) groups. No major differences were seen due to smoking (Figure 3.3.4) and/or drinking habits (Figure 3.3.5).

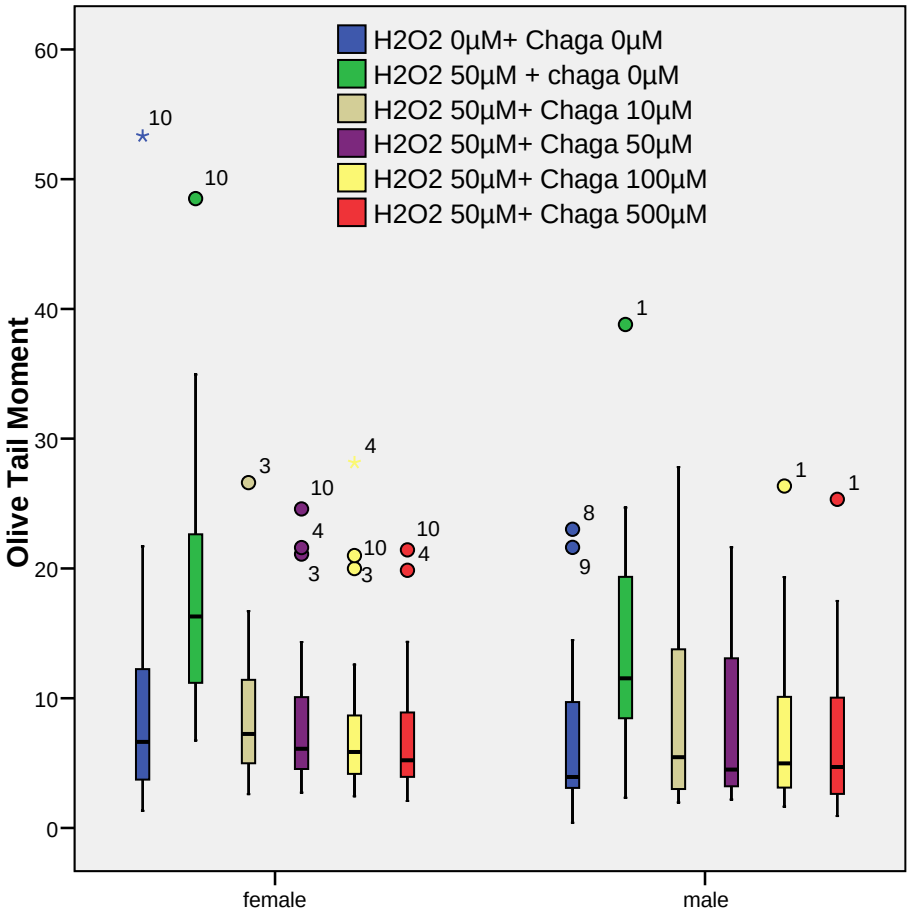


Figure 3.3.3. DNA damage in patients and controls relating to gender: male (n = 19) and female (n = 21). The ∗ and ° symbols followed by a number indicate individual outliers with the respective patient number (Kruskal-Wallis (K-W) test).

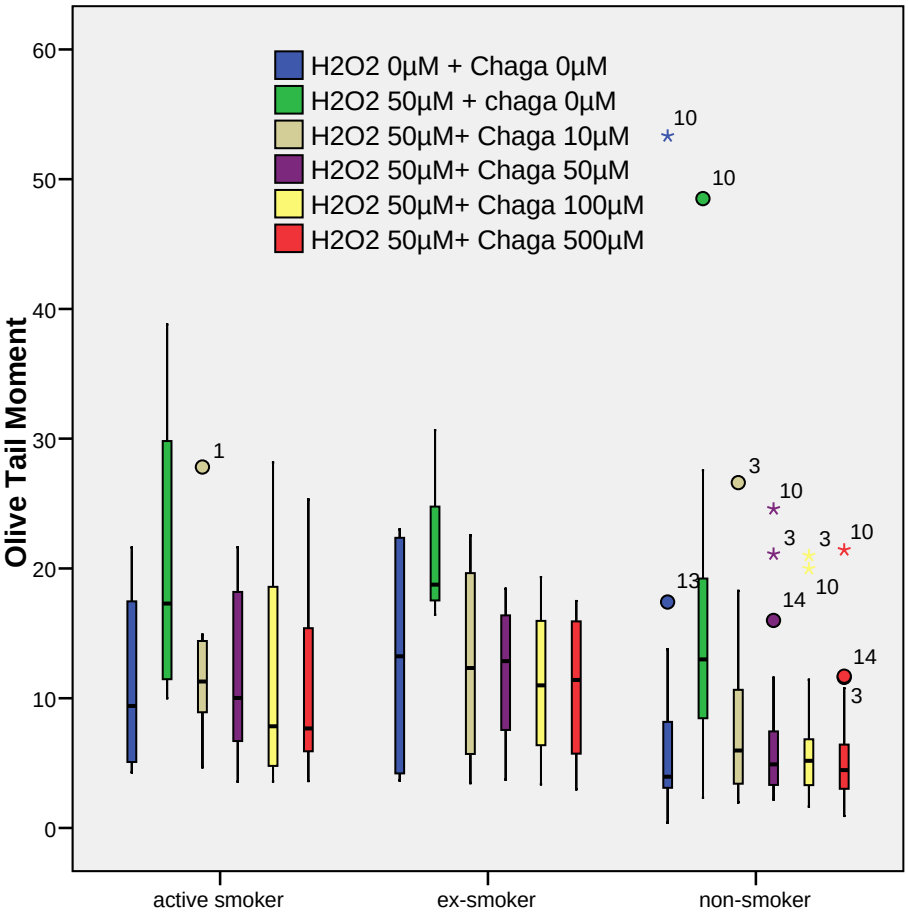


Figure 3.3.4. DNA damage in patients and controls relating to smoking: active smokers (n = 19), ex-smokers (n = 2) and non smokers (n = 19). The ∗ and ° symbols followed by a number indicate individual outliers with the respective patient number (Kruskal-Wallis (K-W) test).

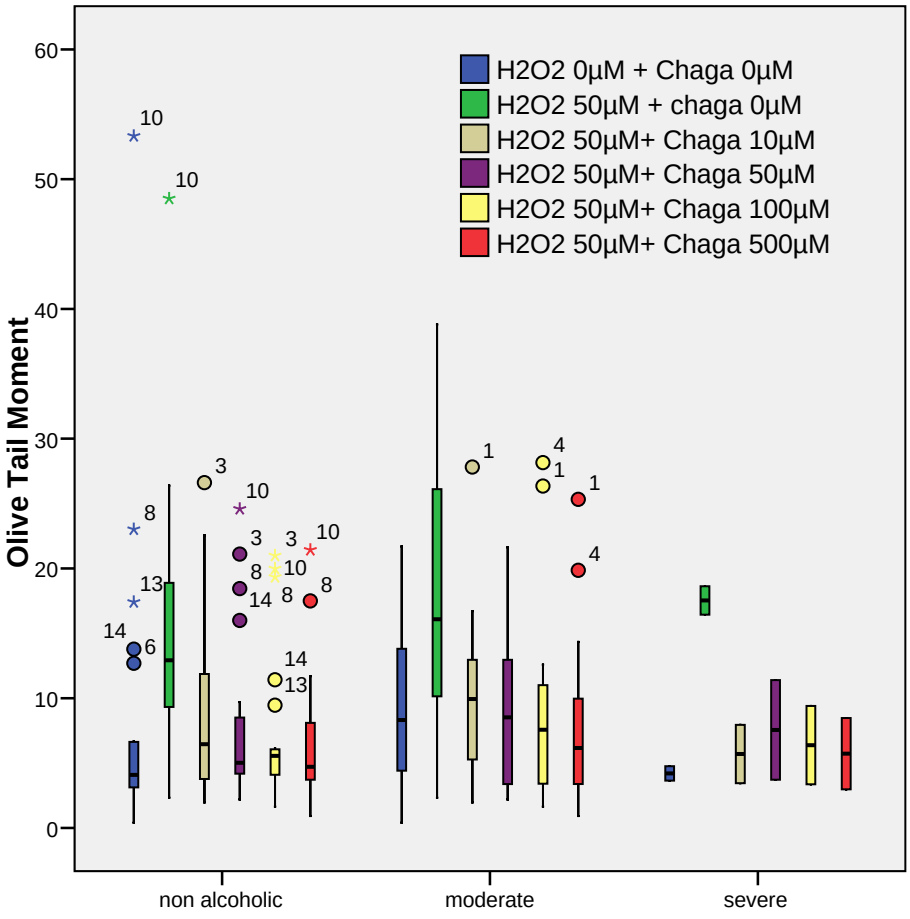


Figure 3.3.5. DNA damage inpatients and controls relating to alcohol intake: non alcoholic (n = 26), moderate (n = 12) and severe (n = 4). The ∗ and ° symbols followed by a number indicate individual outliers with the respective patient number (Kruskal-Wallis (K-W) test).

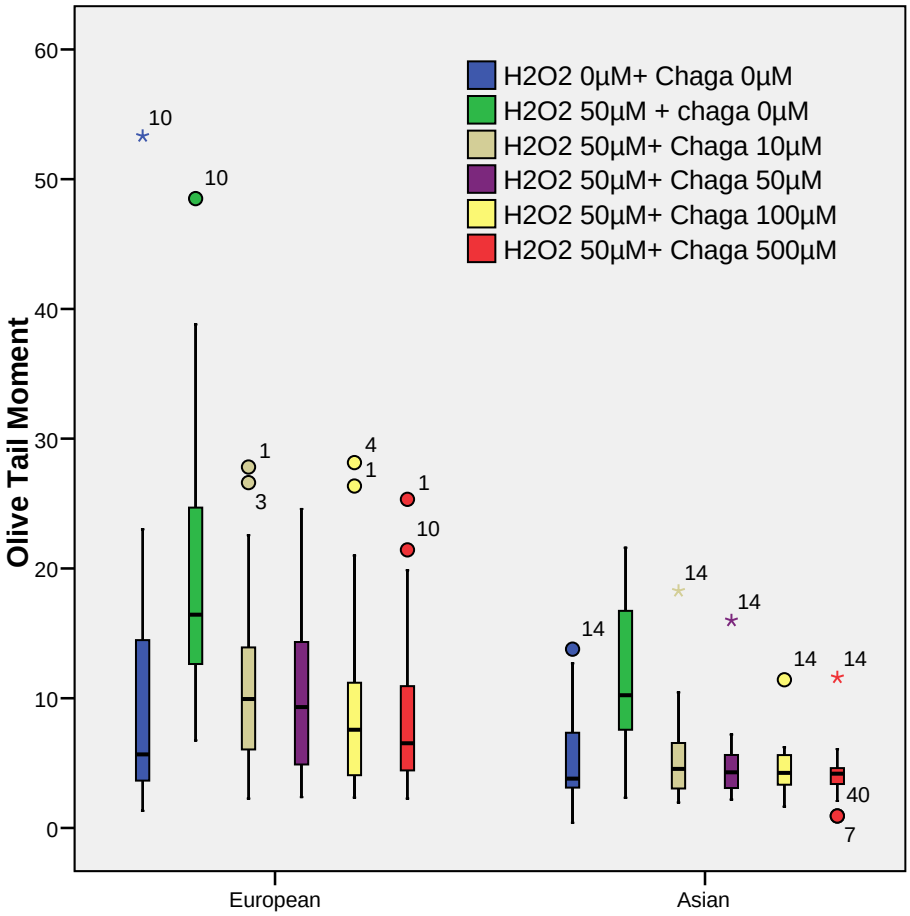


Figure 3.3.6. DNA damage in patients and controls relating to ethnicity: European (n = 27) and Asian (n = 13). The ∗ and ° symbols followed by a number indicate individual outliers with the respective patient number (Kruskal-Wallis (K-W) test).

b) Previous medication in the IBD group as a confounding factor

As shown in figure 3.3.7 patients had been treated with a range of drugs for IBD, namely, methotrexate azathioprine, mesalazine and pantasa, asacol, prednisolone, mercaptopurine alone or in combination prior to taking part in the study [balsalazide & calcium (n = 2); azathioprine & pentasa, azathioprine & mesalazine, mercaptopurine & balsalazide (n = 10); asacol (n = 4); pentasa & prednisolone, prednisolone & mesalazine (n = 3); methotrexate & mesalazine (n = 1)]. Within the treatment groups, there appeared to be differences but because of small sample sizes they were not significant (Figure 3.3.7).

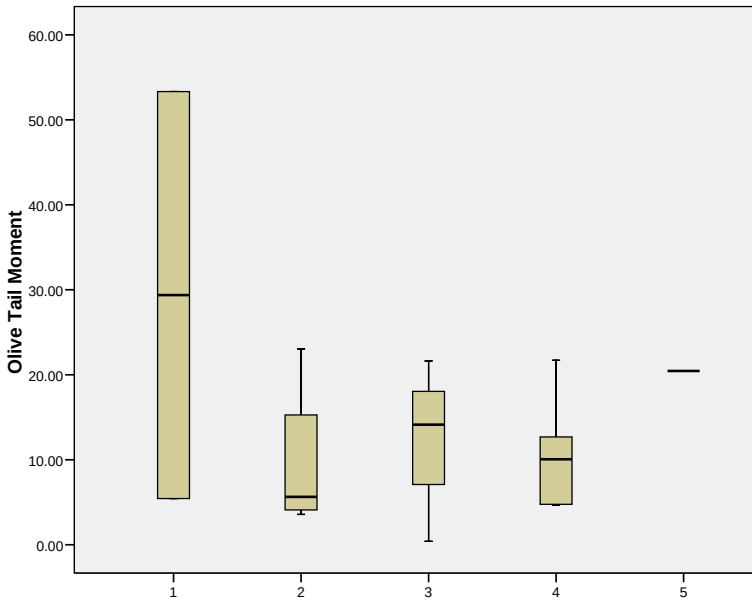


Figure 3.3.7.

1. Methotrexate, azathioprine, mesalazine and pantasa, asacol, prednisolone, mercaptopurine alone or in combination prior to taking part in the study balsalazide & calcium (n = 2)
2. Azathioprine & pentasa, azathioprine & mesalazine, mercaptopurine & balsalazide (n = 10)
3. Asacol (n = 4)
4. Pentasa & prednisolone, prednisolone & mesalazine (n = 3)
5. Methotrexate & mesalazine (n = 1)]

3.4. Discussion

The Chaga mushroom is a wood-rotting fungus used as a folk medicine in Russia and East-European countries to treat tuberculosis, gastritis and cancers. In some Asian countries, a large number of herbal and mushroom extracts has been used traditionally for treatment of inflammatory disease. These mushrooms, such as *Ganoderma lucidum* (Reishi), *Lentinus edodes* (Shiitake), *Grifola frondosa* (Maitake), *Hericium erinaceum* (Yamabushitake) and *Inonotus obliquus* (Chaga) have been collected throughout China, Korea and Japan, but they are almost unknown as treatment options for patients in the West. The pharmacological importance of these mushrooms is very high in the Far East as a traditional medicine in treating various diseases (Lee, 2007 ;Saar, 1991). For the Chaga mushroom, it has been ascribed with a number of biologically active polysaccharides and immuno-stimulatory properties, which seem to protect cells also against free radicals (Allgayer, 1991; Kim, 2007;Lee, 2007; Nakata, 2007). It has as well been recognised that oxidative stress is universally accepted as an important factor in the pathogenesis of IBD (Allgayer, 1991).

Inhibition of iNOS and COX-2 expression via the down-regulation of NF-kappa B binding activity may explain the anti-inflammatory and anti-nociceptive properties of a methanol extract from *Inonotus obliquus* (Park, 2005; Van, 2009). A previous study using the Comet assay in human lymphocytes after *in vitro* treatment with Chaga mushroom extract has shown cellular protection against endogenous DNA damage produced by H_2O_2 (Park, 2004; Zheng, 2009). *Inonotus obliquus* supplementation in the form of an ethanol extract to human

lymphocytes and macrophages *in vitro* was able to markedly inhibit H_2O_2-induced oxidative damage to cellular DNA (Kim, 2007;Park, 2004).

In the present *in vitro* study, for the first time a reduction of induced DNA damage is reported in H_2O_2-treated lymphocytes obtained from IBD patients and healthy volunteers after treatment with an ethanolic extract of the Chaga mushroom (Figure 3.3.1). This protective effect has been shown to be statistically significant. H_2O_2 alone is known to cause destabilisation of the DNA structure by breaking DNA strands in a dose-dependent manner (Limoli, 2003). An *in vitro* treatment of isolated lymphocytes from IBD patients and healthy volunteers with 50 µg/ml H_2O_2 created a maximum of oxidative stress but left the cells still viable so that cytotoxicity did not confound effects (Henderson, 1998). This damage significantly levelled off with increasing doses of the Chaga extract.

Interestingly, also in the present study lymphocytes from CD patients appeared to have a greater level of basic DNA damage than those from UC patients when compared to the whole patient group ($p < 0.001$), suggesting that there may be more oxidative stress involved with Crohn's disease (Figure 3.3.2). This is a very important new finding using the Comet assay in peripheral lymphocytes serving as surrogate cells for colonic mucosa cells. It seems that a misbalanced production of pro-inflammatory and anti-inflammatory cytokines is characteristic of IBD and severely affects the immune homeostasis in peripheral blood cells, even more in CD than in UC patients (Sventoraityte, 2008). Increased free radical production as well as a simultaneously reduced antioxidative potential

(Loguercio, 2003;Koch, 2000) contribute to the DNA damage not only *in situ* but also in peripheral lymphocytes.

In our study, we found no differences for the level of DNA damage between patient and control groups regarding confounding effects such as smoking and drinking alcohol. However, it is known that smoking increases oxidative DNA damage by approximately 50% (Loft, 1992). For smokers, the higher 8-OHdG excretion might be related to a higher rate of metabolism with increased availability of reactive oxygen species (Chen, 2007). The apparent 7-fold individual variation in oxidative DNA damage carries implications regarding ageing and the risk of cancer and other degenerative diseases (Loft, 1992; Takeuchi, 1993; Cooke, 2003). Also when the effects of gender and ethnicity were examined, both showed trends towards being more sensitive to induced DNA damage for females (n = 24) compared to males (n = 16), and for the Caucasian group (n = 25) compared to the Asian group (n = 15), but neither was significant. Differences in life style, diet and possibly genetic background, e.g. different isoforms of metabolising enzymes, might contribute to such an increased trend without reaching significance. Nevertheless, Baypajee and colleagues found significantly higher basal level of DNA damage between males and females in the normal healthy South Indian population where males showed higher levels of damage levels than females (Bajpayee, 2002). Furthermore, as IBD patients who volunteered for this study had been on medication against bowel inflammation, which was also considered a confounding factor possibly contributing to the background DNA damage. The statistical significance in the difference of baseline values between the control and the study group might

partly be explained by such medication causing increased damage as well as increased inflammatory responses in the patients.

CHAPTER FOUR

HETEROCYCLIC AMINES;

FLAVONOIDS: QUERCETIN, EPICATECHIN

4. Introduction

In this chapter lymphocytes from patients with IBD were exposed to a heterocyclic amine and flavonoids and compared to lymphocytes from healthy control individuals also exposed to these compounds.

4.1. Heterocyclic amines and flavonoids

The generation of DNA damage by environmental, medical or life style factors is considered to be an important initial event in carcinogenesis. Despite various cellular mechanisms to counteract these detrimental events the sheer number of potentially carcinogenic compounds leading to oxidative stress can negatively affect the cells' DNA integrity.

4.2. Heterocyclic Amines

A very important family of promutagenic/carcinogenic chemicals, the heterocyclic amines (HCA), is widely produced when cooking food, especially during the pyrolysis of creatinine, amino acids and proteins (Schut, 1999). Major subclasses of HCA found in the human diet comprise of aminoimidazoazaarenes (AIA), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline

(DiMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Schut, 1999; Sutandyo, 2010). There is increasing evidence for the involvement of HCA as pyrolysis products of high protein diets in the aetiology of human cancer {Adamson, 1996; Bogen, 1994; Metry, 2010), which has stimulated strong efforts to identify exogenous and endogenous factors that modify health risks caused by HCA. More than 600 individual compounds and complex dietary mixtures have been studied for protective effects towards HCA (Schwab, 2000) and numerous articles have been published regarding mammalian enzymes involved in the bioactivation and detoxification of these compounds (Eisenbrand, 1993; Haza, 2010).

In addition to the formation of DNA adducts as a major causal factor in the carcinogenesis of HCA, oxidative stress plays a crucial role in further damaging the DNA (Murata, 1999).

4.3. Flavonoids

Dietary flavonoids acting as antioxidants (Rice-Evans, 2001) have been identified to be capable of counteracting these adverse oxidative effects. Flavonoids are classified as polyphenolic compounds that are ubiquitous in nature and categorised according to their chemical structure into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. So far, thousands of flavonoids have been found and identified in plants showing antioxidant qualities (Rice-Evans, 2001; Mochizuki, 2010). The antioxidant potency of several widespread dietary flavonoids showed a dose-dependent reduction of induced oxidative DNA damage *in vitro*, highlighting an even higher protective effect than vitamin C (Noroozi, 1998). It has also been shown

that flavonoids intake can lower the mortality rate caused by coronary heart disease (Kaur, 2007).

Ulcerative colitis and Crohn's disease are inflammatory disorders of the gastrointestinal tract, which are unevenly distributed within the populations throughout the world. Although the exact cause of IBD remains unknown, the epidemiology of IBD has provided an insight into the pathogenesis of the disease by examining geographic, ethnic and other IBD risk factors (genetic, environmental, etc.) as well as their natural history (Danese, 2006). Interestingly, reactive oxygen species (ROS) are produced in abnormally high levels in cells from IBD patients (Rezaie, 2007) leading to oxidative stress and thus to DNA damage due to an imbalance between innate and exogenous antioxidants and ROS (Hemnani, 1998; Soffler, 2007) (Figure 4.3.1).

Oxidative stress has been linked to cancer, aging, atherosclerosis, ischaemic injury, inflammation and neurodegenerative diseases (Davies, 1995; Tudek, 2010).

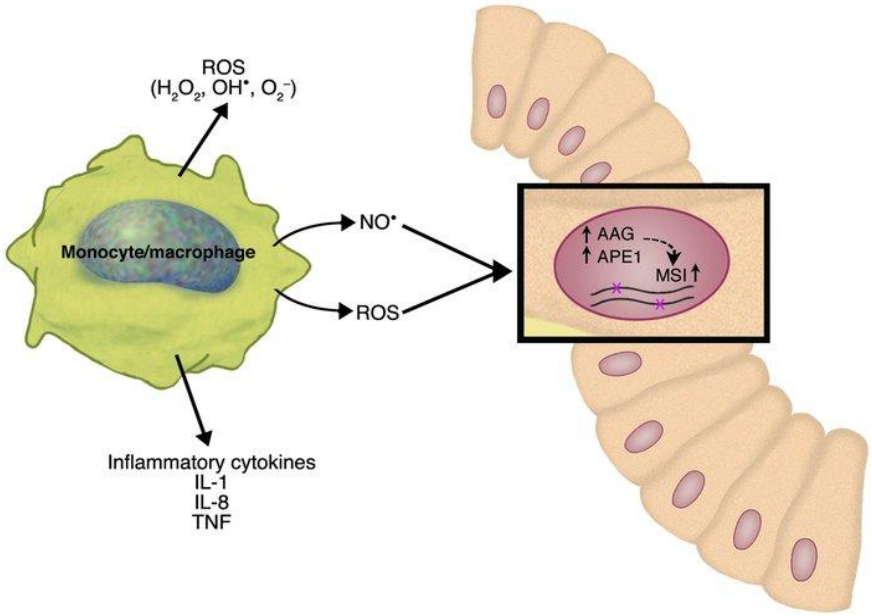


Figure 4.3.1. Schematic illustrating the proposed mechanism of how chronic inflammation may induce microsatellite instability in Ulcerative colitis. Among the factors released by inflammatory cells in Ulcerative colitis are proinflammatory cytokines, ROS, and NO. MSI (microsatellite instability), AAG (alkyladenine DNA glycosylase), APE1 (apurinic/apyrimidinic endonuclease 1) Hofseth et al 2003.

Medically, IBD is characterised by the infiltration of CD4+ T-lymphocytes and other mononuclear cells into inflamed mucosal regions (Seegert, 2001; Cai, 2010). During this process interleukin 16 (IL-16) exerts a strong chemo-attractant activity towards CD4+ cells. Moreover, IL-16 activates the expression and production of pro-inflammatory cytokines such as IL-1 beta, IL-6, IL-15 and tumour necrosis factor alpha (TNF-alpha) in human monocytes (Seegert, 2001). The cytokine TNF-alpha plays a crucial role in the pathogenesis of IBD (Spoettl, 2007). To inhibit TNF-alpha activity biotechnologically manufactured agents such as the chimeric monoclonal anti-TNF antibody (infliximab), a human monoclonal anti-TNF antibody (CDP571) and a recombinant TNF receptor fusion protein (etanercept) have been used (Ardizzone, 2005; van Assche, 2010).

Important insights have also been gained into the function of “caspase-activating and recruitment domain-15” (CARD15)/NOD2, the first cloned susceptibility gene for CD (Rogler, 2004; Rigoli, 2008). An inflammatory reaction of the intestinal mucosa as a response of the innate immune system may be essential for the maintenance of gut homeostasis (Rogler, 2004). CD may therefore be seen as a defective immune response, no longer only as hyper-responsiveness of the mucosal immune system. Data on CARD15/NOD2 expression suggest that macrophages and epithelial cells could be the site of a primary pathophysiologic defect, and T-cell activation might just be a secondary effect inducing chronic inflammation, perhaps as a backup mechanism to a defective innate immunity (Rogler, 2004; Rigoli, 2008). Nevertheless, additional "innate" pathways by which commensal and pathogenic bacteria are able to directly interact with cells of the intestinal mucosa exist (e.g., toll-like receptors).

In this present study, we used the Comet assay, as a fast and reliable method that is able to detect genotoxicity in virtually any mammalian cell type without the requirement for cell culture (Moller, 2006), in human lymphocytes from 20 IBD patients and 20 healthy individuals. The antioxidant effect of the flavonoids quercetin and epicatechin (Sachse, 2002; Haza, 2010) was tested in the presence of an exogenous oxidative insult (H_2O_2) to show that these two flavonoids are able to reliably protect cells against the damaging effects of reactive oxygen species, even in the context of a disease like IBD where levels of ROS are already highly increased.

4.3. Materials and methods

The materials and methods used in this study have been described in section 2.1.

4.3.1. Treatment

Isolated lymphocytes from 20 new IBD patients and 20 controls were treated for 30 minutes at 37 ºC either with different doses of quercetin (0, 50, 100, and 200 µg/ml) in the presence of hydrogen peroxide (50 µg/ml) or with different doses of epicatechin (0, 25, 50, 100 µg/ml) together with IQ (50 µg/ml) (i.e. 10 patients in each patient group) . Lymphocytes from healthy individuals served as a control group. After incubation, the cells were pelleted (5 minutes at 900 g) and resuspended in 100 µl of the remaining supernatant. For DNA damage studies, the cell suspension was mixed with the same volume of 1% low melting point agarose for the Comet assay.

4.3.2. Statistical analysis

The statistical methods have been applied, described in section 2.2.1. 50 cells were analyzed for individuals making in a total 500 observations per experimental IBD group and 1000 for the control group and everything high statistical power throughout the study.

4.4. Results

4.4.1. Patient versus control group

As shown in Figures 4.4.1 and 4.4.2, there was a significant difference in basic DNA damage within lymphocytes of IBD patients when comparing them with

healthy individual controls (2.5- fold in the groups treated with H_2O_2 and quercetin and 5.2-fold in the groups treated with IQ and epicatechin) (M-W, $p < 0.001$) before *in vitro* treatment.

The study groups as well as the control groups showed significant increases in DNA damage induced by H_2O_2 (study group = 2.1-fold and control group = 3.2-fold) ($p < 0.001$) and IQ (study group = 2.0-fold and control group = 3.6-fold) ($p < 0.001$). The induced damage caused by the *in vitro* treatment with H_2O_2 or IQ decreased significantly in both groups when co-treated with the flavonoids quercetin or epicatechin, respectively (Figures 4.4.1 and 4.4.2).

Flavonoids supplementation at the highest concentration (250 μM quercetin or 100 μM epichatechin) caused an overall significant reduction of the induced DNA damage within the patient group and the control groups. This resulted in a 48.6% ($p < 0.001$) reduction of H_2O_2 induced DNA damage and a 43% reduction of IQ induced DNA damage within the patient groups. For both control groups, reductions in DNA damage of 35.2% and 57.1%, respectively, were observed (both, $p < 0.001$) (Figures 4.4.1 and 4.4.2). As expected, the two different control groups showed the same basic DNA damage (M-W, $p < 0.174$. t-test $p < 0.134$).

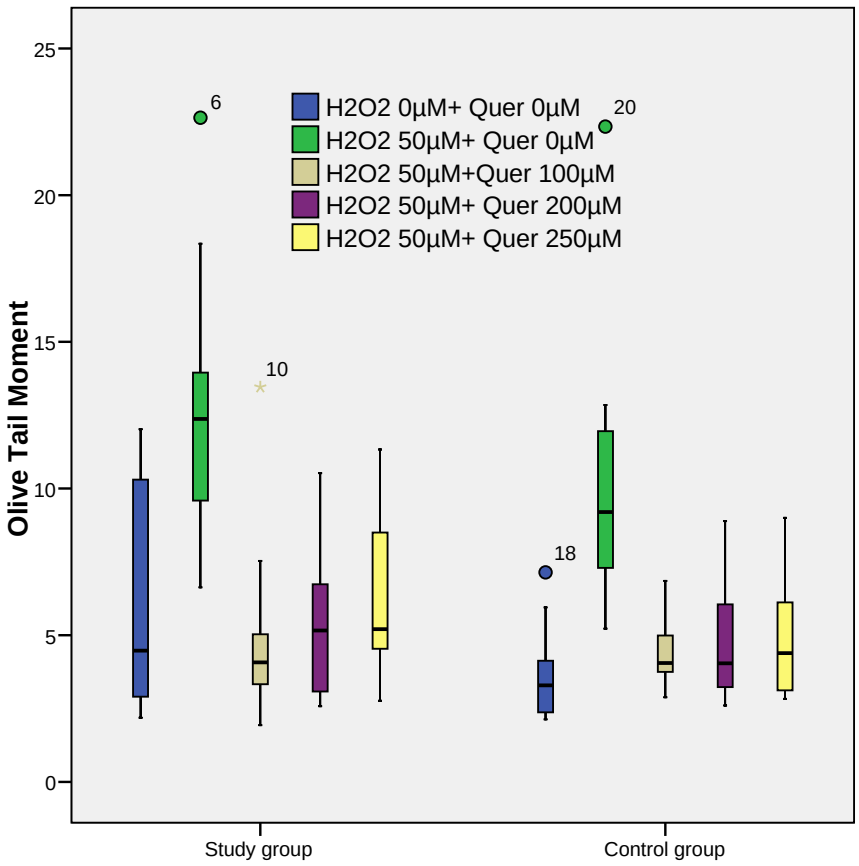


Figure 4.4.1. IBD patient group and control group (n = 10 each) after *in vitro* treatment with H_2O_2 (50 µg/ml) and supplementation with the flavonoid quercetin at different concentration levels, showing median levels of DNA damage and 75% quartiles. The ∗ and ° symbols followed by a number indicate individual outliers with the respective patient number (Kolmogorov-Smirnov test, $p < 0.001$).

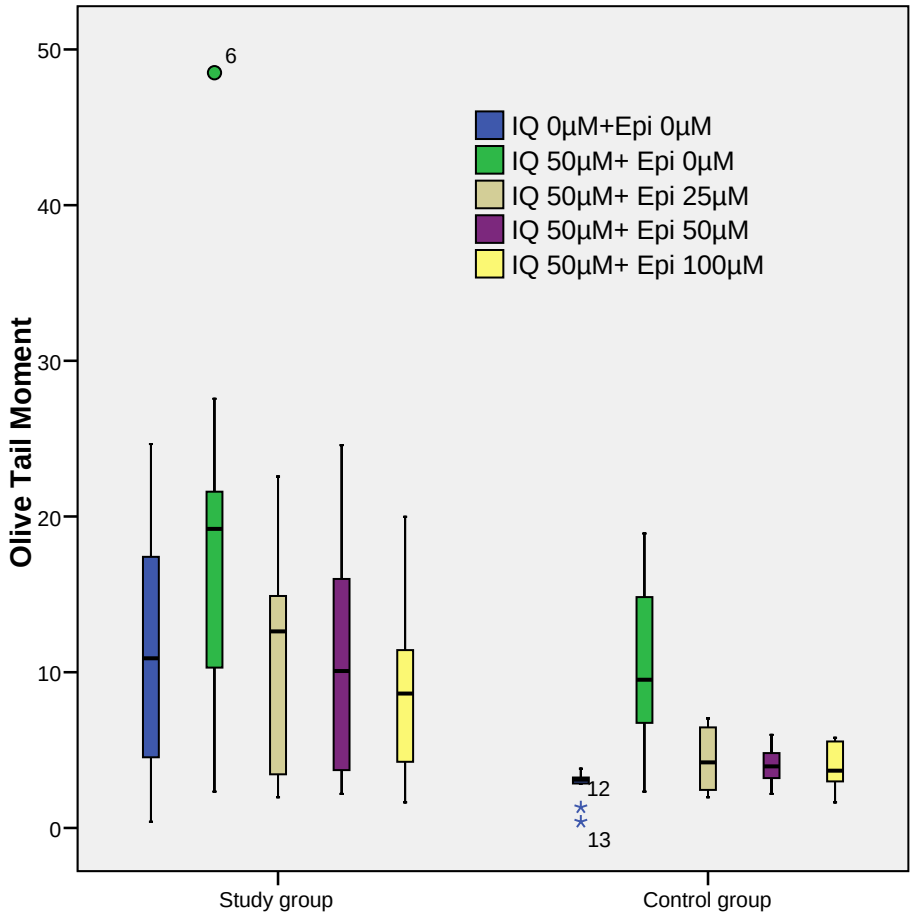


Figure 4.4.2. IBD patient group and control group (n = 10 each) after *in vitro* treatment with IQ (50 µg/ml) and supplementation with the flavonoid epicatechin at different concentration levels, showing median levels of DNA damage and 75% quartiles. The ∗ and ° symbols followed by a number indicate individual outliers with the respective patient number (Kolmogorov-Smirnov test, $p < 0.001$).

4.4.2. Differences in IBD sub-groups

As shown in Figures 4.4.1 and 4.4.2 there was less baseline damage in the UC patient group (n = 4) than in the CD group (n = 4) each being significantly different ($p < 0.001$) (2.0-fold in the study group which was treated with H_2O_2 and quercetin and 3.8-fold in study group treated with IQ and epicatechin) when compared with the combined patient groups, which also included the indeterminate group where it was difficult to differentiate into UC or CD (n = 2). Also there was less induced DNA damage in the study group treated with H_2O_2 and quercetin compared with the study group treated with IQ and epicatechin although the patients were selected randomly (Figures 4.4.3 and 4.4.4). There was less induced damage of DNA in the newly selected lymphocytes from UC patients compared to CD patients in both series of experiments (H_2O_2 with quercetin and IQ with epicatechin ($p < 0.001$) (Figures 4.4.3 and 4.4.4).

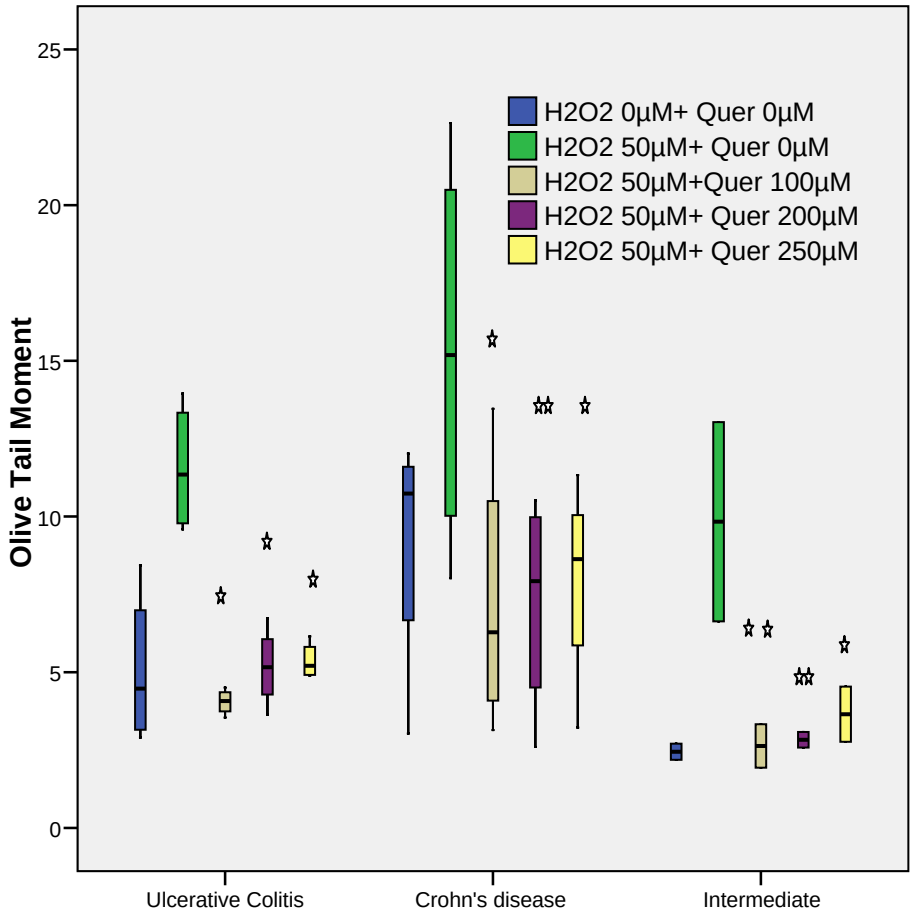


Figure 4.4.3. DNA damage within three IBD subgroups after *in vitro* treatment with H_2O_2 (50µg/ml) and supplementation with the flavonoid quercetin at different concentration levels: the first two were diagnosed with Crohn's disease (n = 4) and Ulcerative colitis (n = 4). The third group was an indeterminate subgroup (n = 2). The ∗ and ° number (Kruskal-Wallis (K-W) test, $p < 0.001$). * ($P < 0.01$) and ** ($P < 0.001$) **.**

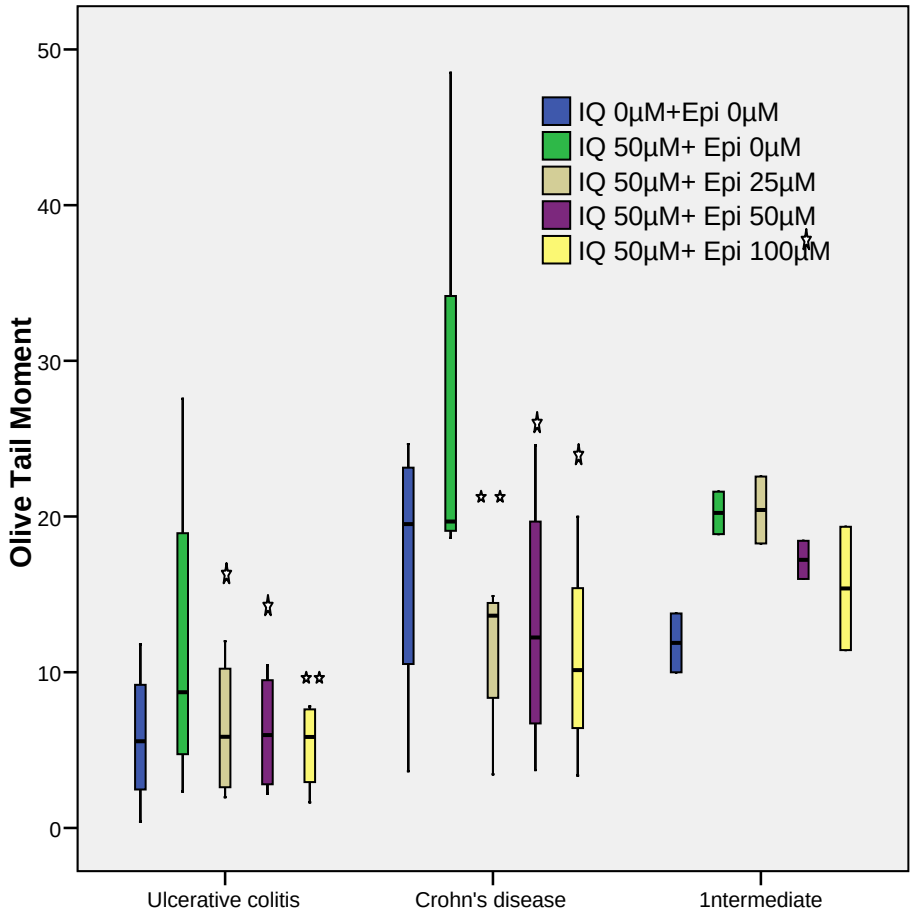


Figure 4.4.4. DNA damage within three IBD subgroups after *in vitro* treatment with IQ (50 µg/ml) and supplementation with the flavonoid epicatechin at different concentration levels: the first two were diagnosed with Crohn's disease (n = 4) and Ulcerative colitis (n = 4). The third group was an indeterminate subgroup (n = 2). * ($P < 0.01$) and ** ($P < 0.001$). (Kruskal-Wallis (K-W) test, $p < 0.001$).

4.4.3. Confounding factors:

a) Ethnicity, age, gender, smoking and drinking habits

As shown in figures 4.5-4.13 there were small differences of median levels of DNA damage in Caucasians (n = 13) and Asians (n = 7) (Figures 4.4.11, 4.4.12) as well as in males and females (Figures 4.4.7, 4.4.8). However, these differences were not found to be statistically significant (M-W, p = 0.170); neither was there a statistically significant difference in the age distributions between patient (mean age = 42.40 years ± SD 11.62) and control (mean age = 28.9 years ± SD 9.00) groups. No major differences were seen due to smoking (Figures 4.4.9, 4.4.10) and/or drinking habits (Figures 4.4.5, 4.4.6).

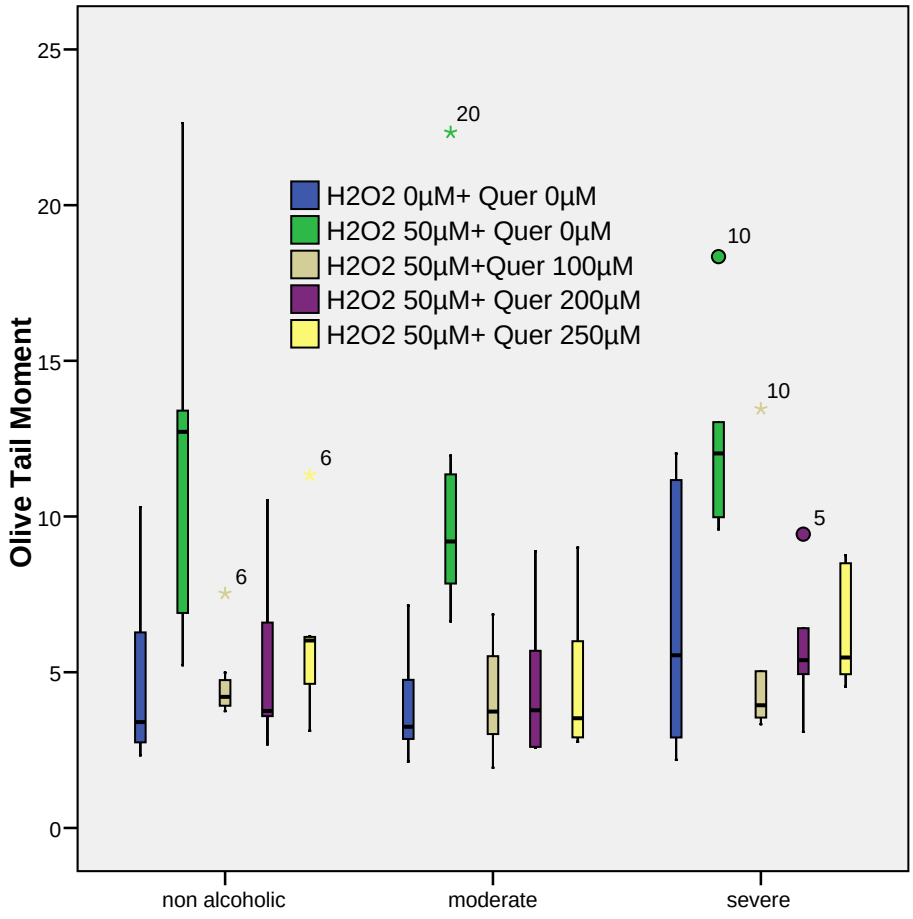


Figure4.4.5. DNA damage in patients and controls (all subjects n = 20, 10 each group) relating to alcohol intake: non alcoholic (n = 7), moderate alcoholic (n = 7) and severe alcoholic (n = 6) groups after *in vitro* treatment with H_2O_2 (50 µg/ml) and supplementation with the flavonoid quercetin at different concentration levels. The ∗ and ° symbols followed by a number indicate individual outliers with the respective patient number (Kruskal-Wallis (K-W) test).

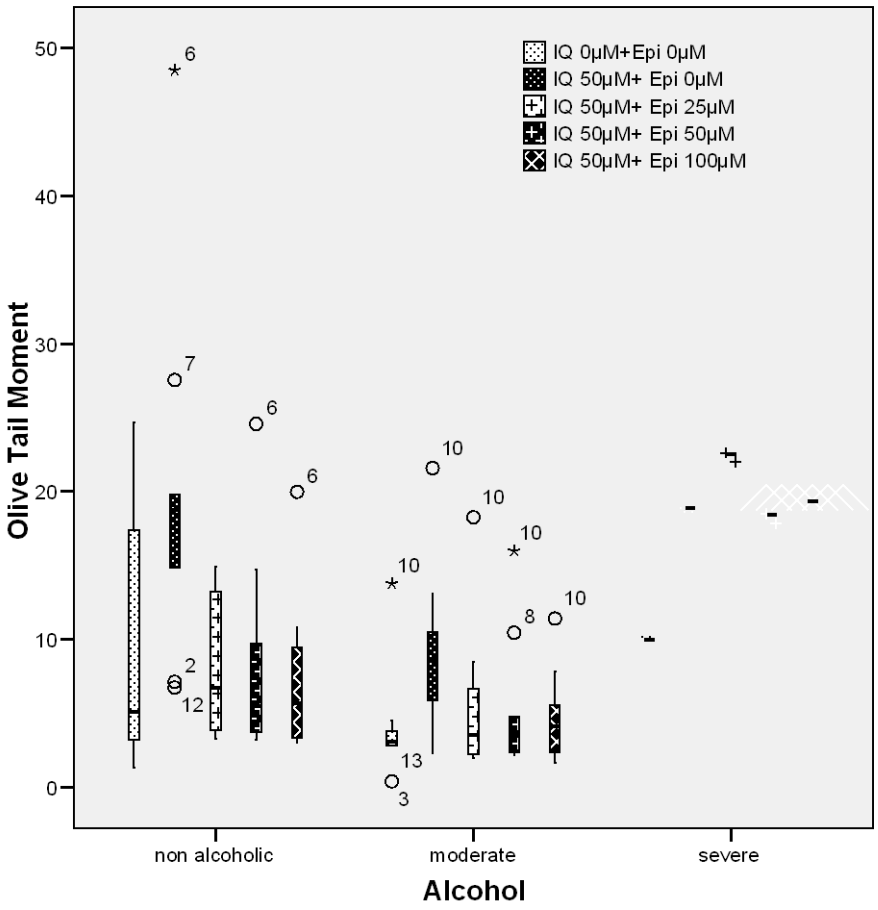


Figure 4.4.6. DNA damage in patients and controls (all subjects n = 20, 10 each group)relating to alcohol intake: non alcoholic (n = 7), moderate alcoholic (n = 7) and severe alcoholic (n = 6) groups after *in vitro* treatment with IQ (50 μg/ml) and supplementation with the flavonoid epicatechin at different concentration levels. The ∗ and ° symbols followed by a number indicate individual outliers with the respective patient number (Kruskal-Wallis (K-W) test).

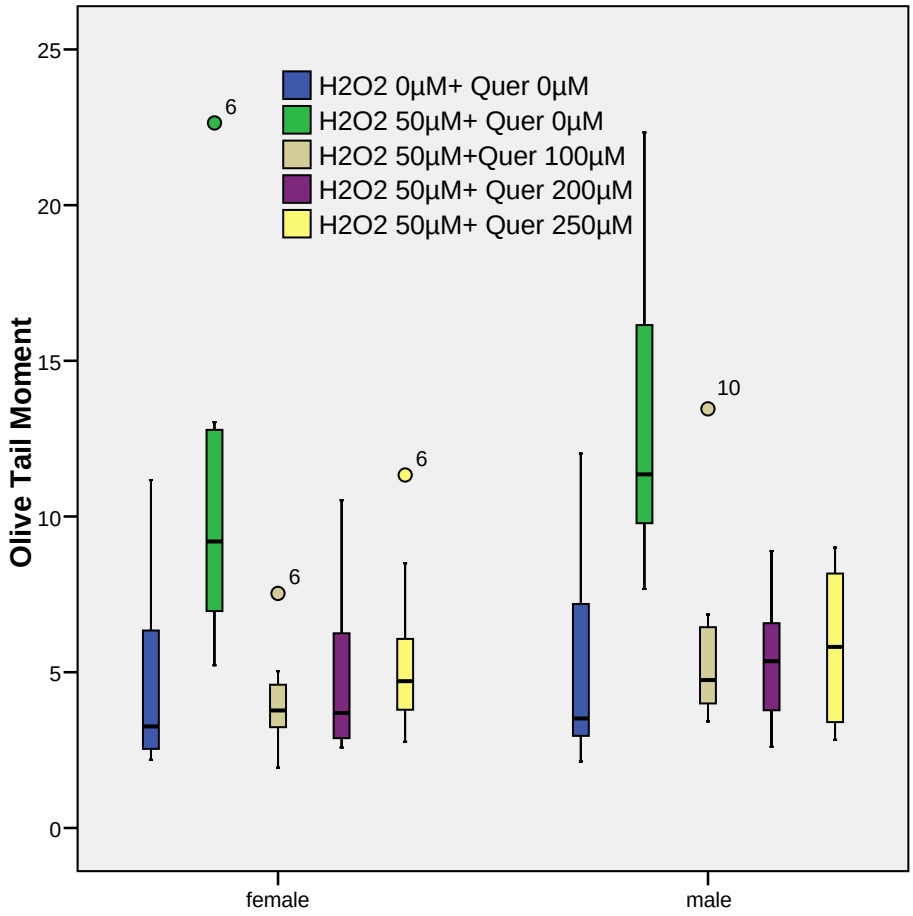


Figure 4.4.7. DNA damage in patients and controls (all subjects n = 20, 10 each group) regarding to gender: male (n = 11) and female (n = 9) groups, after *in vitro* treatment with H_2O_2 (50 µg/ml) and supplementation with the flavonoid quercetin at different concentration levels. The ° symbol followed by a number indicate individual outliers with the respective patient number (Kruskal-Wallis (K-W) test).

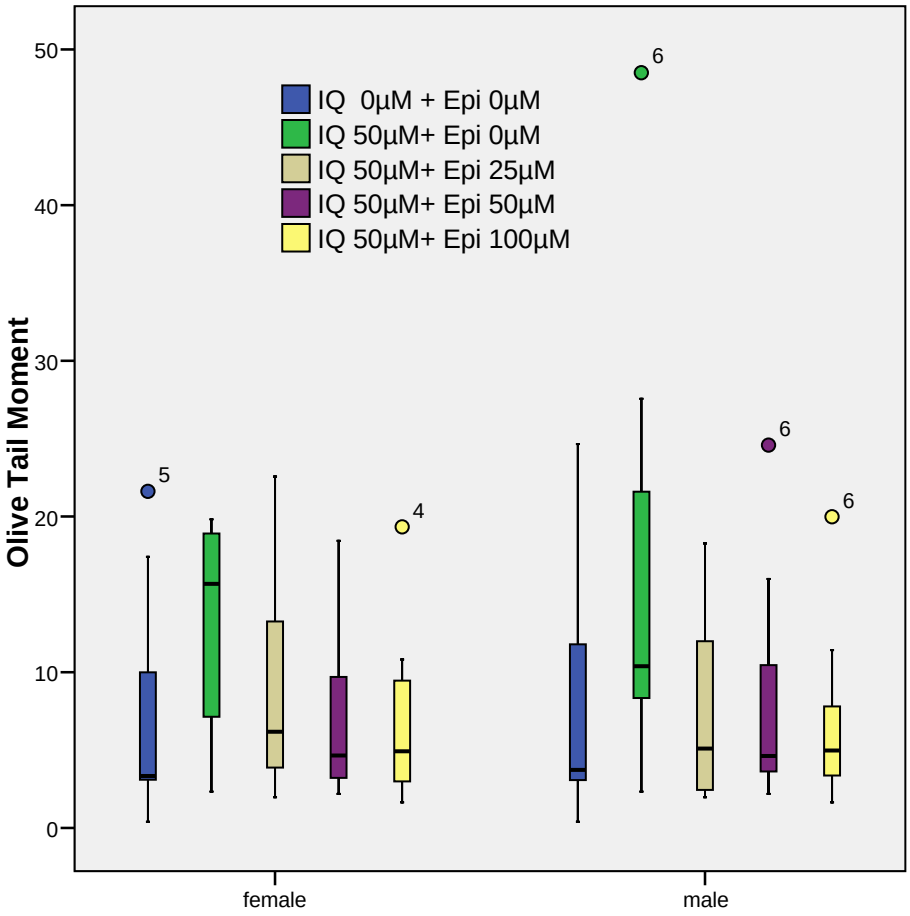


Figure 4.4.8. DNA damage in patients and controls (all subjects n = 20, 10 each group) relating to gender: male (n = 11) and female (n = 9) groups, after *in vitro* treatment with IQ (50 μg/ml) and supplementation with the flavonoid epicatechin at different concentration levels. The ° symbol followed by a number indicate individual outliers with the respective patient number (Kruskal-Wallis (K-W) test).

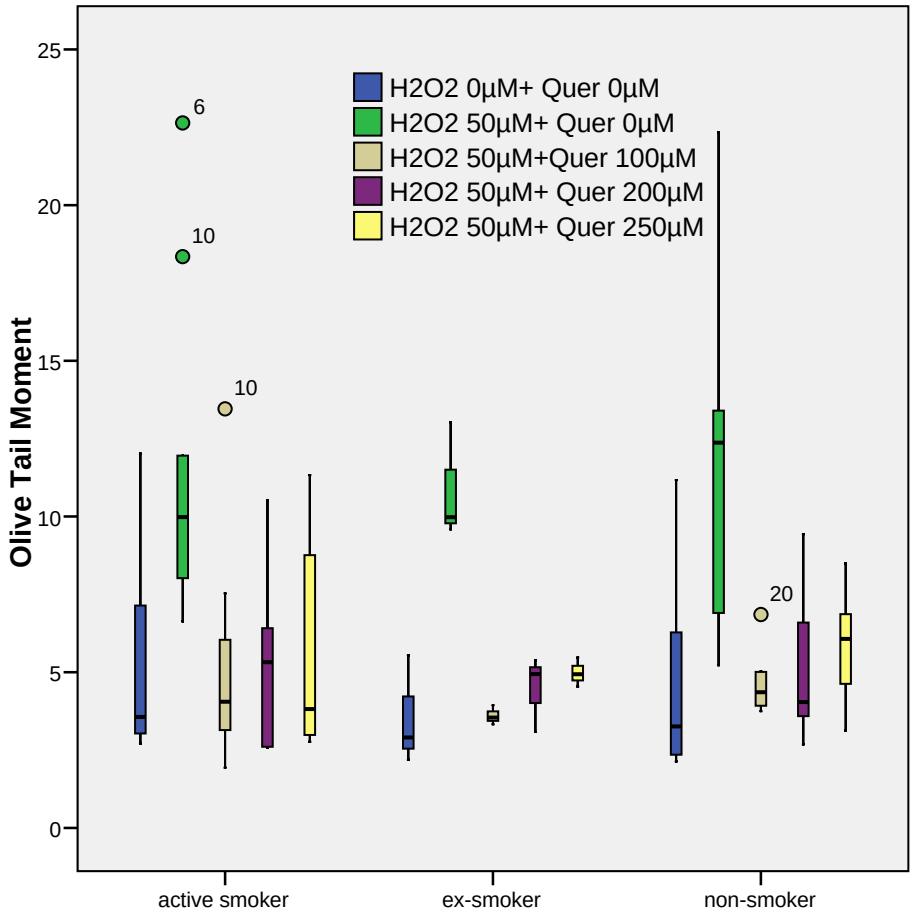


Figure 4.4.9. DNA damage in patients and controls (all subjects n = 20, 10 each group) relating to smoking: active smoker (n = 8), ex-smoker (n = 3) and non-smoker (n = 9) groups, after *in vitro* treatment with H_2O_2 (50 µg/ml) and supplementation with the flavonoid quercetin at different concentration levels. The ° symbol followed by a number indicate individual outliers with the respective patient number (Kruskal-Wallis (K-W) test).

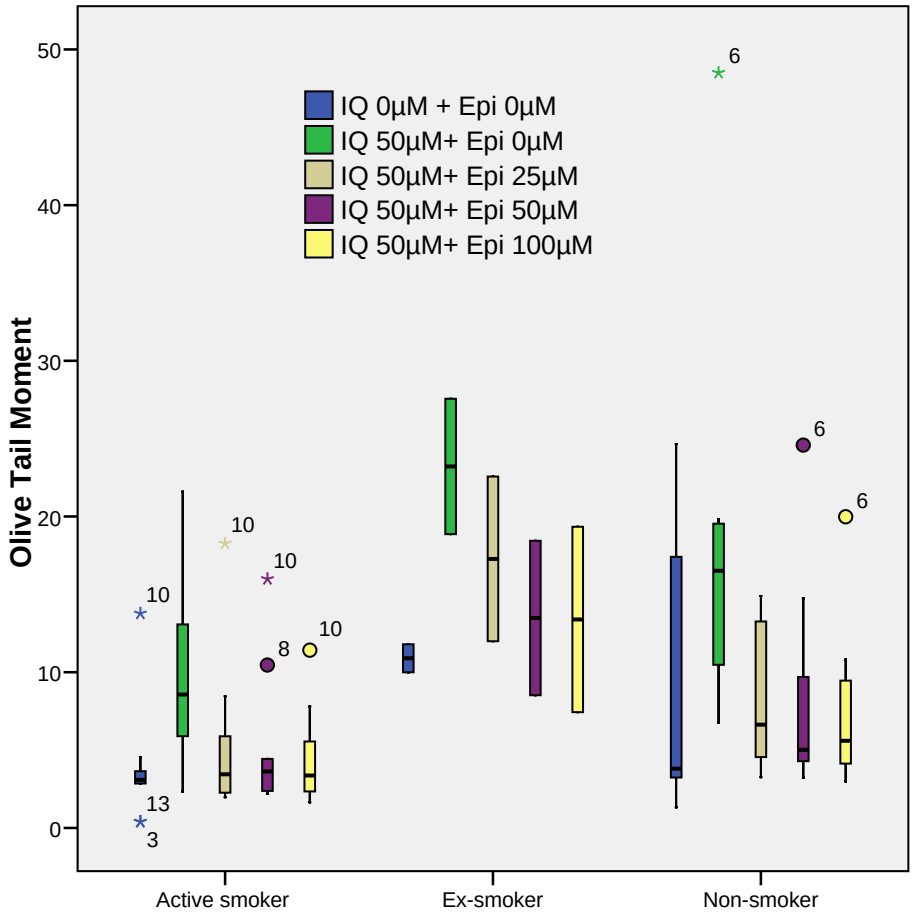


Figure 4.4.10. DNA damage in patients and controls (all subjects n = 20, 10 each group) relating to smoking: active smoker (n = 8), ex-smoker (n = 3) and non-smoker (n = 9) groups, after *in vitro* treatment with IQ (50 µg/ml) and supplementation with the flavonoid epicatechin at different concentration levels. The ∗ and ° symbols followed by a number indicate individual outliers with the respective patient number (Kruskal-Wallis (K-W) test).

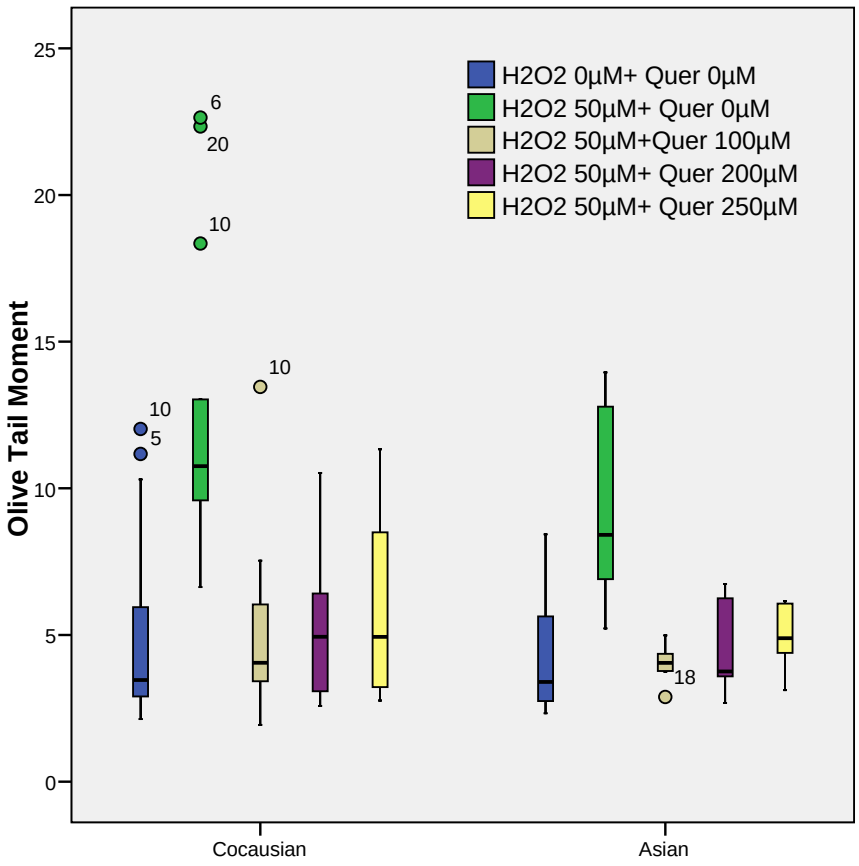


Figure 4.4.11. DNA damage in patients and controls (all subjects n = 20, 10 each group) relating to ethnicity: Caucasian (n = 12) and Asian (n = 8) groups, after *in vitro* treatment with H_2O_2 (50 µg/ml) and supplementation with the flavonoid quercetin at different concentration levels. The ° symbol followed by a number indicate individual outliers with the respective patient number (Kruskal-Wallis (K-W) test).

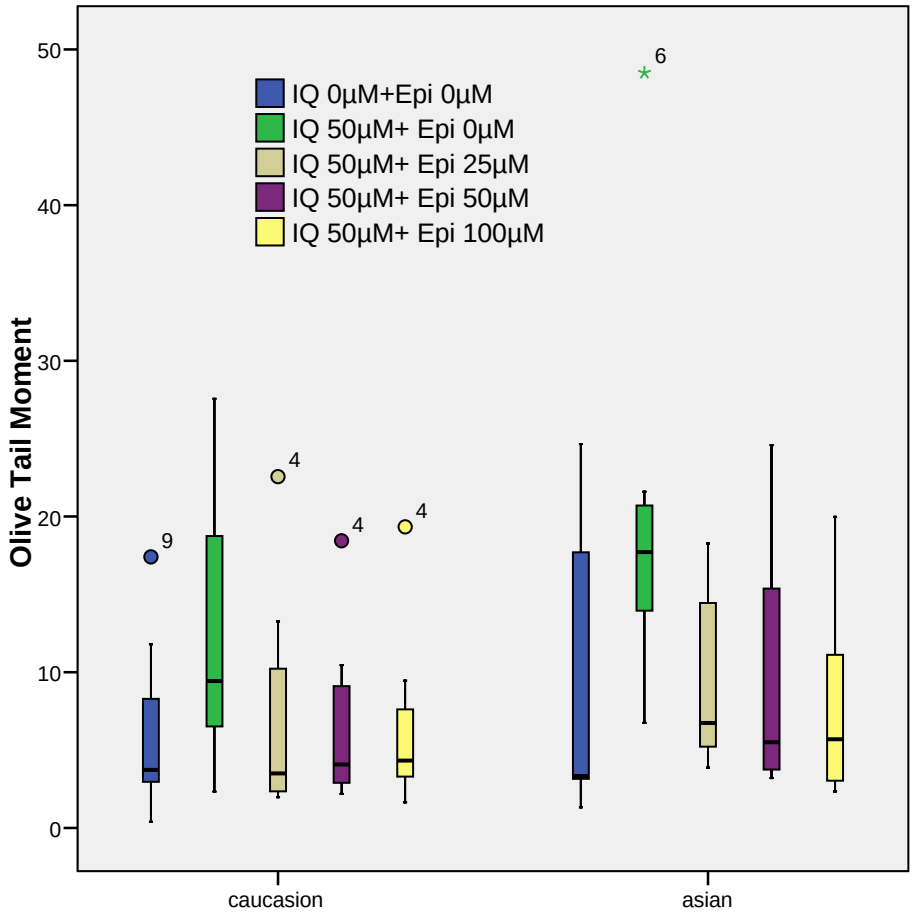


Figure 4.4.12. DNA damage in patients and controls (all subjects n = 20, 10 each group) relating to ethnicity: Caucasian (n = 12) and Asian (n = 8) groups, after *in vitro* treatment with IQ (50 µg/ml) and supplementation with the flavonoid epicatechin at different concentration levels. The ∗ and ° symbols followed by a number indicate individual outliers with the respective patient number (Kruskal-Wallis (K-W) test).

b) Previous medication in the IBD group as a confounding factor

As shown in figure 4.4.13 patients had been treated with a range of drugs for IBD, namely, azathioprine, mesalazine and pentasa, asacol, prednisolone, mercaptopurine alone or in combination prior to taking part in the study azathioprine & pentasa, azathioprine & mesalazine, mercaptopurine &

balsalazide (n = 6); asacol (n = 1); pentasa & prednisolone, prednisolone & mesalazine (n = 2);. Within the treatment groups, there appeared to be differences but they were not significant (figure 4.4.13).

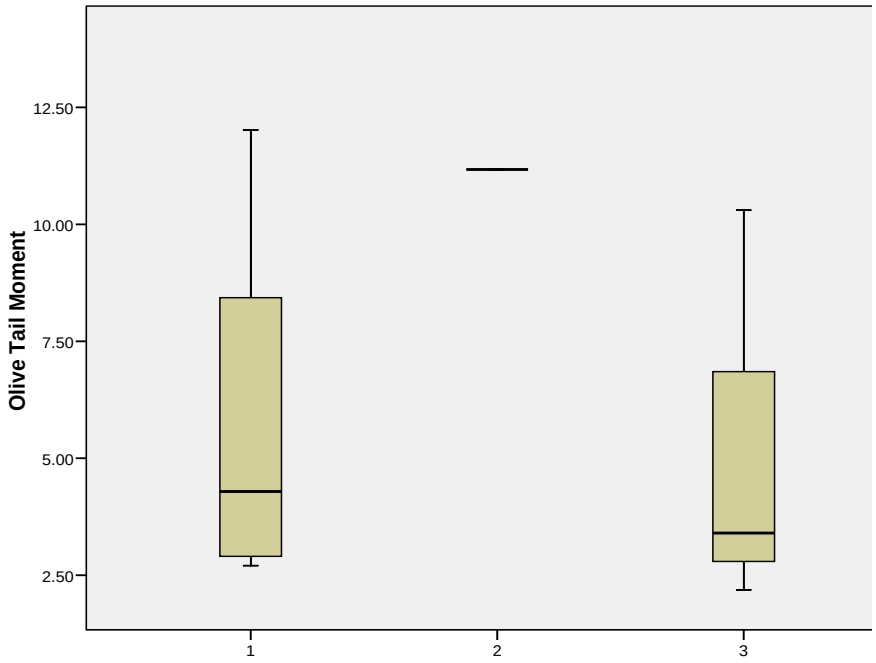


Figure 4.4.13. 1. Azathioprine, mesalazine and pentasa, asacol, prednisolone, mercaptopurine alone or in combination prior to taking part in the study azathioprine & pentasa, azathioprine & mesalazine, mercaptopurine & balsalazide (n = 6)

2. Asacol (n = 1)

3. pentasa & prednisolone, prednisolone & mesalazine (n = 2)

4.5. Discussion

Crohn's disease (CD) and Ulcerative colitis (UC), known as inflammatory bowel disease (IBD), are fairly common chronic inflammatory conditions of the gastrointestinal tract. Although the exact aetiology of IBD remains uncertain, dysfunctional immunoregulation of the gut is believed to be the main cause. Amongst the immunoregulatory factors, reactive oxygen species (ROS) are produced in abnormally high levels in IBD (Rezaie, 2007). An imbalance between antioxidants and ROS results in oxidative stress, leading to cellular damage (Rezaie, 2007).

Food-derived heterocyclic amines (HCA) like IQ have been shown to be mutagenic in the Ames test inducing gene mutations and tumours *in vivo* (Adamson, 1995; Knize, 1995). Food mutagens may cause different types of DNA damage from chromosomal aberrations to subtle nucleotide alterations. Most food mutagens like HCA are able to form reactive DNA adducts by covalently binding to nucleotides. However, the effect of food mutagens in carcinogenesis can be modified by heritable traits, namely, low-penetrant genes that affect exposure of the mutagen to DNA through metabolic activation and detoxification or other cellular responses to DNA damage. Also DNA damage seems to be indirectly triggered by oxidative stress. When considering the human diet, it should be recognized that food contains both, mutagens and components that decrease cancer risk such as antioxidants (Goldman, 2003; Maeda, 1999).

The present study demonstrates that H_2O_2 and IQ are capable of inducing significant DNA damage as a result of oxidative stress (Figures 4.4.1, 4.4.2). There was a significant increase of DNA damage after treating lymphocytes with H_2O_2 and IQ, while a significant protective effect was found in the presence of the flavonoids quercetin and epicatechin (Figures 4.4.1 and 4.4.2). Flavonoids are known to have antioxidative properties *in vivo* (Rice-Evans, 2001) and modulate effects of food mutagens *in vitro* in human lymphocytes and sperm (Anderson, 1998). Cooking fish and beef inevitably generate HCA especially at high temperatures (Schut, 1999), which are carcinogenic in mice, rats and monkeys producing hepatic, intestinal and mammary tumours (Schoeffner, 2000). For instance, AIA (aminoimidazoazaarene), categorised as a subclass of HCA, can be found in the human diet (Schut, 1999; Haza, 2010; Najafzadeh, 2009) and is only genotoxic after being activated to electrophilic derivatives that form DNA adducts (Hatch, 2001). A variety of host drug-metabolising enzymes are able to activate and detoxify heterocyclic amines including enzymes like CYP1A2, *N*-acetyltransferase, sulfotransferase, prolyl tRNA synthetase, phosphorylase and COX isomers (Wolz, 2000; Huycke, 2004). In a case-control study, no associations were found between CRC risk and polymorphisms within the genes of those enzymes (Sachse, 2002). This comprehensive analysis, however, failed to consider commensal bacteria and their potential impact on HCA activation, an effect independent of the host genotype. The pro-carcinogen IQ is predominantly produced through the pyrolysis of creatinine with sugars and becomes significantly mutagenic in the presence of hepatic microsomes (Sugimura, 1983; Turesky, 2007). Anaerobic colonic bacteria can convert IQ to 2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline-7-one (HOIQ), a direct-acting

mutagen (Bashir, 1987). Intestinal anaerobic bacteria like *Eubacterium* spp. and *Clostridium* spp. specifically metabolise IQ to HOIQ along with yet undefined commensal bacteria in mice, rats, and humans (Carman, 1988; Van Tassell, 1990; Rumney, 1993). These commensal bacteria can strongly influence IQ-induced DNA damage in colonic cells and also in hepatocytes as measured by the alkaline Comet assay (Knasmuller, 2001). DNA from axenic rats exhibited significantly fewer alkaline-labile breaks than rats colonised with conventional murine or human commensal bacteria. In contrast, other intestinal commensal bacteria including *Bifidobacterium longum* and lactobacilli appear to be antagonistic to the mutagenic effects of IQ (Reddy, 1993; Knasmuller, 2001). Mechanisms underlying these observations are unclear but may involve inactivation of IQ or direct binding of IQ to bacteria (Rumney, 1993). Modulating effects of two flavonoids, quercetin and rutin, on the mutagenic anticancer drug mitomycin C have been found using the Comet assay with human lymphocytes (Undeger, 2004). Quercetin significantly reduced DNA strand breakage induced by mitomycin C displaying protective effects supporting other findings where flavonoids modulated effects of food mutagens (Anderson, 1998). Flavonoids have a long history of use in preventing capillary fragility and bruising, but also some of the research has focused on their anticancer benefits (de Whalley, 1990; Ren, 2003; Mochizuki, 2010). Green tea and, to a lesser extent, black tea are a rich source of still another group of flavonoids called catechins. These catechins - including the closely related epigallocatechin-3 gallate (EGCG), epigallocatechin (EGC) and epicatechin-3 gallate (ECG) - form about 30 percent of the dry weight of tea leaves (de Whalley, 1990). It was found that drinking green tea could prevent cancer

(Jankun, 1997; Romier, 2009): EGCG in green tea has been identified as a potent inhibitor of urokinase, an enzyme used by cancer cells to invade and metastasise. A single cup of green tea contains enough EGCG to temporarily inhibit urokinase activity - and is safer than synthetic drugs that block urokinase activity.

In conclusion, flavonoids significantly reduce oxidative stress *in vitro* in lymphocytes of IBD patients and more so in CD than UC patients as well as healthy individuals. Thus, a diet containing flavonoids could be very effective in reducing baseline and exogenously induced oxidative DNA damage of IBD patients.

CHAPTER FIVE

UVA & PRE CANCEROUS AND CANCER

STATES

5. Introduction

In this chapter lymphocytes from patients with cancerous and pre-cancerous states were exposed to UVA and compared with lymphocytes after UVA exposure in healthy control individuals.

5.1.1. UVA exposure

UVA (315-400 nm) (Figures 5.1.1, 5.1.2) radiation can have significant effects on the cells. DNA is certainly one of the key targets for UV-induced damage in a variety of organisms ranging from bacteria to humans. The most prevalent DNA lesions induced by UVB and UVA are the cyclobutane pyrimidine dimers (CPDs) and the pyrimidine (6-4) pyrimidine photoproducts ((6-4) PPs). However, cells have developed a number of repair or tolerance mechanisms to counteract the DNA damage caused by UV or any other stressors (Sinha, 2002).

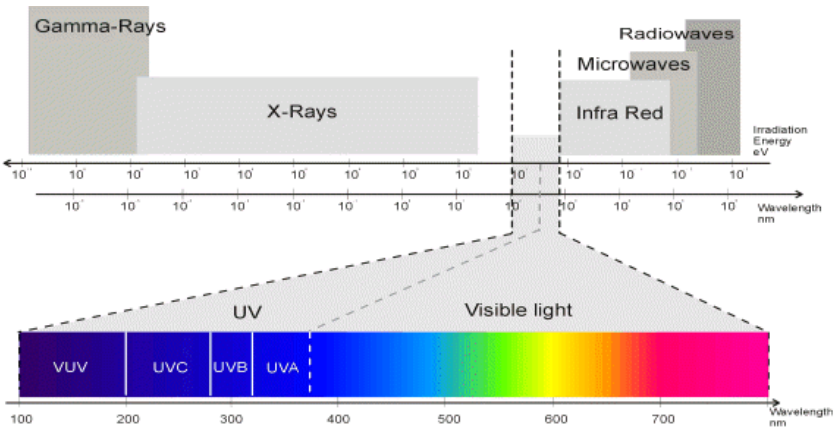


Figure 5.1.1.Electromagnetic spectrum 1

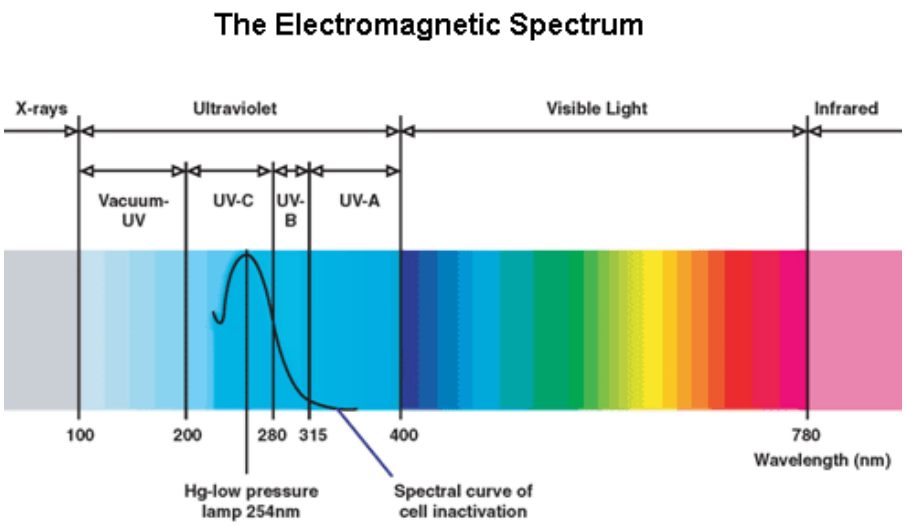


Figure 5.1.2.Electromagnetic spectrum 2

Intracellular and extracellular oxidative stress initiated by reactive oxygen species (ROS) advances skin aging, which is characterized by wrinkles and atypical pigmentation. Because UV enhances ROS generation in cells, skin aging is usually discussed in relation to UV exposure (Masaki, 2010).

Constitutive skin pigmentation dramatically affects the incidence of skin cancer, and the photoprotective function of melanin in skin is highly significant. The rate of malignant melanoma even squamous and basal cell carcinomas is higher in Caucasians than in African Americans. Cyclobutanate-pyrimidine dimmers (CPDs) and 6-4 photoproducts (6-4 PPs) are the products resulting from UV damage on DNA lesions which accelerates production in Caucasians because of the lack of melanin (Tadokoro, 2003).

In a study by Kimura, Lee et al 2010, UV-induced cancer cell death was wave-length and dose dependent, as well as cell-line dependent. After UVA exposure, most cells were viable even when the UV dose was increased up to 200 J/m. With UVB irradiation, cell death was observed with irradiation at 50 J/m (Kimura, Lee et al. 2010).

The repair of damaged DNA takes place in G1/G2 phases of cell cycle, the wild type p53 plays a major role in the induction of transient G1 and/or G2 arrests, and the expression of gadd45 (growth arrest and DNA damage gene) and gadd153 is also associated with the cell cycle arrest and DNA damage. Transient cell cycle arrest and the expression of p53, gadd45 and gadd153 in normal human oral keratinocytes, HPV-immortalized oral keratinocytes, and an oral cancer cell line expressing mutant p53 were investigated after exposing cells to UV light. The level of gadd45 transcripts was enhanced in all tested cells, but normal cells demonstrated a higher increase in the level of gadd45 after UV-exposure compared to other tested cells. The level of gadd153 gene transcripts was only increased in normal oral keratinocytes after UV-irradiation. These data indicate that UV-induced transient G1 arrest in normal oral keratinocytes may be associated with both enhanced levels of intranuclear wild type p53 (Gujuluva, 1994).

Protein damage is incredible in the process of inactivation by sunlight. Protein damage in UVA-irradiated *Escherichia coli* cells has been evaluated by an immunoblot method for carbonylated proteins and an aggregation assay based on semi-quantitative proteomics. A wide spectrum of structural and enzymatic proteins within the cell is affected by carbonylation and aggregation. Vital cellular functions like the transcription and translation apparatus, transport systems, amino acid synthesis and degradation, respiration, ATP synthesis, glycolysis, the TCA cycle, chaperone functions and catalase are targeted by UVA irradiation. The protein damage pattern caused by SODIS (Summary Solar disinfection) strongly resembles the pattern caused by reactive oxygen stress.

Hence, sunlight probably accelerates cellular senescence and leads to the inactivation and finally death of UVA-irradiated cells (Bosshard, 2010).

5.1.2. Oxidative stress and cancers

Oxidative DNA damage is an unavoidable consequence of cellular metabolism (Cooke, 2003) and it affects constantly the carcinoma cells *in vivo* and *in vitro* (Brown, 2001) (Figure 5.1.3).

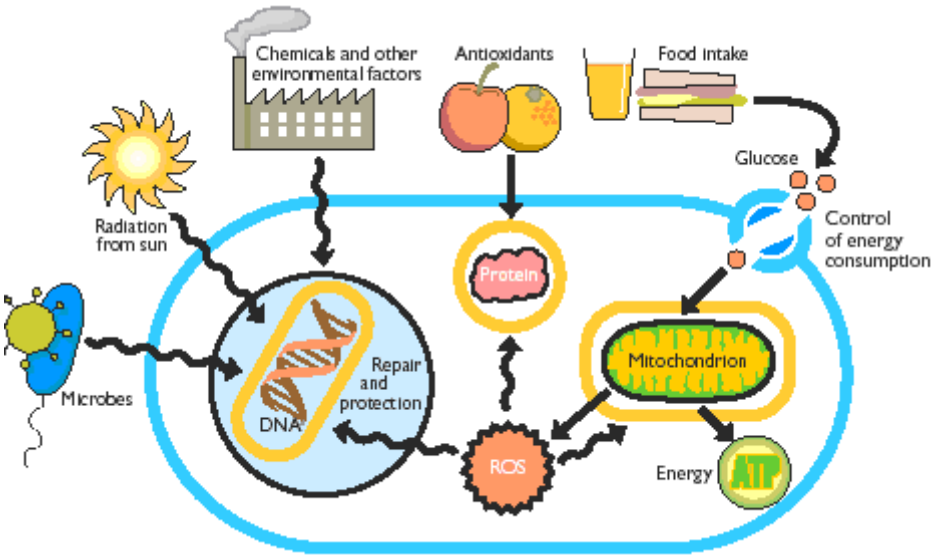


Figure 5.1.3. Oxidative stress and environment

Oxidative stress results from cellular metabolism and interaction with cells of exogenous sources such as carcinogenic compounds, redox-cycling drugs and ionizing radiation (Cooke, 2003). Superoxide and a significant amount of reactive oxygen species (ROS) are generated during mitochondrial oxidative phosphorylation (Yakes, 1997). There are some assays showing that mtDNA is a

critical cellular target for ROS and cancer and chronic inflammatory diseases generate ROS as part of the pathophysiological mechanism (Moller, 2002 ; Yakes, 1997). Cells have developed a complex network of defence barriers to neutralize the generation of ROS and protect against the oxidation of macromolecules by scavenging ROS (Moller, 2002). Ultraviolet A (UVA) and hydrogen peroxide (H_2O_2) both generate reactive oxygen species (ROS) making them relevant in the study of skin cell responses to oxidative stresses (Hazane-Puch, 2010). This effect may be the result of a two-faced character of ROS acts as secondary messengers in intracellular signalling cascades, which induce and maintain the oncogenic phenotype of cancer cells and also induce cellular senescence and apoptosis (Valko, 2006). A novel feature of our study is that we used peripheral lymphocytes as target cells instead of cancer cells directly, since the genome damage to the lymphocytes of peripheral blood has been widely used as a biomarker of genotoxic environmental factors, and long-term studies have demonstrated its validity and high clinical productivity (Hagmar, 2004) (Figure 5.1.4). Possibly both oxidative stress and antigen-mediated preferential cell death of antigen-experienced memory cytolytic T lymphocytes (CTLs) may be a major contributor to tumour-induced immune dysfunction and phagocyte-derived ROS have been ascribed a suppressive role in immunoregulation by inducing dysfunction and apoptotic cell death in lymphocytes (Thoren, 2007).

The oxygen radicals have been projected to contribute to the dysfunction of cytotoxic lymphocytes characteristic of malignant disorders and chronic

infections (Hellstrand, 2003). The oxygen radical-induced inactivation of lymphocytes may constitute a significant mechanism of immunosuppression and the role of cytotoxic lymphocytes in malignant diseases (Gelderman, 2006). Although oxygen radical-induced inhibition of lymphocyte function thus may contribute to protection against autoimmunity, a reverse situation appears the approach in malignant diseases. Thus, oxygen radicals, formed by phagocytic cells at the site of malignant expansion, are believed to significantly contribute to the characteristic state of energy (effectively freezing T cell responses pending a "second signal" from the antigen-presenting cell) of cytotoxic lymphocytes in, e.g., colorectal cancer, malignant melanoma, renal cell carcinoma, and certain hemopoietic cancers, in addition to chronic viral infections such as HIV and hepatitis C (Samlowski, 2003; Hellstrand, 2003). In the origin of several diseases as well as cancer, oxidative damage to the cellular macromolecules occurs (Chatterjee, 1989). Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged and there are several enzyme systems within the body that scavenge free radicals. The same as for other cancers, imbalance in the redox status of oral cancer patients may be due to enhanced lipid peroxidation and compromised antioxidant defences (Subapriya, 2003). The biomarkers of oxidative stress and inflammation can be produced and modulateed by an antioxidant micronutrient cocktail in humans (Hopkins, 2010). ROS are independent of TP-triggered (thymidine phosphorylase) signalling transduction and are associated with increased tumour invasion. Thus ROS may provide improved therapeutic results as well as a preventative effect on carcinogenesis of the colorectum (Inokuma, 2009).

Antioxidants are intimately involved in the prevention of cellular damage; the common pathway for cancer, aging, and a variety of diseases (Thompson, 2004). The modifications caused by ROS and RNS may result in gene mutation and membrane alterations, as it has been shown for the tumour suppressor gene p53 (Pacher, 2007) and ceramide release (Grether-Beck, 2000), respectively, following UVA irradiation. A recent study showed the vulnerable cancer cells can be more sensitive to UVA radiation than normal skin cells because they are unable to repair themselves as efficiently (Tang, 2010).

5.1.3. Malignant melanoma and suspected melanoma

As mentioned before cutaneous malignant melanoma, is the most lethal form of skin cancer, which has become an important public health issue because in the U.K. over 8,000 cases are currently diagnosed each year and the numbers are steadily rising. Worldwide, the number of new cases of melanoma is increasing faster than any other cancer and melanoma mortality is also rising (Vasquez-Moctezuma, 2010). These figures make melanoma an important cancer for study. The majority of melanomas arise sporadically but in about 5% of cases there is a history of melanoma in two or more close relatives. Cases of familial cancer have been very informative in learning more about the genetics of sporadic cases in other cancers, e.g. colon cancer (Psaty, 2010). Two high-risk genes have been implicated in the development of malignant melanoma. Germ line mutations of the CDKN2A gene are found in < 25% of melanoma-prone families and there are

only seven families with mutations of the CDK4 gene reported to date. The CDKN2A locus encodes two gene products involved in cell growth regulation (Peric, 2008). It is identified germ line CDKN2A mutations in 31.3% of families; this figure rises to 50% in families with three or more first-degree relatives. By collaborating with international colleagues to demonstrate that the M53I mutation in the CDKN2A gene, which is present in a proportion of melanoma families' worldwide, appears to have arisen in the North of England, and it is clear that there remain two-thirds of melanoma families who must also have a gene abnormality (Lang, 2007). Exons and introns refer to specific nucleotide base sequences in the genetic code that are involved in producing proteins.

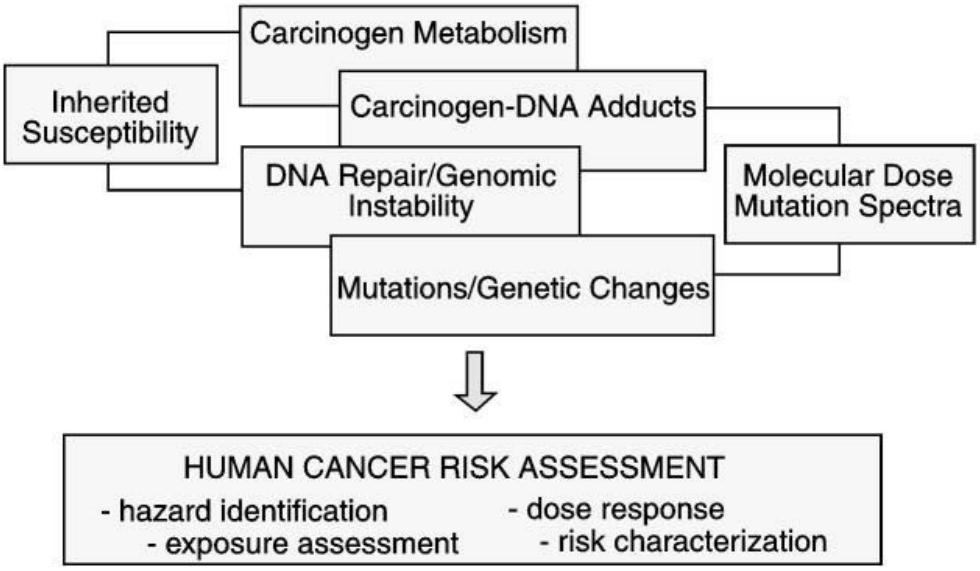


Figure 5.1.4.Cancer and risk factors

Exons are the DNA bases that are transcribed into mRNA and eventually code for amino acids in the proteins and transcription from exon 2 and 3 is shared by

p16 and p14, in different reading frames. P16 initiates within unique exon 1 and p14 also within exon 1, which is located approximately 20 kbp upstream of exon 1. Melanocytes are producing melanin after UV irradiation as a defence mechanism. However, UV-induced damage is involved in melanoma initiation, depending on skin photo type. Melanocytes seem to be extremely susceptible to free radicals. Their main enzymatic antioxidants are superoxide dismutase and catalase (Baldea, 2009).

As the MMR enzyme (DNA mismatch repair) hMSH2 displays a p53 target gene, is induced by UVB radiation and is involved in NER pathways, studies have now been initiated to elucidate the physiological and pathophysiological role of MMR in malignant melanoma and nonmelanoma skin cancer development (Rass, 2008). Also, it is considered that UVA and UVB are equally critical players in melanoma formation. Whereas UVA can indirectly damage DNA through the formation of reactive oxygen radicals, UVB can directly damage DNA causing the apoptosis of keratinocytes by forming the sunburn cells. Besides action through mutations in critical regulatory genes, UV radiation may promote cancer through indirect mechanisms, e.g. immunosuppression and dysregulation of growth factors. The carcinogenic process probably involves multiple sequential steps, some, but not all of which involve alterations in DNA structure (Situm, 2007).

5.1.4. Colorectal cancer and polyposis coli

Colorectal cancer is a major cause of morbidity and mortality among various types of cancer in the Western world (Yang, 1999). Sporadic disease, in which there is no family history, accounts for approximately 70 percent of all CRCs. It is most common over the age of 50, and dietary and environmental factors have been etiologically implicated. Age is a major risk factor for sporadic CRC. It is a rare diagnosis before the age of 40, the incidence begins to increase significantly between the ages of 40 and 50, and age-specific incidence rates increase in each succeeding decade thereafter (Neugut 1990). One of the most common diagnosed cancers in human is colorectal cancer (Ozdemirler Erata, 2005). Colorectal cancer is associated with oxidative stress and also Ihsp70 (inducible heat shock protein 70) expression is suppressed under induced oxidative stress conditions in malignant tissues of patients with colorectal cancer (Ozdemirler Erata, 2005; Chang, 2008). The most common sort of polyp is a metaplastic polyp which can be very similar in appearance to adenomatous polyps also it has the potential to become malignant. In addition, familial polyposis coli develops multiple adenomatous polyps bears a very high risk of colon cancer (Stigliano, 2008).

Reactive oxygen and nitrogen species created by inflammatory cells can interact with key genes involved in carcinogenic pathways such as p53, DNA mismatch repair genes, and even DNA base excision-repair genes although NF-κB and cyclooxygenases may also contribute (Itzkowitz, 2004). The gene with mutations that result in familial adenomatous polyposis (FAP) has been identified as

adenomatous polyposis coli (APC). Similarly, mutations in several genes that normally function in DNA mismatch repair result in HNPCC. Colorectal cancer is the result of accumulated mutations in several additional oncogenes or tumour suppressor genes, and this information leads to the formulation of a genetic model for the disease. Recent studies have also identified a relatively prevalent polymorphism in the APC gene in Ashkenazi Jews that is associated with an increased risk for colorectal cancer. The risk factors for CRC are both environmental and inherited. These studies present a paradigm based on the APC mutation (APC I1307K) for the screening of cancer susceptibility genes in the population at large. Significant progress has been made in understanding the molecular mechanisms that lead to it (Yang, 1999). The connection between inflammation and tumourigenesis is well-established and in the last decade has received a great deal of supporting evidence from genetic, pharmacological, and epidemiological data. Inflammatory bowel disease is an important risk factor for the development of colon cancer. Inflammation is also likely to be involved with other forms of sporadic as well as heritable colon cancer (Terzic, 2010). One study has elucidated the role of distinct immune cells, cytokines, and other immune mediators in virtually all steps of colon tumorigenesis, including initiation, promotion, progression, and metastasis (Terzic, 2010). The "serrated neoplastic pathway" describes the progression of serrated polyps, including sessile serrated adenomas and traditional serrated adenomas, to colorectal cancer. In the past, all serrated polyps (Terzic, 2010) were classified simply as hyperplastic polyps and were considered to have no malignant potential. Reappraisal of this view was largely driven by increasing recognition of the malignant potential of hyperplastic polyposis (Terzic, 2010). The acquisition of

genomic instability is a crucial feature in tumour development and there are at least 3 distinct pathways in colorectal cancer pathogenesis: the chromosomal instability (CIN), microsatellite instability, and CpG island methylator phenotype pathways. Most cases of colorectal cancer arise through the CIN pathway, which is characterized by widespread imbalances in chromosome number (aneuploidy) and loss of heterozygosity (Pino, 2010). Between 2% to 5% of all colon cancers arise in the setting of well-defined inherited syndromes, including Lynch syndrome, familial adenomatous polyposis, MUTYH-associated polyposis, and certain hamartomatous polyposis conditions (Jasperson, 2010). In addition to the syndromes, up to one-third of colon cancers exhibit increased familial risk, likely related to inheritance. A number of less penetrant, but possibly more frequent susceptibility genes have been identified for this level of inheritance. Clarification of predisposing genes allows for accurate risk assessment and more precise screening approaches (Jasperson, 2010).

In the present study peripheral blood lymphocytes from cancerous and precancerous states were exposed to UVA and compared with similarly exposed lymphocytes from healthy control individuals. They were examined with the micronucleus and Comet assays in order to determine if there was any differential sensitivity to exposure within the different groups.

5.2. Material and methods

5.2.1. Blood samples

Peripheral whole blood was collected by venepuncture from 80 (male and female) individuals (5 groups: MM, SM, and CRC, PC and healthy control individuals, 20 individuals per group). Ages range from 20 to 78 years old for the patients' groups and from 24 to 58 years old for the healthy control group. MM and SM patients have received treatment at the Dermatology Department of the St Luke's Hospital in Bradford, UK. CRC and PC patients have been treated at the Colorectal Surgery Department of St. Luke's Hospital and Bradford Royal Infirmary (BRI), Bradford, West Yorkshire, UK. Twenty healthy volunteers were recruited within the Division of Biomedical Sciences at the University of Bradford (West Yorkshire, UK). Ethical permission was obtained from both the BRI Local Ethics Committee (reference numbers 04/QI202/132 and 04/Q1202/15) and the University of Bradford's sub-committee of Research Ethics involving Human Subjects (reference number 0405/8).

5.2.2. Questionnaire for patients and controls

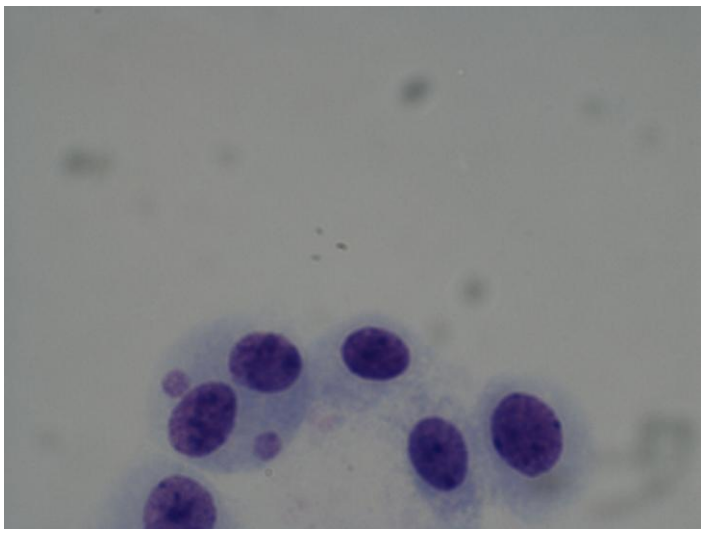
A questionnaire was administered to each donor immediately after taking the blood sample. The completed questionnaire for the patient and control groups provided essential information about lifestyle, confounding factors and medical treatment or any other drug intake. (The sample of questionnaire is in the appendix1)

5.2.3. UVA source

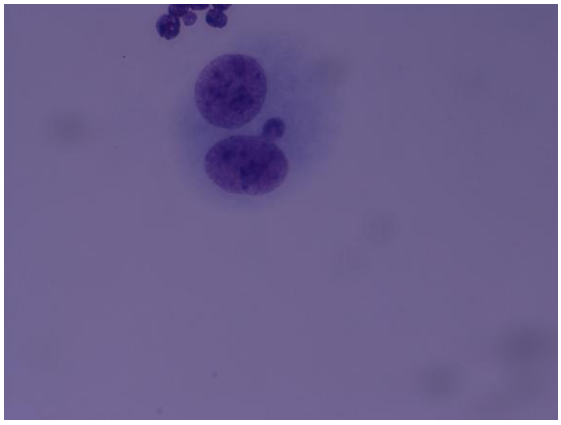
For UVA exposure, a table-top lamp housing two 15W PUVA tubes (Waldmann, Villingen-Schwenningen, Germany; bought from Athrodax Healthcare International Ltd, UK) was used. The spectrum of the PUVA tube ranged from 320-410 nm with a maximum at 351 nm. The lamp was positioned 15 cm above the cell layer. For the micronucleus assay, the cells were irradiated in suspension in Corning 25 cm^2 plastic culture flasks (VWR, UK), placed horizontally on the bench under the UV lamp. For the Comet assay, cells were exposed embedded in 0.5% agarose on slides.

5.2.4. The cytokinesis block micronucleus (CBMN) assay

The materials and methods used in this study have been described in section 2.2.



Photograph 5.2.1.Two BiMNis in binucleated cell in SM patient



Photograph 5.2.2. A BiBud in polyposis coli patient

5.2.5. The alkaline Comet assay

Isolated lymphocytes were isolated from whole blood of all patients and healthy individuals using Lymphoprep according to the manufacturer's protocol (Axis Shield, Norway). Lymphocytes were treated with UVA for 15 minutes at 37 ºC at a mean sample surface intensity of 1.53 ± 0.01 $mWcm^{-2}$. Untreated lymphocytes served as a negative control. 20 patients of each SM, MM; and 20 patients of each PC, CRC; and 20 control individuals were used for the investigation.

For each replicate slide, 50 cells were scored (100 cells in total from every sample). The % Tail DNA and Olive tail moment, a measure of tail moment length and the fraction of DNA in the Comet tail, was used to assess DNA damage (Tice, 2000).

5.3. Results

5.3.1. Micronucleus assay

5.3.1.1. Healthy control individual group (HCI)

After UVA treatment, lymphocytes of healthy individuals showed a statistically significant reduction in CBPI from 1.84 to 1.62 for UVA ($p < 0.001$) and to 1.67 for MMC ($p < 0.01$) due to a significant decrease in binucleated cells (BiNC) as seen in Table 5.3.1. Additionally, UVA treatment significantly induced micronuclei in cytokinesis-blocked lymphocytes almost to the levels of the positive control MMC. The number of binucleated cells containing MN increased 2.8-fold (BiMN; $p < 0.001$) (Figure 5.3.1), but also the number of mononucleated cells with MN increased a significant 4-fold (MonoMN; $p < 0.01$). However, there were no statistically significant differences in the number of nucleoplasmic bridges and nuclear buds (BiNPB and BiBud) found in BiNC (Table 5.3.1).

	Treatment	CBPI	%BiNC	BiMN	BiNPBs	BiBuds	MonoMN
Healthy control	Untreated	1.84 ± 0.08	56.03 ± 5.46	9.86 ± 2.64	0.00 ± 0.00	0.93 ± 0.97	2.71 ± 0.97
	UVA	1.62 ± 0.04 ***	41.80 ± 3.84 ***	27.50 ± 1.02 ***	0.60 ± 1.05	0.86 ± 0.67	10.79 ± 5.47 **
	MMC	1.67 ± 0.02 **	47.60 ± 3.26 **	32.71 ± 3.54 **	0.14 ± 0.87	1.43 ± 0.98*	12.00 ± 6.89 **
SM patients	Untreated	1.61 ± 0.02 §§	49.70 ± 2.03 §§	20.00 ± 3.40 §§	0.00 ± 0.00	0.00 ± 0.00	2.00 ± 1.76
	UVA	1.47 ± 0.03 ‡‡ ¥	37.90 ± 1.42 ‡‡	23.00 ± 1.57	0.00 ± 0.00	0.00 ± 0.00	7.00 ± 5.42 ‡
	MMC	1.36 ± 0.02 ‡‡‡ ¥¥	30.30 ± 3.45 ‡‡‡ ¥	44.00 ± 2.46 ‡‡	0.00 ± 0.00	4.00 ± 1.56 **‡‡	2.50 ± 0.42
MM patients	Untreated	1.73 ± 0.04 §	52.30 ± 3.75 §§	22.00 ± 1.89 §§§	0.20 ± 1.42	4.20 ± 0.87 §	4.20 ± 3.64 §§
	UVA	1.52 ± 0.03 ‡‡	46.50 ± 1.78 ‡‡	40.67 ± 2.16 ‡‡ ¥	0.20 ± 0.78	6.40 ± 1.35* ‡	6.444 ± 2.63
	MMC	1.67 ± 0.03 ‡	49.70 ± 2.34 ‡	45.89 ± 2.85 ‡‡‡ ¥	0.40 ± 0.93	6.50 ± 1.12 *‡	10.50 ± 4.85 ‡
PC patients	Untreated	1.63 ± 0.06 §§	43.43 ± 4.46 §§§	13.41 ± 4.25 §	0.30 ± 1.23	1.60 ± 1.33 §	4.80 ± 2.67 §
	UVA	1.38 ± 0.03 ‡‡‡ ¥¥	31.13 ± 3.02 ‡‡‡ ¥	33.17 ± 3.93 ‡	0.30 ± 0.75	4.00 ± 2.02** ‡‡	16.50 ± 11.33 ‡‡
	MMC	1.45 ± 0.03 ‡‡ ¥¥	37.35 ± 2.87 ‡‡	33.08 ± 3.27 ‡	0.17 ± 1.14	0.17 ± 0.56***	15.00 ± 6.89 ‡
CRC patients	Untreated	1.61 ± 0.04 §§	42.88 ± 2.56 §§§	23.89 ± 1.66 §§§	0.20 ± 0.00	2.50 ± 1.79 §§	3.60 ± 2.63 §
	UVA	1.31 ± 0.03 ‡‡‡ ¥¥	26.04 ± 1.65 ‡‡‡ ¥	34.40 ± 2.43 ‡	0.00 ± 0.00	1.60 ± 1.34* ‡	22.60 ± 12.43 ‡‡‡
	MMC	1.51 ± 0.02 ‡ ¥	30.30 ± 1.73 ‡‡ ¥	51.00 ± 1.45 ‡‡‡ ¥¥	0.00 ± 0.00	0.20 ± 0.12***	21.40 ± 14.63 ‡‡

Table 5.3.1. The effect of UVA treatment on peripheral blood lymphocytes from suspected melanoma (SM), malignant melanoma (MM), polyposis coli (PC) and colorectal cancer (CRC) patients compared to healthy control individuals.

*, ‡, §, ¥ - $p < 0.05$, , ‡‡, §§, ¥¥ - $p < 0.01$, , ‡‡‡, §§§, ¥¥¥ - $p < 0.001$;

* indicates the level of statistical significance when comparing lymphocytes from healthy control individuals after treatment with UVA and MMC to untreated lymphocytes;

‡ indicates the level of statistical significance when comparing lymphocytes from patients after treatment with UVA and MMC to untreated lymphocytes;

§ shows the level of statistical significance when comparing untreated lymphocytes from patients to healthy control individuals;

¥ Shows the level of statistical significance when comparing treated lymphocytes from patients to treated healthy control individuals;

5.3.1.2. Suspected melanoma (SM) and malignant melanoma (MM) patients

5.3.1.2.1. Groups

For both groups, the mitotic index CBPI was significantly decreased from 1.61 (SM group) and 1.73 (MM group) to 1.47 and 1.52, respectively, after UVA treatment (both $p < 0.01$) and to 1.36 ($p < 0.001$) and 1.67 ($p < 0.05$), respectively, after MMC treatment (Table 5.3.1). Similarly, this was also observed for the percentage of BiNC in 1000 scored cells (mono-, bi- and, multinucleated). UVA treatment also induced cytogenetic damage significantly increasing the frequency of BiNC carrying MN. The significant increase of 1.1-fold ($p < 0.05$) for the SM group and 1.9-fold ($p < 0.01$) for the MM-group did not reach the increase in damage afflicted by the positive control compound MMC of more than 2-fold ($p < 0.05$ and $p < 0.001$, respectively) (Table 5.3.1). For SM patients the frequency of MonoMN significantly increased by 3.5-fold ($p < 0.05$). This was not seen for lymphocytes from MM patients after UVA treatment. MMC treatment resulted only in a significant increase in MonoMN in the MM group (2.5-fold, $p < 0.05$). Although there was no statistically significant increase in observed BiNPB, the nuclear buds observed in BiNC increased significantly. For the SM group, while the UVA treatment did not induce any BiBud, MMC treatment did (4 in 1000 BiNC, $p < 0.01$). For the MM group, UVA as well as MMC treatment resulted in significant increases in frequencies of BiBud from 4 per 1000 BiNC to around 6.5 per 1000 BiNC ($p < 0.05$).

When both groups were compared to the group of healthy control individuals (Table 5.3.1, Figure 5.3.1), untreated lymphocytes from suspected melanoma and the malignant melanoma patients showed significantly lower CBPI frequencies, from 1.84 to 1.61 ($p < 0.01$; SM group) and to 1.73 ($p < 0.05$; MM group), which can also be seen in the reduction of the percentage of BiNC. Additionally, the basic

cytogenetic damage (induced BiMN) was significantly increased in untreated lymphocytes from SM and MM patients by 2-fold ($p < 0.01$) and 2.2-fold ($p < 0.001$), respectively (Table 5.3.1, Figure 5.3.1). Neither frequencies of BiNPB, BiBud and MonoMN were significantly increased in the SM group; however, BiBud and MonoMN frequencies were significantly increased in the MM group by 4.5-fold ($p < 0.05$) and 1.5-fold ($p < 0.01$), respectively.

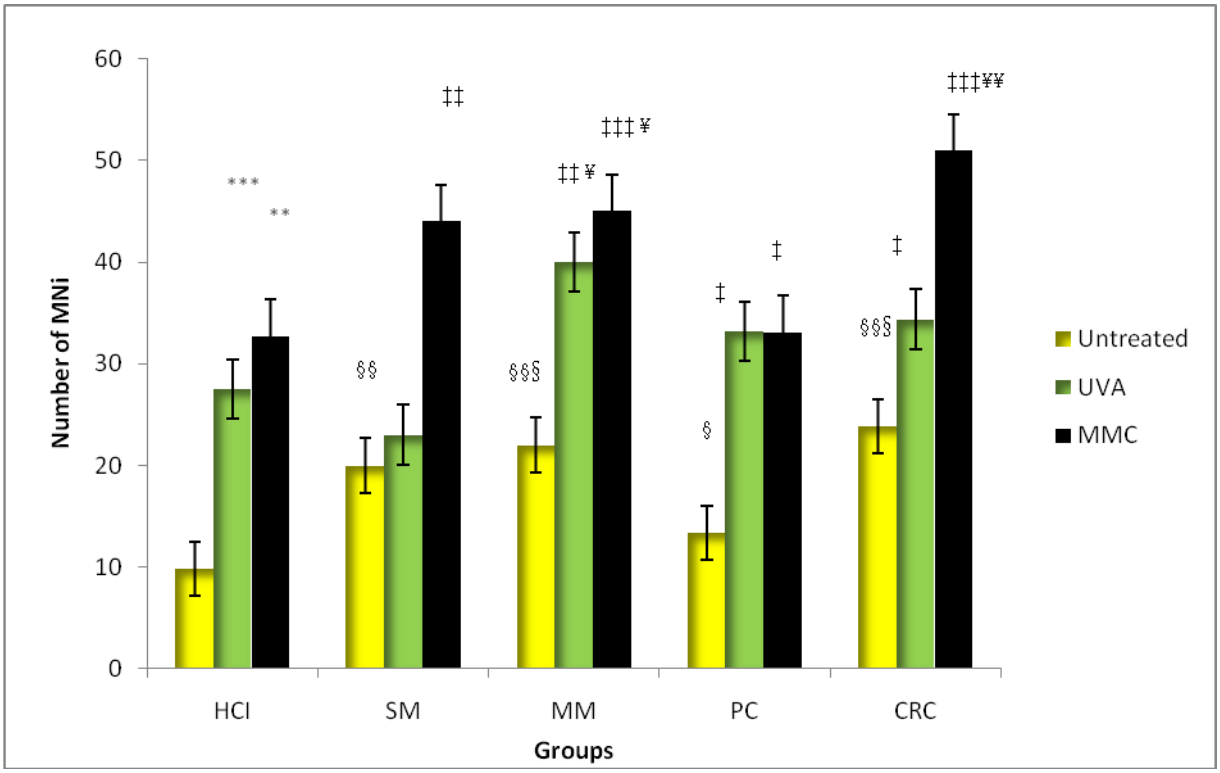


Figure 5.3.1: The number of binucleated micronuclei (MNBN) in peripheral lymphocytes from MM, SM and CRC, polyposis compared to HCI group before and after treatment with UVA or mitomycin C *in vitro* using the MN assay.

*, ‡, §, ¥ - $p < 0.05$, , ‡‡, §§, ¥¥ - $p < 0.01$, , ‡‡‡, §§§, ¥¥¥ - $p < 0.001$;

* indicates the level of statistical significance when comparing lymphocytes from healthy individuals after treatment with UVA and MMC to untreated lymphocytes;

‡ indicates the level of statistical significance when comparing lymphocytes from patients after treatment with UVA and MMC to untreated lymphocytes;

§ shows the level of statistical significance when comparing untreated lymphocytes from patients to healthy controls;

¥ Shows the level of statistical significance when comparing treated lymphocytes from patients to treated healthy controls;

5.3.1.3. Polyposis coli (PC) and colorectal cancer (CRC) patients groups

For both groups, the mitotic index CBPI was significantly decreased from 1.63 (PC group) and 1.61 (CRC group) to 1.38 and 1.31, respectively, after UVA treatment (both $p < 0.001$) and to 1.45 ($p < 0.01$) and 1.51 ($p < 0.05$), respectively, after MMC treatment (Table 5.3.1). Similarly, this was also observed for the percentage of BiNC in 1000 scored cells (mono-, bi- and, multinucleated). Treatment with UVA also generated cytogenetic damage significantly increasing the frequency of BiNC carrying MN by 2.5-fold for the PC group and 1.4-fold for the CRC-group (both $p < 0.05$). The significant increase in damage shown by MMC was 2.5-fold ($p < 0.05$) for the PC group and 2.1-fold ($p < 0.001$) for the CRC group (Table 5.3.1). Additionally, the frequencies of MonoMN significantly increased in both groups after UVA treatment by 3.4-fold ($p < 0.01$; PC group) and 6.3-fold ($p < 0.001$; CRC group), respectively, being in the same range as after the MMC treatment. There was no statistically significant increase in observed BiNPB. Nuclear buds found in BiNC after UVA treatment significantly increased for the PC group (from 1.6 to 4 per 1000 BiNC; $p < 0.01$) and interestingly significantly decreased for the CRC group (from 2.5 to 1.6 per 1000 BiNC; $p < 0.05$). After MMC treatment the frequency of BiBud significantly decreased for both groups.

When both groups were compared to the group of healthy individuals (Table 5.3.1, Figure 5.3.1), untreated lymphocytes from polyposis coli and colorectal cancer showed significantly lower CBPI frequencies, from 1.84 to 1.63 ($p < 0.01$; PC group) and to 1.61 ($p < 0.01$; CRC group), which can also be seen in the reduction of the

percentage of BiNC. Additionally, the basic cytogenetic damage (induced BiMN) was significantly increased in untreated lymphocytes from PC and CRC patients by 1.4-fold ($p < 0.05$) and 2.4-fold ($p < 0.001$), respectively (Table 5.3.1, Figure 5.3.1). Frequencies of BiNPB did not show any differences to those of the healthy individual group. However, BiBud and MonoMN were significantly increased in both groups by 1.7-fold ($p < 0.05$) and 2.7-fold ($p < 0.01$), respectively, for the PC group and by 1.8-fold ($p < 0.05$) and 1.3-fold ($p < 0.01$), respectively, for the CRC group.

5.3.2. Comet assay

5.3.2.1. Suspected melanoma and malignant melanoma patients groups

UVA treatment of lymphocytes from patients with SM and MM showed significantly increased DNA damage, 1.9-fold and 2.3-fold (both $p < 0.001$), respectively, when compared to untreated lymphocytes (Figure 5.3.2). In addition, before treatment with UVA, the lymphocytes from SM and MM patients compared to HC there is a significant increase of DNA damage , 1.3-fold (both $p < 0.05$) (Figure 5.3.2). In addition, we detect significant differences DNA damage in lymphocytes from SM and MM after treatment with UVA in contrast to HC ($P < 0.001$).

DNA damage within SM and MM compared to HCI group after *in vitro* treatment with PUVA using the Comet assay.

*, §, - $p < 0.05$, , §§, - $p < 0.01$, , §§§, - $p < 0.001$;

* indicates the level of statistical significance when comparing lymphocytes from healthy individuals after treatment with UVA and MMC to untreated lymphocytes;

§ shows the level of statistical significance when comparing untreated lymphocytes from patients to healthy controls;

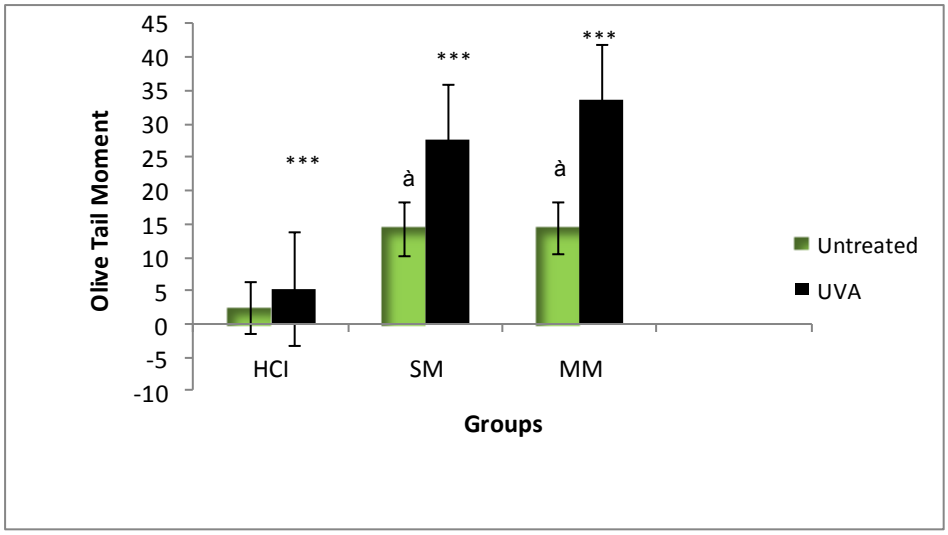


Figure 5.3.2.DNA damage within SM and MM compared to HC group after *in vitro* treatment with UVA using the Comet assay.

*, §, - $p < 0.05$, , §§, - $p < 0.01$, , §§§, - $p < 0.001$;

* indicates the level of statistical significance when comparing lymphocytes from healthy individuals after treatment with UVA and MMC to untreated lymphocytes;

§ shows the level of statistical significance when comparing untreated lymphocytes from patients to healthy controls;

5.3.2.2. Polyposis coli and colorectal cancer patients groups

UVA treatment significantly induced DNA damage ($p < 0.001$) in lymphocytes from all three groups (healthy individuals, PC and CRC). The measured OTM (Olive tail moment) increase were 3.2-fold for the PC group, 3-fold for the CRC group and 3.3-fold for the healthy individual group. The general DNA damage in lymphocytes from CRC patients before UVA treatment was highest followed by PC patients and healthy control individuals, after treatment with UVA (Figure 5.3.3). After inducing the DNA damage to lymphocytes from CP and CRC patients compared to HCI the OTM of the patients groups increased significantly 1.38- fold for PC and 1.48-fold for CRC ($p < 0.05$) (Figure 5.3.3). Additionally, the lymphocytes of the patients groups (PC and

CRC) before treatment with UVA by comparing to HC showed increased 1.43-fold for PC and 1.6- fold for CRC ($P < 0.05$) (Figure 5.3.3).

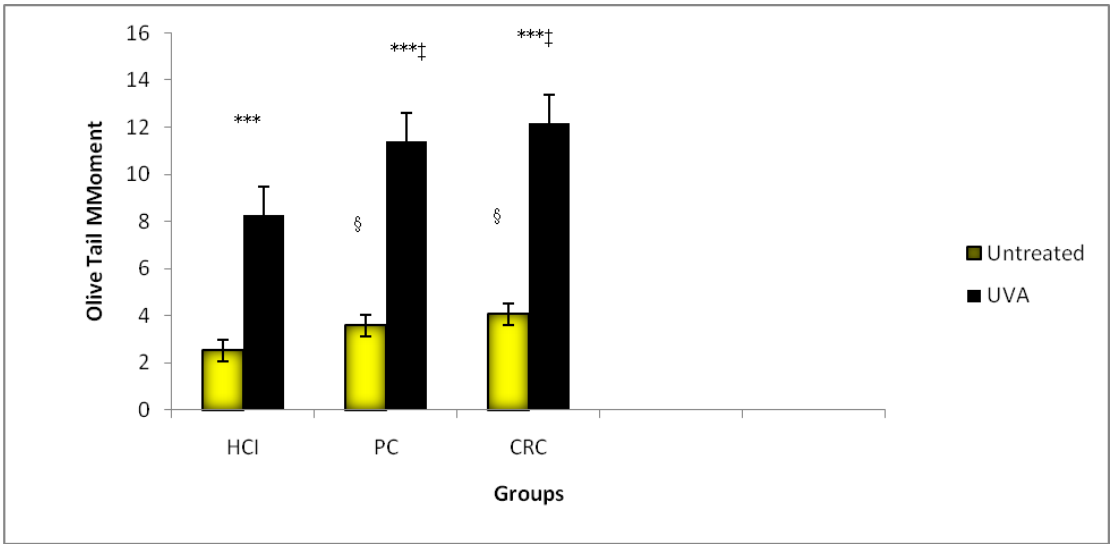


Figure 5.3.3. DNA damage within PC and CRC compared to HC group after *in vitro* treatment with UVA using the Comet assay.

*, ‡, § - $p < 0.05$, , ‡‡, §§- $p < 0.01$, , ‡‡‡, §§§ - $p < 0.001$;

* indicates the level of statistical significance when comparing lymphocytes from healthy individuals after treatment with UVA and MMC to untreated lymphocytes;

‡ indicates the level of statistical significance when comparing lymphocytes from patients after treatment with UVA and MMC to untreated lymphocytes;

§ shows the level of statistical significance when comparing untreated lymphocytes from patients to healthy controls;

5.4. Discussion

Different sensitivities of body cells to genotoxins can be determined in peripheral blood lymphocytes. These cells are ideal to be used as surrogates for all other body cells even DNA is basically the same in all cells. Even though they have failed to be surrogates of cancer cells in the prediction repair activities of cancer cells {Herrera, 2009 #89}, employing them for DNA damage induction studies they can be crucial to determine base line and induced DNA damage e.g. in the Comet assay. As peripheral lymphocytes are exposed to various environments inside the body by travelling in the blood stream, they reflect a common denominator of endogenously and exogenously induced damage from chemical and physical genotoxic insults such as food mutagens or oxidative stress. We have previously shown that treatment with hydrogen peroxide and the food mutagen IQ (2-amino-3-methylimadazo[4,5-f]-quinoline) induced more DNA damage in lymphocytes from patients with IBD, a disease prone to high levels of ROS, than in those from healthy individuals (Najafzadeh, 2009). Irrespective of the underlying condition patients with IBD on the other hand have an increased risk of developing CRC (Itzkowitz, 2004).

In this study we assessed *in vitro* the different sensitivities of peripheral lymphocytes from patients diagnosed with two very common types of cancers, MM and CRC, or with their pre-cancerous states, SM and PC. Our results showed that lymphocytes from selected pre-cancerous states (SM and PC) and cancers (MM and CRC) had significantly decreased CBPI values, hence a lower mitotic index, as well as a significantly increased frequency of induced micronuclei in binucleated cells, i.e.

genetic damage, when compared to healthy control individuals (Table 5.3.1, Figures 5.3.1). This effect was confirmed by examining the lymphocytes from SM, MM and PC, CRC and treating them with UVA and compared to healthy controls individuals *in vitro* in the Comet assay (Figures 5.3.2 & 5.3.3).These findings have been recently supported as newly diagnosed cancer patients were found to have elevated levels of DNA damage in peripheral blood lymphocytes (Vodicka, 2010. Levels of reactive oxidative and reactive nitrogen species in relation to the antioxidant status are most likely to be one of the reasons for this observed damage as they can transiently or permanently damage nucleic acids, lipids and proteins and therefore can increase cancer risk (Thompson, 2004 ;Chatterjee, 1989).

To induce DNA damage *in vitro* in lymphocytes from our selected patient groups, UVA was used as a generic mutagen. Compared to a chemical genotoxin, the advantage of using UVA light was the exact setting of exposure time and strength of the genotoxic insult. UVA is part of the sunlight with its electromagnetic spectrum at sea level (290-5000 nm) not only including the visible (56%) and infrared (39%) part but also ultraviolet (UV) light (5%) (Gonzalez, 2008). Mostly UVA (320-400 nm) and to a lesser extent due to atmospheric absorption UVB (290-320 nm) reaches the earth's surface, while the germicide UVC part is completely filtered off. The UVA/B light has been commonly characterised as an environmental human carcinogen being also responsible for erythema (sun-burn), tanning, photo-aging and immune-suppression. However, artificial UV sources, mainly emitting UVA, can also be found in tanning studios and for the treatment of psoriasis (Matsumura, et al., 2004; Volkmar, 2010). Absorption of UVA in tissue results in the generation of reactive oxygen and nitrogen species and labile iron which can in turn damage other bio-

molecules such as DNA (McMillan, 2008; Reelfs, 2004). The most frequent type of DNA damage after UV exposure are cyclobutane pyrimidine dimers (CPD) being mostly contributed by marginal amounts of UVB while UVA induced oxidative DNA (Woollons, 1999).

For the DNA damage examined after *in vitro* UVA treatment, our results showed that peripheral lymphocytes from patients diagnosed with the pre-cancerous states and the associated cancer have a significantly higher sensitivity to the genotoxic insult when compared to healthy individuals (Table 5.3.1, Figures 5.3.1, 5.3.2 and 5.3.3). The mitotic index CBPI significantly dropped by up to 19% compared to 12% for the group of healthy individuals. Interestingly, the decline in CBPI was smaller for the groups of pre-cancerous states and the highest for the CRC group (9% SM vs. 12% MM, 15% PC vs. 19% CRC). For the PC/CRC groups the decline in CBPI was even lower than that for the positive control (MMC treatment). After inducing DNA damage by UVA in peripheral lymphocytes from SM, MM patients groups and healthy control individuals, no differences were detected between patient groups and the HCI group. In contrast the PC and CRC patient groups compared to the HI group a reasonable increase after treatment with UVA was shown in the Comet assay ($p < 0.05$) (Figures 5.3.2 & 5.3.3).

Malignant melanoma is a life threatening type of skin cancer. It originates from UV induced DNA damage in specialised skin cells, the melanocytes. This initial damage causes mutations which can lead to melanoma. However, the mechanism underlying the role of UV light exposure from sunlight in the aetiology of cutaneous MM is unclear. In xeroderma pigmentosum, a disease with severe sensitivity to UV, due to a

defect in nucleotide excision repair, there is a high incidence of MM, suggesting that DNA repair capacity (DCR) plays a role in sunlight-induced MM (Wei, 2003). Also, DCR reduction is one of the risk factors for MM and may have a separate role in susceptibility to sunlight-induced MM among healthy people (Wei, 2003). The interplay between genetic factors and the ultraviolet (UV) spectrum of sunlight such as specific signal transduction pathways that regulate cell cycling, differentiation and apoptosis is one of the mechanisms of UV induced skin cancers. Another mechanism includes mutations in genes coding for proteins in the Hedgehog pathway and in the p53 gene (de Gruijl, 2001).

For the induced genetic damage in our study, the induction of BiMN after UVA treatment was higher in lymphocytes from cancer and pre-cancerous state patients (1.85-fold, $p < 0.01$, for the MM group, 2.47-fold, $p < 0.05$, for the PC group and 1.44-fold, $p < 0.05$, for the CRC group) when compared to the control group (1.34-fold, $p < 0.001$). Only those from patients of the pre-cancerous SM group was lower (1.05-fold, $p < 0.05$) but still significantly increased. When compared to the genotoxic impact of MMC, UVA treatment produced generally lower frequencies of induced micronuclei (Table 5.3.1). Especially for the PC/CRC group of patients the significantly increased induction of micronuclei in mononucleated cells was obvious (Table 5.3.1) reaching 6.3-fold ($p < 0.001$) in the CRC group after UVA induction. Diseases like cancer have been shown to cause cellular instability increasing chromosomal damage seen in an increase in induced micronuclei using the MN assay (Yildirim, 2006). The genomic damage to the lymphocytes of peripheral blood has been widely used as a biomarker of genotoxic environmental factors, and long-term studies have demonstrated its validity and high clinical productivity (Hagmar, 2004).

In our study peripheral blood lymphocytes were used as surrogate cells to characterise induced DNA damage after a genotoxic insult. Induced micronuclei, the change in the mitotic index and the change in DNA integrity as assessed by the Comet parameters, served as biomarkers for genotoxic damage. This study shows that peripheral lymphocytes from patients diagnosed with pre-cancerous states and with the associated cancer treated *in vitro* with UVA are expressing different sensitivities to a genotoxic insult when compared to a healthy control group. Thus, the damage afflicted by UVA was significantly higher in lymphocytes which originated from individuals with cancer or a pre-cancerous state. In conclusion, our findings suggest that cells not in close proximity to the cancer or the pre-cancerous lesions might carry higher intrinsic damage due for instance to increased oxidative stress and are therefore more sensitive to genotoxic insults. This feature of differential sensitivity of lymphocytes from healthy individuals and patients with cancers or pre cancerous states may be used as an important biomarker in the screening and diagnosis of pre cancerous states and cancers in the early stage.

CHAPTER SIX

GENERAL DISCUSSION

6.1. General discussion

A significant protective effect of the Chaga mushroom as an ethanolic extract against oxidative DNA damage has been demonstrated after oxidative stress has been induced *in vitro* by H_2O_2 in lymphocytes obtained from IBD patients and healthy donors. This protection seems to be based on the antioxidative action of the Chaga extract by inhibiting oxygen radicals and therefore protecting against DNA damage. Furthermore, significant differences in response to DNA damage were also shown between treated and untreated lymphocytes from Crohn's disease and Ulcerative colitis. Lymphocytes from patients with Crohn's disease had increased basic levels of DNA damage. Overall this study suggests that the Chaga mushroom extract may be a potent antioxidant. Therefore, it could be a possible therapeutic agent or an adjunct to treatment in patients or a useful supplement in healthy individuals to generally inhibit excessive oxidative stress (Najafzadeh, Reynolds et al. 2007).

We report for the first time the protective *in vitro* effect of quercetin and epicatechin against oxidative stress in lymphocytes from IBD patients and healthy individuals (Figures. 4.4.1 and 4.4.2). We were able to show that untreated lymphocytes from IBD patients had significantly increased DNA damage when compared to healthy individuals (Najafzadeh, 2008). Flavonoids dramatically reduced the basic DNA damage in lymphocytes from IBD patients treated with H_2O_2 and IQ. *In vitro* treatment with H_2O_2 and IQ significantly induced DNA damage by oxidative stress in both groups. When co-treated with flavonoids, a significant protective effect was shown against free radical damage to the DNA generated by H_2O_2 or IQ. There was a very high level of damage in the patient group without any treatment because of their background inflammation and IBD therapeutic drugs which they had taken, but both

patients and controls showed a parallel and gradual reduction in DNA damage after treating with flavonoids (Figures 4.4.1 and 4.4.2).

Also in the present investigation lymphocytes from CD patients in two series of studies groups appeared to have a greater level of baseline DNA damage than those from UC patients when compared to the whole patient group ($p < 0.001$), suggesting that lymphocytes from CD patients are more exposed to oxidative stress than other IBD subgroups (Figures 3.4.2 and 4.4.2). It becomes obvious that an excessive production of ROS and radical nitrogen metabolites occur during the inflammation of the intestine from IBD patients (Kruidenier, 2003). It seems that a misbalanced production of pro-inflammatory and anti-inflammatory cytokines is characteristic of IBD and severely affects the immune homeostasis in peripheral blood cells, even more in CD than in UC patients (Sventoraityte, 2008). However, all subgroups react in the same way towards exogenous oxidative stressors as well as towards the inhibition of oxidative stress by flavonoids.

By considering the definition of inflammation as the body's natural reaction to invasion by an infectious agent, toxin or physical, chemical or traumatic damage, it is known that chronic inflammation and infection are major causes of cancer (Schetter, 2010).

In our final project we investigated the sensitivity of peripheral lymphocytes from two different types of pre cancerous state and cancer patients to the induced DNA damaging effect of UVA and compared then to lymphocytes from healthy control individuals in the Comet and micronucleus assays. The determination the affect on tumour cells can be carried out by measuring indirectly of the effect of chemicals or physical agents (radiation) on evaluating DNA damage in surrogate cells, such as

peripheral blood lymphocytes of cancer patients (Herrera, 2009).The understanding of the pathogenesis and progression of cancer requires the establishment of the altered genetic/metabolic factors that are essential to the development, growth, and proliferation of the malignant cells (Costello, 2006). Generally, our data showed clearly that the peripheral lymphocytes from suspected melanoma and malignant melanoma, polyposis coli and colorectal cancer patients are highly sensitive to UVA exposure compared to the healthy control individuals (HCI) group in the micronucleus and Comet assays ($p < 0.001$) (Table 5.3.1, Figure 5.3.1). This would suggest that baseline frequencies of different diseases compared to controls could be an important biomarker in the diagnosis of pre-cancers and early stage cancers. Also peripheral lymphocytes are a useful surrogate for cancers and pre-cancerous states since blood is present in all organs and tissues and DNA is basically the same in all cells.

6.2. Future work

Further work needs to be undertaken to determine if the responses to the treatment with UVA seen in the present study can be replicated with other cancer and pre-cancerous states in larger numbers compared to healthy control individuals.

This would strengthen the conclusions.

CHAPTER SEVEN

REFRENCES

References

AAD., A. A. o. D. (2008). " http://www.aad.org/public/publications/pamphlets/common_moles.html. ."

Abreu, M. T., K. D. Taylor, et al. (2002). "Mutations in NOD2 are associated with fibrostenosing disease in patients with Crohn's disease." Gastroenterology **123**(3): 679-688.

Abbasi, N. R., H. M. Shaw, et al. (2004). "Early diagnosis of cutaneous melanoma: revisiting the ABCD criteria." JAMA **292**(22): 2771-2776.

Adamson, R. H. and U. P. Thorgeirsson (1995). "Carcinogens in foods: heterocyclic amines and cancer and heart disease." Adv Exp Med Biol **369**: 211-220.

Adamson, R. H., U. P. Thorgeirsson, et al. (1996). "Extrapolation of heterocyclic amine carcinogenesis data from rodents and nonhuman primates to humans." Arch Toxicol Suppl **18**: 303-318.

Allgayer, H. (1991). "Clinical relevance of oxygen radicals in inflammatory bowel disease--facts and fashion." Klin Wochenschr **69**(21-23): 1001-1003.

Amerio, P., L. Manzoli, et al. (2009). "Epidemiology and clinical and pathologic characteristics of cutaneous malignant melanoma in Abruzzo (Italy)." Int J Dermatol **48**(7): 718-722.

Aoi, W., Y. Naito, et al. (2010). "Regular exercise reduces colon tumorigenesis associated with suppression of iNOS." Biochem Biophys Res Commun.

Anderson, D., M. M. Dobrzynska, et al. (1998). "Flavonoids modulate comet assay responses to food mutagens in human lymphocytes and sperm." Mutat Res **402**(1-2): 269-277.

Anderson, D., T. E. Schmid, et al. (2003). "Oestrogenic compounds and oxidative stress (in human sperm and lymphocytes in the Comet assay)." Mutat Res **544**(2-3): 173-178.

Andus, T. and V. Gross (2000). "Etiology and pathophysiology of inflammatory bowel disease--environmental factors." Hepatogastroenterology **47**(31): 29-43.

Ardizzone, S. and G. Bianchi Porro (2005). "Biologic therapy for inflammatory bowel disease." Drugs **65**(16): 2253-2286.

Aust, D. E. and G. B. Baretton (2010). "Serrated polyps of the colon and rectum (hyperplastic polyps, sessile serrated adenomas, traditional serrated adenomas, and mixed polyps)-proposal for diagnostic criteria." Virchows Arch.

Bajpayee, M., A. Dhawan, et al. (2002). "Gender-related differences in basal DNA damage in lymphocytes of a healthy Indian population using the alkaline Comet assay." Mutat Res **520**(1-2): 83-91.

Barros, K. V., R. A. Xavier, et al. (2010). "Soybean and fish oil mixture increases IL-10, protects against DNA damage and decreases colonic inflammation in rats with dextran sulfate sodium (DSS) colitis." Lipids Health Dis **9**(1): 68.

Bashir, M., D. G. Kingston, et al. (1987). "Anaerobic metabolism of 2-amino-3-methyl-3H-imidazo[4,5-f]quinoline (IQ) by human fecal flora." Mutat Res **190**(3): 187-190.

Bastian, B. C., M. Kashani-Sabet, et al. (2000). "Gene amplifications characterize acral melanoma and permit the detection of occult tumor cells in the surrounding skin." Cancer Res **60**(7): 1968-1973.

Bayless, T. M. T., M. Kaufman, H. et al (2008). "Crohn's disease." Gastroentrology & Hepatology resource centre, The Johns Hopkinse Medical Institutions

Bean, S. M., D. C. Chhieng, et al. (2010). "Anal-rectal cytology: correlation with human papillomavirus status and biopsy diagnoses in a population of HIV-positive patients." J Low Genit Tract Dis **14**(2): 90-96.

Bettega, D., P. Calzolari, et al. (2003). "Differential effectiveness of solar UVB subcomponents in causing cell death, oncogenic transformation and micronucleus induction in human hybrid cells." Int J Radiat Biol **79**(3): 211-216.

Blum, A. (2010). "[Severely dysplastic nevus: atypical Spitz nevus or melanoma in situ?]." Hautarzt **61**(2): 151-152.

Bogen, K. T. (1994). "Cancer potencies of heterocyclic amines found in cooked foods." Food Chem Toxicol **32**(6): 505-515.

Boni, V., N. Bitarte, et al. (2010). "miR-192/miR-215 Influence 5-Fluorouracil Resistance through Cell Cycle-Mediated Mechanisms Complementary to its Posttranscriptional Thymidilate Synthase Regulation." Mol Cancer Ther.

Boughton-Smith, N. K., S. M. Evans, et al. (1993). "Nitric oxide synthase activity in ulcerative colitis and Crohn's disease." Lancet **342**(8867): 338-340.

Buettner, P. G. and C. Garbe (2000). "Agreement between self-assessment of melanocytic nevi by patients and dermatologic examination." Am J Epidemiol **151**(1): 72-77.

Burkhart, C. G. (2003). "Dysplastic nevus declassified: even the NIH recommends elimination of confusing terminology." Skinmed **2**(1): 12-13.

Byrd, K. M., D. C. Wilson, et al. (2004). "Advanced presentation of melanoma in African Americans." J Am Acad Dermatol **50**(1): 21-24; discussion 142-143.

Califano, J. and M. Nance (2009). "Malignant melanoma." Facial Plast Surg Clin North Am **17**(3): 337-348.

Cancer, N. (2009). "The Genetics of Cancer " Journal of Clinical Oncology

Cao, Q., J. M. Si, et al. (2005). "Clinical presentation of inflammatory bowel disease: a hospital based retrospective study of 379 patients in eastern China." Chin Med J (Engl) **118**(9): 747-752.

Carman, R. J., R. L. Van Tassell, et al. (1988). "Conversion of IQ, a dietary pyrolysis carcinogen to a direct-acting mutagen by normal intestinal bacteria of humans." Mutat Res **206**(3): 335-342.

Campbell, P. T., A. Deka, et al. (2010). "Prospective study reveals associations between colorectal cancer and type 2 diabetes mellitus or insulin use in men." Gastroenterology.

Capasso, M., F. Ayala, et al. (2010). "MDM2 SNP309 and p53 Arg72Pro in cutaneous melanoma: association between SNP309 GG genotype and tumor Breslow thickness." J Hum Genet.

Cappell, M. S. (2007). "From colonic polyps to colon cancer: pathophysiology, clinical presentation, screening and colonoscopic therapy." Minerva Gastroenterol Dietol **53**(4): 351-373.

Carrion, S., I. Marin, et al. (2010). "[Appropriateness of colonoscopy indications according to the new EPAGE II criteria.]." Gastroenterol Hepatol.

Castellsague, E., S. Gonzalez, et al. (2010). "Allele-Specific Expression of APC in Adenomatous Polyposis Families." Gastroenterology.

Chan, O. T. and P. Haghighi (2006). "Hamartomatous polyps of the colon: ganglioneuromatous, stromal, and lipomatous." Arch Pathol Lab Med **130**(10): 1561-1566.

Chang, N. B., R. Feng, et al. (2010). "Skin cancer incidence is highly associated with ultraviolet-B radiation history." Int J Hyg Environ Health.

Chien, J. C., D. M. Niu, et al. (2010). "Giant congenital melanocytic nevi in neonates: report of two cases." Pediatr Neonatol **51**(1): 61-64.

Chen, H. I., S. H. Liou, et al. (2007). "Oxidative DNA damage estimated by plasma 8-hydroxydeoxyguanosine (8-OHdG): influence of 4, 4'-methylenebis (2-chloroaniline) exposure and smoking." J Occup Health **49**(5): 389-398.

Coelho, S. G. and V. J. Hearing (2010). "UVA tanning is involved in the increased incidence of skin cancers in fair-skinned young women." Pigment Cell Melanoma Res **23**(1): 57-63.

Colombel, J. F., G. Vernier-Massouille, et al. (2007). "[Epidemiology and risk factors of inflammatory bowel diseases]." Bull Acad Natl Med **191**(6): 1105-1118; discussion 1118-1123.

Cooke, M. S., M. D. Evans, et al. (2003). "Oxidative DNA damage: mechanisms, mutation, and disease." Faseb J **17**(10): 1195-1214.

Coras, B., M. Landthaler, et al. (2010). "Dysplastic Melanocytic Nevi of the Lower Leg: Sex- and Site-Specific Histopathology." Am J Dermatopathol.

Cress, R. D. and E. A. Holly (1997). "Incidence of cutaneous melanoma among non-Hispanic whites, Hispanics, Asians, and blacks: an analysis of california cancer registry data, 1988-93." Cancer Causes Control **8**(2): 246-252.

Cui, Y., D. S. Kim, et al. (2005). "Antioxidant effect of Inonotus obliquus." J Ethnopharmacol **96**(1-2): 79-85.

de la Fuente-Garcia, A. and J. Ocampo-Candiani (2010). "[Cutaneous melanoma]." Gac Med Mex **146**(2): 126-135.

D'Odorico, A., S. Bortolan, et al. (2001). "Reduced plasma antioxidant concentrations and increased oxidative DNA damage in inflammatory bowel disease." Scand J Gastroenterol **36**(12): 1289-1294.

Danese, S. and C. Fiocchi (2006). "Etiopathogenesis of inflammatory bowel diseases." World J Gastroenterol **12**(30): 4807-4812.

Davies, K. J. (1995). "Oxidative stress: the paradox of aerobic life." Biochem Soc Symp **61**: 1-31.

Debinski, H. S., S. Love, et al. (1996). "Colorectal polyp counts and cancer risk in familial adenomatous polyposis." Gastroenterology **110**(4): 1028-1030.

Decarlo, K., S. Yang, et al. (2010). "Oncogenic BRAF-positive dysplastic nevi and the tumor suppressor IGFBP7--challenging the concept of dysplastic nevi as precursor lesions?" Hum Pathol **41**(6): 886-894.

Demierre, M. F., C. Chung, et al. (2005). "Early detection of thick melanomas in the United States: beware of the nodular subtype." Arch Dermatol **141**(6): 745-750.

Demierre, M. F. and L. Nathanson (2003). "Chemoprevention of melanoma: an unexplored strategy." J Clin Oncol **21**(1): 158-165.

de Whalley, C. V., S. M. Rankin, et al. (1990). "Flavonoids inhibit the oxidative modification of low density lipoproteins by macrophages." Biochem Pharmacol **39**(11): 1743-1750.

Doxiadis, II, J. M. Smits, et al. (1996). "Association between specific HLA combinations and probability of kidney allograft loss: the taboo concept." Lancet **348**(9031): 850-853.

Duquesnoy, R. J. and M. Trucco (1988). "Genetic basis of cell surface polymorphisms encoded by the major histocompatibility complex in humans." Crit Rev Immunol **8**(2): 103-145.

Edlich, R., C. L. Cross, et al. (2009). "Revolutionary advances in the diagnosis and treatment of Familial Adenomatous Polyposis." J Environ Pathol Toxicol Oncol **28**(1): 47-52.

Eisenbrand, G. and W. Tang (1993). "Food-borne heterocyclic amines. Chemistry, formation, occurrence and biological activities. A literature review." Toxicology **84**(1-3): 1-82.

Erickson, C. and S. J. Miller (2010). "Treatment options in melanoma in situ: topical and radiation therapy, excision and Mohs surgery." Int J Dermatol **49**(5): 482-491.

Farrell, R. J. and M. A. Peppercorn (2002). "Ulcerative colitis." Lancet **359**(9303): 331-340.

Ferrara, N. and W. J. Henzel (1989). "Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells." Biochem Biophys Res Commun **161**(2): 851-858

Fenech, M.Crott, J. W.(2002). "Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes-evidence for breakage-fusion-bridge cycles in the cytokinesis-block micronucleus assay" **504** (1-2): (131-6)

Fenech, M., W. P. Chang, et al. (2003). "HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures." Mutat Res **534**(1-2): 65-75.

Fenech, M. (2005)." In vitro micronucleus technique to predict chemosensitivity" Methods Mol Med **111:** 3-32

Fiocchi, C. (2005). "Inflammatory bowel disease phatogenesis: therapeutic implications." Chin j Dig Dis **6**(1): 6-9.

Gao, M., Q. Cao, et al. (2005). "[NOD2/CARD15 gene polymorphisms and susceptibility to Crohn's disease in Chinese Han population]." Zhonghua Nei Ke Za Zhi **44**(3): 210-212.

Galiatsatos, P. and W. D. Foulkes (2006). "Familial adenomatous polyposis." Am J Gastroenterol **101**(2): 385-398.

Geller, A. C., D. R. Miller, et al. (2002). "Melanoma incidence and mortality among US whites, 1969-1999." JAMA **288**(14): 1719-1720.

Giatromanolaki, A., E. Sivridis, et al. (2003). "Hypoxia-inducible factors 1alpha and 2alpha are related to vascular endothelial growth factor expression and a poorer prognosis in nodular malignant melanomas of the skin." Melanoma Res **13**(5): 493-501.

Gitnick, G. (1994). "Current views of the etiology of inflammatory bowel disease." Semin Pediatr Surg **3**(1): 2-7.

Goldman, R. and P. G. Shields (2003). "Food mutagens." J Nutr **133 Suppl 3**: 965S-973S.

Goel, A. and C. R. Boland (2010). "Recent insights into the pathogenesis of colorectal cancer." Curr Opin Gastroenterol **26**(1): 47-52.

Grob, J. J. and J. J. Bonerandi (1998). "The 'ugly duckling' sign: identification of the common characteristics of nevi in an individual as a basis for melanoma screening." Arch Dermatol **134**(1): 103-104.

Guindi, M. and R. H. Riddell (2004). "Indeterminate colitis." J Clin Pathol **57**(12): 1233-1244.

Gutierrez, M. P., M. Barengo, et al. (2009). "[Cutaneous melanoma associated with previous nevus]." Medicina (B Aires) **69**(5): 536-540.

Halloran, P. F., A. Wadgymar, et al. (1986). "The regulation of expression of major histocompatibility complex products." Transplantation **41**(4): 413-420.

Hall, G., A. Clarkson, et al. (2010). "Immunohistochemistry for PMS2 and MSH6 alone can replace a four antibody panel for mismatch repair deficiency screening in colorectal adenocarcinoma." Pathology **42**(5): 409-413.

Hanauer, S. B. (2006). "Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities." Inflamm Bowel Dis 12 Suppl 1: S3-9.

Hatch, F. T., M. G. Knize, et al. (2001). "Extended quantitative structure-activity relationships for 80 aromatic and heterocyclic amines: structural, electronic, and hydropathic factors affecting mutagenic potency." Environ Mol Mutagen **38**(4): 268-291.

Hemnani, T. and M. S. Parihar (1998). "Reactive oxygen species and oxidative DNA damage." Indian J Physiol Pharmacol **42**(4): 440-452.

Henderson, L., A. Wolfreys, et al. (1998). "The ability of the Comet assay to discriminate between genotoxins and cytotoxins." Mutagenesis **13**(1): 89-94.

Hosono, K., H. Endo, et al. (2010). "Metformin suppresses azoxymethane-induced colorectal aberrant crypt foci by activating AMP-activated protein kinase." Mol Carcinog **49**(7): 662-671.

Hu, S., Y. Parmet, et al. (2009). "Disparity in melanoma: a trend analysis of melanoma incidence and stage at diagnosis among whites, Hispanics, and blacks in Florida." Arch Dermatol **145**(12): 1369-1374.

Hu, S., R. M. Soza-Vento, et al. (2006). "Comparison of stage at diagnosis of melanoma among Hispanic, black, and white patients in Miami-Dade County, Florida." Arch Dermatol **142**(6): 704-708.

Ibarrola-Villava, M., L. P. Fernandez, et al. (2010). "Genetic analysis of three important genes in pigmentation and melanoma susceptibility: CDKN2A, MC1R and HERC2/OCA2." Exp Dermatol.

Institute, N. C. (2008). "What You Need To Know About Melanoma - Melanoma: Who's at Risk?"." National Cancer Institute.

Jahanshahi, G., V. Motavasel, et al. (2004). "Alterations in antioxidant power and levels of epidermal growth factor and nitric oxide in saliva of patients with inflammatory bowel diseases." Dig Dis Sci **49**(11-12): 1752-1757.

Jahanshahi, G. M., Vian1; Rezaie, Ali1; Hashtroudi, Ali2; Daryani, Naser2; Abdollahi, Mohammad (November 2004). "Alterations in Antioxidant Power and Levels of Epidermal Growth Factor and Nitric Oxide in Saliva of Patients with Inflammatory Bowel Diseases " Scandinavian Journal of Gastroenterology **49**(11-12): 1752-1757(1756).

Jankun, J., S. H. Selman, et al. (1997). "Why drinking green tea could prevent cancer." Nature **387**(6633): 561.

Jenab-Wolcott, J. and B. J. Giantonio (2010). "Antiangiogenic therapy in colorectal cancer: where are we 5 years later?" Clin Colorectal Cancer **9**: S7-S15.

Kaur, G., M. Roberti, et al. (2007). "Suppression of human monocyte tissue factor induction by red wine phenolics and synthetic derivatives of resveratrol." Thromb Res **119**(2): 247-256.

Keshavarzian, A., A. Banan, et al. (2003). "Increases in free radicals and cytoskeletal protein oxidation and nitration in the colon of patients with inflammatory bowel disease." Gut **52**(5): 720-728.
Kim, H. G., D. H. Yoon, et al. (2007). "Ethanol extract of Inonotus obliquus inhibits lipopolysaccharide-induced inflammation in RAW 264.7 macrophage cells." J Med Food **10**(1): 80-89.
Kim, Y. O., H. W. Park, et al. (2006). "Anti-cancer effect and structural characterization of endo-polysaccharide from cultivated mycelia of Inonotus obliquus." Life Sci **79**(1): 72-80.
Kimura, H., C. Lee, et al. (2010). "UV light killing efficacy of fluorescent protein-expressing cancer cells in vitro and in vivo." J Cell Biochem **110**(6): 1439-1446.
Kirsner, J. B. "Inflammatory bowel disease. Part II: Clinical and therapeutic aspects." SO - Dis Mon. 1991 Nov;37(11):669-746.
Kirsner, J. B. (1991). "Inflammatory bowel disease. Part II: Clinical and therapeutic aspects." Dis Mon **37**(11): 669-746.
Klink, M., A. Swierzko, et al. (2001). "Nitric oxide generation from hydroxylamine in the presence of neutrophils and in the cell-free system." Apmis **109**(7-8): 493-499.
Knasmuller, S., H. Steinkellner, et al. (2001). "Impact of bacteria in dairy products and of the intestinal microflora on the genotoxic and carcinogenic effects of heterocyclic aromatic amines." Mutat Res **480-481**: 129-138.
Knize, M. G., F. A. Dolbeare, et al. (1995). "Mutagenic activity and heterocyclic amine content of the human diet." Princess Takamatsu Symp **23**: 30-38.
Koch, T. R., L. X. Yuan, et al. (2000). "Induction of enlarged intestinal lymphoid aggregates during acute glutathione depletion in a murine model." Dig Dis Sci **45**(11): 2115-2121.
Koester, M. P., O. Muller, et al. (2007). "Adenomatous polyposis coli is differentially distributed in growth cones and modulates their steering." J Neurosci **27**(46): 12590-12600.
Koyama, M., M. Ito, et al. (1999). "Inactivation of both alleles of the DPC4/SMAD4 gene in advanced colorectal cancers: identification of seven novel somatic mutations in tumors from Japanese patients." Mutat Res **406**(2-4): 71-77.
Krensky, A. M. and C. Clayberger (2005). "Granulysin: a novel host defense molecule." Am J Transplant **5**(8): 1789-1792.
Krensky, A. M., A. Weiss, et al. (1990). "T-lymphocyte-antigen interactions in transplant rejection." N Engl J Med **322**(8): 510-517.
Kruidenier, L., I. Kuiper, et al. (2003). "Intestinal oxidative damage in inflammatory bowel disease: semi-quantification, localization, and association with mucosal antioxidants." J Pathol **201**(1): 28-36.
Kumaravel, T. S. and A. N. Jha (2006). "Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals." Mutat Res **605**(1-2): 7-16.
Kundu, R. V., M. Kamaria, et al. (2010). "Effectiveness of a knowledge-based intervention for melanoma among those with ethnic skin." J Am Acad Dermatol **62**(5): 777-784.
Kupfer, S. S., S. McCaffrey, et al. (2006). "Racial and gender disparities in hereditary colorectal cancer risk assessment: the role of family history." J Cancer Educ **21**(1 Suppl): S32-36.

LeAnder, R., P. Chindam, et al. (2010). "Differentiation of melanoma from benign mimics using the relative-color method." Skin Res Technol **16**(3): 297-304.
Lee, H. Y., S. Y. Na, et al. (2010). "A malignant melanoma associated with a blue nevus of the lip." Ann Dermatol **22**(1): 119-124.
Lee, I. K., Y. S. Kim, et al. (2007). "New antioxidant polyphenols from the medicinal mushroom Inonotus obliquus." Bioorg Med Chem Lett **17**(24): 6678-6681.
Lefevre J.H.Colas, C. C., F.Bonilla, C.Mourra, N.Flejou, J. F.Tiret, E.Bodmer, W.Soubrier, F.Parc, Y. (2010). "MYH biallelic mutation can inactivate the two genetic pathways of colorectal cancer by APC or MLH1 transversions." Fam Cancer.
Le Marchand, L., L. R. Wilkens, et al. (1999). "Independent and joint effects of family history and lifestyle on colorectal cancer risk: implications for prevention." Cancer Epidemiol Biomarkers Prev **8**(1): 45-51.
Lewis, J. D., F. N. Aberra, et al. (2004). "Seasonal variation in flares of inflammatory bowel disease." Gastroenterology **126**(3): 665-673.
Limoli, C. L. and E. Giedzinski (2003). "Induction of chromosomal instability by chronic oxidative stress." Neoplasia **5**(4): 339-346.
Limsui, D., R. A. Vierkant, et al. (2010). "Cigarette Smoking and Colorectal Cancer Risk by Molecularly Defined Subtypes." J Natl Cancer Inst.
Lin, J., Q. Yang, et al. (2010). "The calcium-binding protein S100B down-regulates p53 and apoptosis in malignant melanoma." J Biol Chem.
Loft, S., K. Vistisen, et al. (1992). "Oxidative DNA damage estimated by 8-hydroxydeoxyguanosine excretion in humans: influence of smoking, gender and body mass index." Carcinogenesis **13**(12): 2241-2247.
Loguercio, C., G. D'Argenio, et al. (1996). "Direct evidence of oxidative damage in acute and chronic phases of experimental colitis in rats." Dig Dis Sci **41**(6): 1204-1211.
Loguercio, C., G. D'Argenio, et al. (2003). "Glutathione supplementation improves oxidative damage in experimental colitis." Dig Liver Dis **35**(9): 635-641. Lee, I. K., Y. S. Kim, et al. (2007). "New antioxidant polyphenols from the medicinal mushroom Inonotus obliquus." Bioorg Med Chem Lett **17**(24): 6678-6681.
Loft, S., K. Vistisen, et al. (1992). "Oxidative DNA damage estimated by 8-hydroxydeoxyguanosine excretion in humans: influence of smoking, gender and body mass index." Carcinogenesis **13**(12): 2241-2247.
Loguercio, C., G. D'Argenio, et al. (1996). "Direct evidence of oxidative damage in acute and chronic phases of experimental colitis in rats." Dig Dis Sci **41**(6): 1204-1211.
Loguercio, C., G. D'Argenio, et al. (2003). "Glutathione supplementation improves oxidative damage in experimental colitis." Dig Liver Dis **35**(9): 635-641.

Maeda, H., T. Sawa, et al. (1999). "Free radical generation from heterocyclic amines by cytochrome b5 reductase in the presence of NADH." Cancer Lett **143**(2): 117-121.
Maldonado, J. L., J. Fridlyand, et al. (2003). "Determinants of BRAF mutations in primary melanomas." J Natl Cancer Inst **95**(24): 1878-1890.
Markowitz, S. D. and M. M. Bertagnolli (2009). "Molecular origins of cancer: Molecular basis of colorectal cancer." N Engl J Med **361**(25): 2449-2460.

Miracco, C., R. Santopietro, et al. (1998). "Different patterns of cell proliferation and death and oncogene expression in cutaneous malignant melanoma." J Cutan Pathol **25**(5): 244-251.
Moller, P. (2006). "The alkaline comet assay: towards validation in biomonitoring of DNA damaging exposures." Basic Clin Pharmacol Toxicol **98**(4): 336-345.
Murata, M., M. Kobayashi, et al. (1999). "Mechanism of oxidative DNA damage induced by a heterocyclic amine, 2-amino-3,8-dimethylimidazo[4,5f]quinoxaline." Jpn J Cancer Res **90**(3): 268-275.
Naba. H (1994). "Current clinical protocol submitted to the N.I.H Scientific Director Cancer Treatment Resaerch Foundation, ." Arlington Heights.
Nagase, S., K. Takemura, et al. (1997). "A novel nonenzymatic pathway for the generation of nitric oxide by the reaction of hydrogen peroxide and D- or L-arginine." Biochem Biophys Res Commun **233**(1): 150-153.
Naito, Y., T. Takagi, et al. (2007). "Molecular fingerprints of neutrophil-dependent oxidative stress in inflammatory bowel disease." J Gastroenterol **42**(10): 787-798.
Najafzadeh, M., P. D. Reynolds, et al. (2007). "Chaga mushroom extract inhibits oxidative DNA damage in lymphocytes of patients with inflammatory bowel disease." Biofactors **31**(3-4): 191-200.
Najafzadeh, M., P. D. Reynolds, et al. (2009). "Flavonoids inhibit the genotoxicity of hydrogen peroxide (H(2)O(2)) and of the food mutagen 2-amino-3-methylimadazo[4,5-f]-quinoline (IQ) in lymphocytes from patients with inflammatory bowel disease (IBD)." Mutagenesis 24(5): 405-411.
Nakata, T., T. Yamada, et al. (2007). "Structure determination of inonotsuoxides A and B and in vivo anti-tumor promoting activity of inotodiol from the sclerotia of Inonotus obliquus." Bioorg Med Chem **15**(1): 257-264.
Nandakumar, G., J. A. Morgan, et al. (2004). "Familial polyposis coli: clinical manifestations, evaluation, management and treatment." Mt Sinai J Med **71**(6): 384-391.
Nannery, W. M., J. G. Barone, et al. (1990). "Familial polyposis coli & Gardner's syndrome." N J Med **87**(9): 731-733.
Navarini, A. A., I. Kolm, et al. (2010). "Trauma as triggering factor for development of melanocytic nevi." Dermatology **220**(4): 291-296.
Neugut, A. I. (1990). "Screening for colorectal cancer." Ann Intern Med **113**(11): 899-900.
Newton-Bishop, J. A., Y. M. Chang, et al. (2010). "Melanocytic Nevi, Nevus Genes, and Melanoma Risk in a Large Case-Control Study in the United Kingdom." Cancer Epidemiol Biomarkers Prev.
Nieuwenhuis, M. H., K. F. Douma, et al. (2010). "Female Fertility After Colorectal Surgery for Familial Adenomatous Polyposis: A Nationwide Cross-sectional Study." Ann Surg.
Nigg, C., U. Kolyvanos Naumann, et al. (2008). "[Crohn disease. Main symptoms: diarrhea, abdominal pain (especially right lower abdomen in ileocolitis), fatigue, weight loss]." Praxis (Bern 1994) **97**(3): 105-113; quiz 113-104.
Noroozi, M., W. J. Angerson, et al. (1998). "Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes." Am J Clin Nutr **67**(6): 1210-1218.
Olive, P. L. (1989). "Cell proliferation as a requirement for development of the contact effect in Chinese hamster V79 spheroids." Radiat Res **117**(1): 79-92.

Pais, R., H. Silaghi, et al. (2009). "Metabolic syndrome and risk of subsequent colorectal cancer." World J Gastroenterol **15**(41): 5141-5148.
Park, J. R., J. S. Park, et al. (2006). "Reversal of the TPA-induced inhibition of gap junctional intercellular communication by Chaga mushroom (Inonotus obliquus) extracts: effects on MAP kinases." Biofactors **27**(1-4): 147-155.
Park Yk Fau - Lee, H. B., E.-J. Lee Hb Fau - Jeon, et al. "Chaga mushroom extract inhibits oxidative DNA damage in human lymphocytes as assessed by comet assay." SO - Biofactors. 2004;21(1-4):109-12.
Park, Y. K., H. B. Lee, et al. (2004). "Chaga mushroom extract inhibits oxidative DNA damage in human lymphocytes as assessed by comet assay." Biofactors **21**(1-4): 109-112.
Park, Y. M., J. H. Won, et al. (2005). "In vivo and in vitro anti-inflammatory and anti-nociceptive effects of the methanol extract of Inonotus obliquus." J Ethnopharmacol **101**(1-3): 120-128.
Park, Y. S. (2007). "[COX-2 inhibitors in inflammatory bowel disease: friends or foes?]." Korean J Gastroenterol **50**(6): 350-355.
Partelli, S., S. Mukherjee, et al. (2010). "Larger hepatic metastases are more frequent with N0 colorectal tumours and are associated with poor prognosis: Implications for surveillance." Int J Surg.
Pasha, S. F. L., J. (2007). "New techniques in the diagnosis of inflammatory bowel disease." Division of gastroenterology and hepathology, Myo clinic college, Scottsdale, Arizona.
Penn, E., D. Garrow, et al. (2010). "Influence of race and sex on prevalence and recurrence of colon polyps." Arch Intern Med **170**(13): 1127-1132.
Physician, A. F. (2000). "Early Detection and Treatment of Skin Cancer." American Family Physician.
Porter, C. K., D. R. Tribble, et al. (2008). "Infectious gastroenteritis and risk of developing inflammatory bowel disease." Gastroenterology **135**(3): 781-786.
Psaty, E. L., A. Scope, et al. (2010). "Defining the patient at high risk for melanoma." Int J Dermatol **49**(4): 362-376.
Reddy, B. S. and A. Rivenson (1993). "Inhibitory effect of Bifidobacterium longum on colon, mammary, and liver carcinogenesis induced by 2-amino-3-methylimidazo[4,5-f]quinoline, a food mutagen." Cancer Res **53**(17): 3914-3918.
Ren, W., Z. Qiao, et al. (2003). "Flavonoids: promising anticancer agents." Med Res Rev **23**(4): 519-534.
Reynolds, P. D., S. T. Rhenius, et al. (1996). "Xanthine oxidase activity is not increased in the colonic mucosa of ulcerative colitis." Aliment Pharmacol Ther **10**(5): 737-741.
Rezaie, A., R. D. Parker, et al. (2007). "Oxidative stress and pathogenesis of inflammatory bowel disease: an epiphenomenon or the cause?" Dig Dis Sci **52**(9): 2015-2021.
Rhodes, J., G. Thomas, et al. (1997). "Inflammatory bowel disease management. Some thoughts on future drug developments." Drugs. **53**(2): 189-194.
Rice-Evans, C. (2001). "Flavonoid antioxidants." Curr Med Chem **8**(7): 797-807.
Rogler, G. (2004). "Update in inflammatory bowel disease pathogenesis." Curr Opin Gastroenterol **20**(4): 311-317.
Rumney, C. J., I. R. Rowland, et al. (1993). "Conversion of IQ to 7-OHIQ by gut microflora." Nutr Cancer **19**(1): 67-76.
Registry, N. I. C. (2010). "Cancer Incidence and Mortality."

Risio, M., L. Casorzo, et al. (2003). "Deletions of 17p are associated with transition from early to advanced colorectal cancer." Cancer Genet Cytogenet **147**(1): 44-49.

Rogers, R. S., 3rd and L. E. Gibson (1997). "Mucosal, genital, and unusual clinical variants of melanoma." Mayo Clin Proc **72**(4): 362-366.

Rouhani, P., P. S. Pinheiro, et al. (2010). "Increasing rates of melanoma among nonwhites in Florida compared with the United States." Arch Dermatol **146**(7): 741-746.

Ruiz-Tovar, J., J. Jimenez-Miramon, et al. (2010). "Endoscopic resection as unique treatment for early colorectal cancer." Rev Esp Enferm Dig **102**(7): 435-441.

Saar, M. (1991). "Fungi in Khanty folk medicine." J Ethnopharmacol **31**(2): 175-179.

Sachse, C., G. Smith, et al. (2002). "A pharmacogenetic study to investigate the role of dietary carcinogens in the etiology of colorectal cancer." Carcinogenesis **23**(11): 1839-1849.

Saro Gismera, C., M. Lacort Fernandez, et al. (2001). "[Epidemiology of chronic inflammatory bowel disease in Gijon, Asturias]." Gastroenterol Hepatol **24**(5): 228-235.

Sasaki, Y., C. Niu, et al. (2004). "BRAF point mutations in primary melanoma show different prevalences by subtype." J Invest Dermatol **123**(1): 177-183.

Schoeffner, D. J. and U. P. Thorgeirsson (2000). "Susceptibility of nonhuman primates to carcinogens of human relevance." In Vivo **14**(1): 149-156.

Schut, H. A. and E. G. Snyderwine (1999). "DNA adducts of heterocyclic amine food mutagens: implications for mutagenesis and carcinogenesis." Carcinogenesis **20**(3): 353-368.

Schwab, C. E., W. W. Huber, et al. (2000). "Search for compounds that inhibit the genotoxic and carcinogenic effects of heterocyclic aromatic amines." Crit Rev Toxicol **30**(1): 1-69.

Seegert, D., P. Rosenstiel, et al. (2001). "Increased expression of IL-16 in inflammatory bowel disease." Gut **48**(3): 326-332.

Sica, A., P. Allavena, et al. (2008). "Cancer related inflammation: The macrophage connection." Cancer Lett **267**(2): 204-215.

Simmonds, N. J. and D. S. Rampton (1993). "Inflammatory bowel disease--a radical view." Gut **34**(7): 865-868.

Singh, N. P., M. T. McCoy, et al. (1988). "A simple technique for quantitation of low levels of DNA damage in individual cells." Exp Cell Res **175**(1): 184-191.

Shrestha, B., J. Bishop, et al. (2010). "Detection of atypical texture features in early malignant melanoma." Skin Res Technol **16**(1): 60-65.

Sjursen, W., B. I. Haukanes, et al. (2010). "Current clinical criteria for Lynch syndrome are not sensitive enough to identify MSH6 mutation carriers." J Med Genet.

Slattery, M. L., J. Herrick, et al. (2010). "Genetic variation in a metabolic signaling pathway and colon and rectal cancer risk: mTOR, PTEN, STK1, RPKAA1, PRKAG2, TSC1, TSC2, PI3K, and Akt1." Carcinogenesis.

Slavin, M., W. Kenworthy, et al. (2009). "Antioxidant properties, phytochemical composition, and antiproliferative activity of Maryland-grown soybeans with colored seed coats." J Agric Food Chem **57**(23): 11174-11185.

Slaper, H., G. J. Velders, et al. (1996). "Estimates of ozone depletion and skin cancer incidence to examine the Vienna Convention achievements." Nature **384**(6606): 256-258.

Soffler, C. (2007). "Oxidative stress." Vet Clin North Am Equine Pract **23**(1): 135-157.
Sonnenberg, A. (1990). "Occupational distribution of inflammatory bowel disease among German employees." Gut **31**(9): 1037-1040.
Spoettl, T., M. Hausmann, et al. (2007). "Serum soluble TNF receptor I and II levels correlate with disease activity in IBD patients." Inflamm Bowel Dis **13**(6): 727-732.
Stephen, B. H., S. B. (2003). "Genetics of IBD: Clinical Relevance." Digestive Disease, IBD/Colorectal Cancer & Liver Disease.
Sugimura, T. and S. Sato (1983). "Mutagens-carcinogens in foods." Cancer Res **43**(5 Suppl): 2415s-2421s.
Sung, B., M. K. Pandey, et al. (2008). "Identification of a novel blocker of IkappaBalpha kinase activation that enhances apoptosis and inhibits proliferation and invasion by suppressing nuclear factor-kappaB." Mol Cancer Ther **7**(1): 191-201.
Suthanthiran, M. and T. B. Strom (1994). "Renal transplantation." N Engl J Med **331**(6): 365-376.
Sventoraityte, J., A. Zvirbliene, et al. (2008). "Immune system alterations in patients with inflammatory bowel disease during remission." Medicina (Kaunas) **44**(1): 27-33.
Statistics, O. f. N. (2010). "Cancer Statistics registrations: registrations of cancer diagnosed in 2007, England.".
Suzuki, T., H. Kumamoto, et al. (2002). "Intramucosal naevus with pseudoepitheliomatous hyperplasia in the gingiva: a case report." Int J Oral Maxillofac Surg **31**(3): 330-333.
Swetter, S. M., J. C. Boldrick, et al. (2005). "Increasing incidence of lentigo maligna melanoma subtypes: northern California and national trends 1990-2000." J Invest Dermatol **125**(4): 685-691.
Takeuchi, T. and K. Morimoto (1993). "Increased formation of 8-hydroxydeoxyguanosine, an oxidative DNA damage, in lymphoblasts from Fanconi's anemia patients due to possible catalase deficiency." Carcinogenesis **14**(6): 1115-1120.
Tanaka, M. S., H.; Kusumi, T.; Fukuda, S.; Shimoyama, T.; Sasaki, Y.; Suto, K.; Munakata, A.; Kudo, H. (December 2001). "Spatial distribution and histogenesis of colorectal Paneth cell metaplasia in idiopathic inflammatory bowel disease " Scandinavian Journal of Gastroenterology **16**(12): 1353-1359(1357).
Taouqi, M., I. Ingrand, et al. (2010). "Determinants of participation in colonoscopic screening by siblings of colorectal cancer patients in France." BMC Cancer **10**(1): 355.
Tice, R. R., E. Agurell, et al. (2000). "Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing." Environ Mol Mutagen **35**(3): 206-221.
Torrens, R. and B. A. Swan (2009). "Promoting prevention and early recognition of malignant melanoma." Dermatol Nurs **21**(3): 115-122; quiz 123.
Tournigand, C., T. Andre, et al. (2004). "FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study." J Clin Oncol **22**(2): 229-237.
Turkmen, A., D. Isik, et al. (2010). "Comparison of Classification Systems for Congenital Melanocytic Nevi." Dermatol Surg.

Ueda N Fau - Shah, S. V. and S. V. Shah "Endonuclease-induced DNA damage and cell death in oxidant injury to renal tubular epithelial cells." SO - J Clin Invest. 1992 Dec;90(6):2593-7.

Undeger, U., S. Aydin, et al. (2004). "The modulating effects of quercetin and rutin on the mitomycin C induced DNA damage." Toxicol Lett **151**(1): 143-149.

van der Meijden, W. A., R. L. van Bruchem-Visser, et al. (2010). "[Melanomas more serious in the elderly]." Ned Tijdschr Geneeskd **154**: A1535.

Zanetti, E., P. Barozzi, et al. (2010). "Common Vascular Endothelial Growth Factor Variants and Risk for Posttransplant Kaposi Sarcoma." Transplantation **90**(3): 337-338.

Zheng, W., M. Zhang, et al. (2009). "Accumulation of antioxidant phenolic constituents in submerged cultures of Inonotus obliquus." Bioresour Technol **100**(3): 1327-1335.

Appendix1

A sample of questionnaire

INFLUENCES ON SUSCEPTIBILITY TO *IN VITRO* DNA DAMAGE BY ENVIRONMENTAL AGENTS ON LYMPHOCYTES FROM Inflammatory bowel disease and cancers PATIENTS AND CONTROL SUBJECTS

HOSPITAL NUMBER		DATE OF SAMPLE	
AGE		FASTING	NON FASTING
SEX (PLEASE TICK)	M / F	CONSENT	Y / N
ETHNIC GROUP		INFORMATION SHEET	Y / N

OCCUPATION

CURRENT SMOKER Y/N PAST SMOKER Y/N HOW MANY/MUCH PER WEEK?

CIGARETTES CIGARS PIPE

ALCOHOL Y/N UNITS PER WEEK

DIET WESTERN | ASIAN | OMNIVORE | VEGETARIAN | VEGAN

VITAMINS / ANTI-OXIDANTS (PLEASE LIST)

PRESCRIBED DRUG USE (PLEASE LIST)

RECREATIONAL DRUG USE Y/N

IF YES PLEASE LIST

COLORECTAL or other CANCER CROHN'S, UC, IBD NO OF YRS

MEDICAL

EXTENT SITE HISTOLOGY SURGERY

COMPLICATIONS OF CANCER
(Primary Sclerosing Cholangitis)
DYSPLASIA ON COLONOSCOPY
OTHER MEDICAL CONDITIONS
(PLEASE LIST)
Family History of Cancer and Colorectal Cancer

MOST RECENT MEASURED

	RESULT	DATE		RESULT	DATE
WEIGHT			FBC		
HEIGHT			CRP		
BMI			LFTs		
CREATININE					

Appendix 2

The list of publications

1. Najafzadeh, M., P. D. Reynolds, et al. (2007). "Chaga mushroom extract inhibits oxidative DNA damage in lymphocytes of patients with inflammatory bowel disease." Biofactors **31**(3-4): 191-200.

2. Najafzadeh, M., P. D. Reynolds, et al. (2009). "Flavonoids inhibit the genotoxicity of hydrogen peroxide (H(2)O(2)) and of the food mutagen 2-amino-3-methylimadazo[4,5-f]-quinoline (IQ) in lymphocytes from patients with inflammatory bowel disease (IBD)." Mutagenesis **24**(5): 405-411.

3. Najafzadeh, M, A. Baumgartner, et al (2010). *In vitro* evaluation of different sensitivities of lymphocytes from patients diagnosed with cancer or pre-cancerous state by using UVA as a generic mutagen and employing the micronuclei assay and the Comet assay. **submitted**

Appendix 3

The list of groups and confounding factors for chapter 4

Group	IBD type	Gender	Class	Age	smoking	Alcohol	Ethnic	IBD treatment
1	3	1	11	27	1	1	1	1
1	2	1	11	22	1	1	1	1
1	1	2	12	58	2	2	1	1
1	1	1	11	31	2	2	1	1
1	2	1	11	38	3	2	1	2
1	2	1	11	65	1	0	1	3
1	1	1	11	30	3	0	2	1
1	3	1	11	39	2	2	1	2
1	1	2	12	59	3	0	2	1
1	2	2	12	55	1	2	1	2
2		2	21	25	1	1	1	
2		1	22	20	3	0	2	
2		2	21	22	1	1	1	
2		2	21	24	1	1	1	
2		2	21	26	1	1	1	
2		1	22	34	3	0	2	
2		1	22	56	3	0	2	
2		1	22	40	1	1	2	
2		1	22	22	3	0	2	
2		2	21	20	3	1	1	

Group 1: Patient group, group 2: Control group
IBD type: 1; UC, 2;CD, 3; Indeterminate
Gender: 1: Male, 2: Female
Smoking: 1; non smoker, 2; smoker, 3; exsmoker
Alcohol: 0; tea total (no alcohol), 1; mild (under 5 U / week), 2; moderate
Ethnic: 1; Caucasian, 2; Asian
IBD treatment: 1. Azathioprine, mesalazine and pentasa, asacol, prednisolone, mercaptopurine alone or in combination prior to taking part in the study azathioprine & pentasa, azathioprine & mesalazine, mercaptopurine & balsalazide (n = 6)
2. Asacol (n = 1)
3. pentasa & prednisolone, prednisolone & mesalazine (n = 2)

Appendix 4
The list of groups and confounding factors for chapter 3

Counter	Group	IBD type	Gender	Age	Smoking	Alcohol	Ethnic	IBD treatment
1	1	3	2	49	1	1	1	3
2	1	1	2	48	1	0	1	7
3	1	2	1	42	3	0	1	1
4	1	2	1	49	1	1	1	6
5	1	3	1	39	2	2	1	2
6	1	1	1	30	3	0	2	5
7	1	1	2	18	3	0	2	3
8	1	2	2	55	2	0	1	2
9	1	2	2	32	1	1	1	3
10	1	1	1	49	3	0	1	1
11	1	1	1	31	3	1	1	5
12	1	2	1	22	1	1	1	2
13	1	2	1	38	3	0	1	2
14	1	1	2	59	3	0	2	3
15	1	3	1	27	2	1	1	4
16	1	1	2	42	2	2	1	8
17	1	1	1	51	3	1	1	5
18	1	2	1	53	1	1	1	2
19	1	2	2	28	3	0	1	2
20	1	3	2	42	3	0	1	8
21	2		1	36	1	0	2	0
22	2		1	30	1	0	1	0
23	2		2	26	1	1	2	0
24	2		1	22	1	1	2	0
25	2		1	39	1	0	2	0
26	2		2	31	1	0	1	0
27	2		2	21	1	0	2	0
28	2		1	41	1	1	2	0
29	2		2	23	1	1	1	0
30	2		1	29	3	1	1	0
31	2		2	26	1	0	1	0
32	2		1	22	3	2	1	0
33	2		1	41	1	2	2	0
34	2		1	41	1	1	2	0
35	2		1	57	1	0	2	0
36	2		1	33	1	0	2	0
37	2		1	30	1	1	1	0
38	2		1	25	1	0	1	0
39	2		2	40	3	1	2	0
40	2		2	34	1	0	2	0

Group 1: Patient group, group 2: Control group
IBD type: 1; UC, 2;CD, 3; Indeterminate
Gender: 1: Male, 2: Female
Smoking: 1; non smoker, 2; smoker, 3; exsmoker
Alcohol: 0; tea total (no alcohol), 1; mild (under 5 U / week), 2; moderate
Ethnic: 1; Caucasian, 2; Asian

IBD treatment: 1. Azathioprine, mesalazine and pentasa, asacol, prednisolone, mercaptopurine alone or in combination prior to taking part in the study azathioprine & pentasa, azathioprine & mesalazine, mercaptopurine & balsalazide (n = 6)

2. Asacol (n = 1)

3. pentasa & prednisolone, prednisolone & mesalazine (n = 2)