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„PMN-mediated Mutagenesis in Ulcerative Colitis“

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Table of Contents

1. Introduction

1.1 Inflammatory bowel disease (IBD)

IBD is considered as an autoimmune disease due to an immune-related intestinal disorder and is comprised of two major types: ulcerative colitis (UC) and Crohn's disease (CD) [1]. These two disorders have distinct clinical and pathological characteristics linked to inflammation and ulceration within the inner lining of the gastrointestinal (GI) tract which can be treated but not cured [1], [2], [3]. The important difference between UC and CD is the nature and location of the inflammatory changes **[Figure1]** [3]. CD has the possibility to affect different parts of the GI tract, while UC is restricted to the innermost lining of large intestine (colon) and rectum [4]. Intestinal inflammation has similarities to CD in that the inside lining of the large intestine is affected, but the injury takes place in the inner lining of the colon and doesn't spread out deeper into intestinal epithelial wall [3], [5].

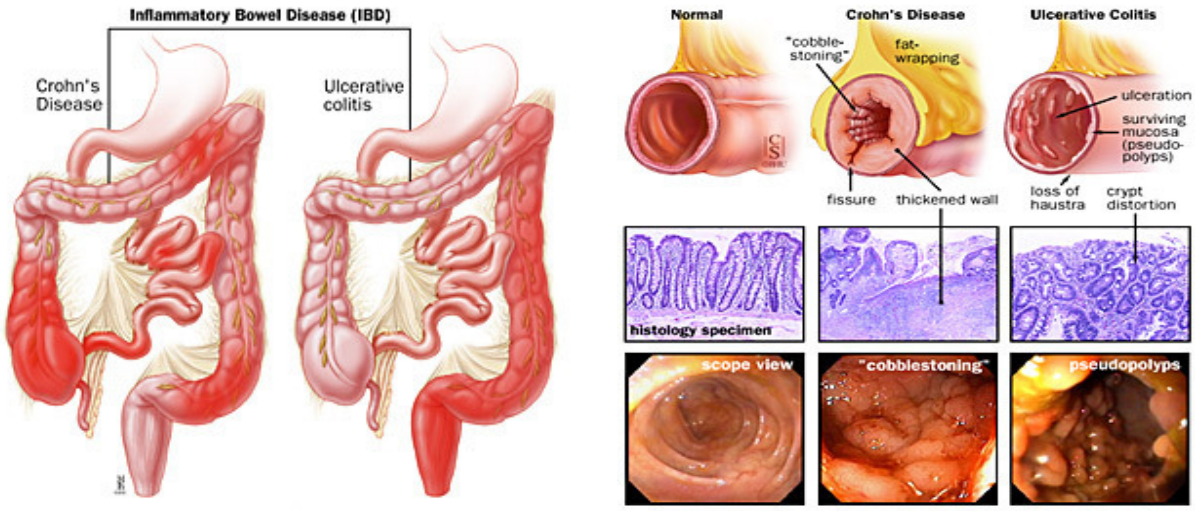


Figure1. In UC large intestine (rectum = proctitis, whole colon = pancolitis) is involved. In CD, whole alimentary tract from mouth to anus can be diseased. Possibility for inflammation through all layers of gut wall (transmural) and skip lesions with normal tissue in between. Artwork is reproduced from the Johns Hopkins Gastroenterology and Hepatology Resource Center. www.hopkins-gi.org. copyright 2006. JH University [6].

The human GI tract (or digestive tract) is important for digestion of food, absorption of nutrients and expels the remaining waste. Chronic inflammation can lead to a dysfunction of affected GI organs and to the occurrence of known symptoms like diarrhea, cramping and further rectal bleeding. Both inflammatory diseases, CD and UC can include symptom-free periods (remission) and disease-active periods (flares) [7]. While the leading cause of IBD is not completely understood, there is evidence that it has to be a consequence of a complex interaction of several factors involving the immune system (IS), environmental factors and genes [5]. The IS of a person with IBD can become activated by environmental factors - or by microorganisms within the GI tract. For example in individuals with IBD, harmless bacteria aiding in digestion can be recognized as harmful invaders and the IS can build up a response where leukocytes **(**white blood cells) travel out of the blood stream into the intestine and try to eliminate the microorganisms leading to inflammation (normal immune response). However, the inflammation does not diminish, chronic inflammation, ulcerations, intestinal wall thickening and other symptoms occur [4], [8].

1.1.2 The genetics behind IBD

It has been shown that there is a genetic predisposition for IBD, and people with this condition are more susceptible for developing malignancy. Genes can play a key role in the development of IBD and several studies demonstrate that children of parents with IBD are at higher risk than general population and that 36% of kids with both parents affected developed this disease [9], [10].

Over the past years, scientific research identified specific genes which contributes to the susceptibility for CD including nucleotide oligomerization domain 2 (NOD2/CARD15) [11], coding for an intracellular receptor of bacterial peptidoglycan which can activate the NFκB pathway, autophagy genes (e.g. ATG16L) [12], [13], [14] and components of the interleukin-23–type 17 helper T-cell (Th17) pathway [15]. Up to 20% of IBD patients in North America and Europe may have a mutation in the NOD2/CARD15 gene [4].

The international IBD genetics consortium (IIBDGC) has taken on the task to identify multiple susceptibility genes and recent studies confirmed 99 loci: 71 linked to CD, 47 to UC, and 28 both **[Figure2]** [16].

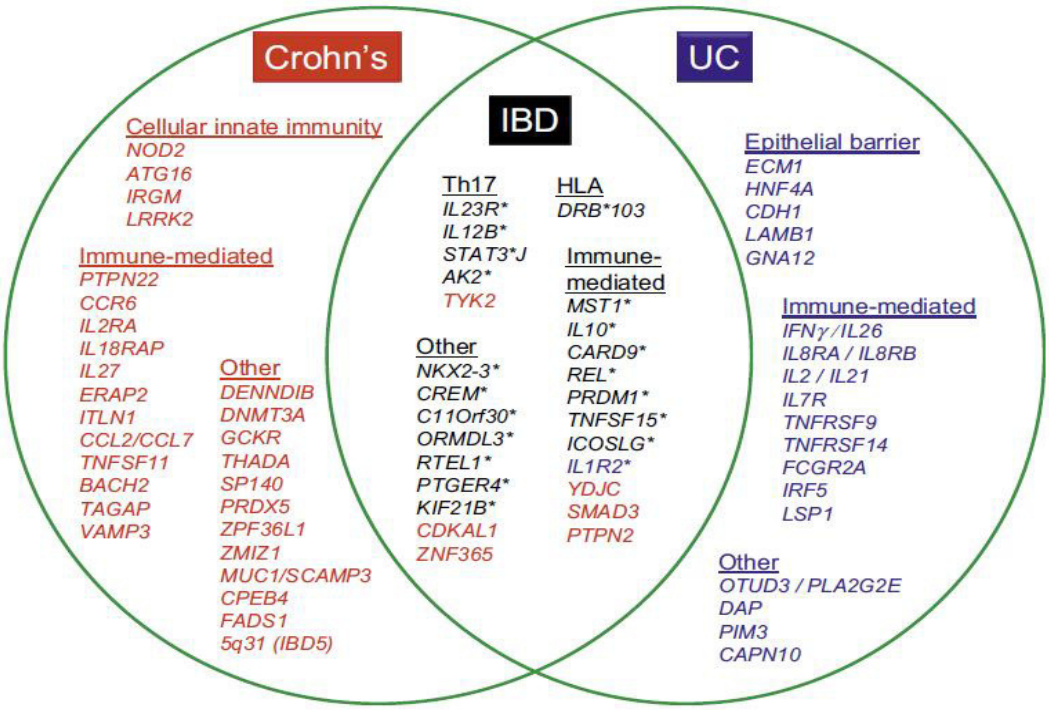


Figure2: IBD susceptibility genes: genome-wide significance is shown for Crohn's disease (red), ulcerative colitis (blue) and IBD (CD and UC; black) [16].

Approximately one-third of genes are associated with both and among those, genes are mainly involved in IL23/Th17 signaling (IL12B, IL23R , JAK2, TYK2 and STAT3), mediating intestinal inflammation and microbial defense [15], [16].

Defective processing of intracellular bacteria plays a key role in the development of CD and genome-wide association (GWA) studies confirmed hot targets in autophagy and innate immunity like NOD2 and ATG16L1 as previously mentioned. Recent GWA studies showed that also epithelial barrier genes (ECM1, CDH1, HNF4A, LAMB1 and GNA12) are involved in the development of UC but not CD [17], [18], [19].

Intracellular receptors like NOD2 and Toll like receptors are responsible for mediating the communication between luminal flora and epithelium which can lead to activity of an immune cell response and stimulation or repression of the well known NFκB pathway, a very important signaling pathway in IBD [3].

Cytokines like IL-10 are known to play a role in T-cell tolerance [8], and IL-10 mutations has been shown to promote the incidence of UC [20]. Infiltration of the lamina propria by immune cells like macrophages or neutrophils is known to be a key factor of active IBD, which can lead to a local increase of known cytokines and chemokines (e.g. tumor necrosis factor α, interferon-γ and IL-8) [8], [21].

1.1.3 Incidence and Prevalence of IBD

The incidence rates and the prevalence for IBD are highest in northern Europe [22], the United Kingdom [23] and North America **[Figure3]** [24], [25] and has increased in the past 50 years up to 8–14/100k and 120–200/100k persons for UC and 6–15/100k and 50–200/100k persons for CD.

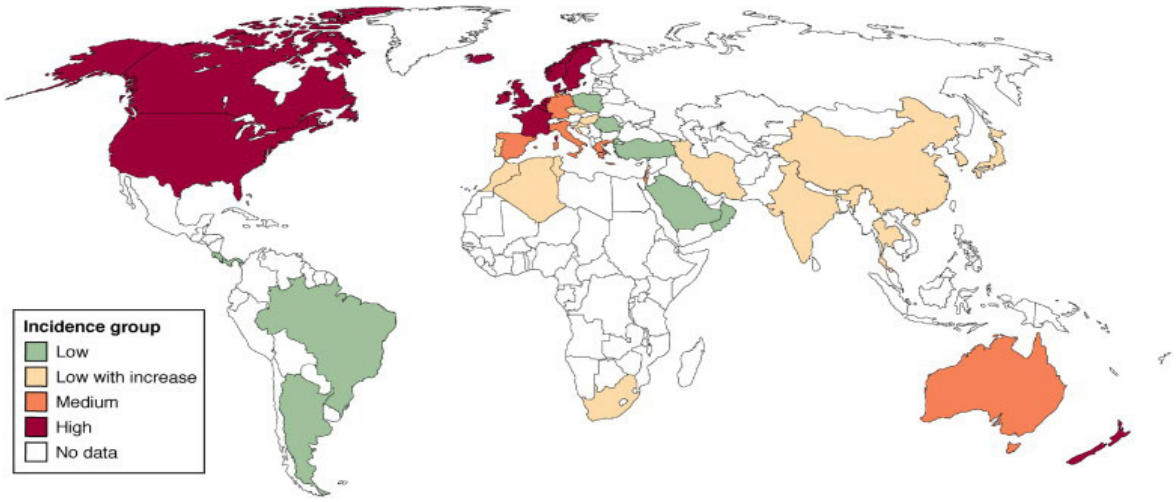


Figure3. The global map of IBD: *red* shows annual incidence greater than $10/10^5$, *orange* incidence of 5–$10/10^5$, *green* incidence less than $4/10^5$, *yellow* to low with increasing. no color indicates absence of data [7].

In those regions, such high incidences may indicate common etiologic factors. The prevalence is more than 200 cases per 100k inhabitants in the West, and it has a high impact on health care resources. Crohn's and UC are incurable; they begin in young adulthood (the peak age for CD occurrence is 20–30 years; for UC, it is 30–40 years) and continue throughout life. The incidence of UC is greater than that of CD, except in Canada and several areas of Europe, although this has been changing over the past 20 years. Canterbury County, New Zealand, has among the highest incidence of CD (16.5/100k people) [7].

1.1.4 A closer look into Ulcerative colitis (UC)

UC is considered as a disease of the large intestine that is noticeable by chronic inflammations and ulcerations of the colon mucosa and affects around 0.3% of Western population [7]. The pathogenesis of this disease is not already clear but includes disorders of the auto-immune system, genetic predisposition and environmental based triggers (e.g. nutrition) leading to an enhanced colorectal cancer (CRC) risk [26]. Using a set of clinical parameters, disease activity can be described as mild, moderate and severe **[Table-1]** [27], [28]:

	Mild	Moderate	Severe
Bowel movement	<4per day	4-6 per day	> 6per day
Blood in stool	Small	Moderate	Severe
Fever	None	<37.5°C mean	>37.5°C mean
Tachycardia	None	<90 mean pulse	>90 mean pulse
Anemia	Mild	>75%	<75%
ESR	<30mm		>30mm
Endoscopic Appearance	Erythema, decreased Vascular pattern, fine granularity	Marked erythema, absent vascular pattern, granularity, contact bleeding, no ulcerations	Spontaneous bleeding, ulcerations

Table-1. Disease classification and severity evaluation based on the criteria of Truelove and Witts, additional endoscopic features [28].

The formation of tiny bleeding ulcers on the surface which are able to release pus and mucus is one phenomenon of UC. Diarrhea and abdominal pain are common symptoms due to the inflammation related frequent emptying of the large intestine [26], [29], [30]. Inflammation in the colon may be limited to the rectum or expand to proximal parts of the large intestine. If the total large intestine is involved the disease is called pancolitis. Backwash ileitis, inflammation of the terminal ileum, occurs in 15 %-20 % of all patients suffering from pancolitis [30].

Patients with severe disease have more than 10 bowel movements daily, continuous bleeding, marked signs of toxicity (fever, tachycardia) and abdominal tenderness as well as distension. They require transfusions and abdominal plain films show colonic dilatation. Mild inflammation leads to an erythematous mucosa with granular surface, severe disease leads to hemorrhage, ulcers and to the development of pseudopolyps [29] **[Figure4]**.

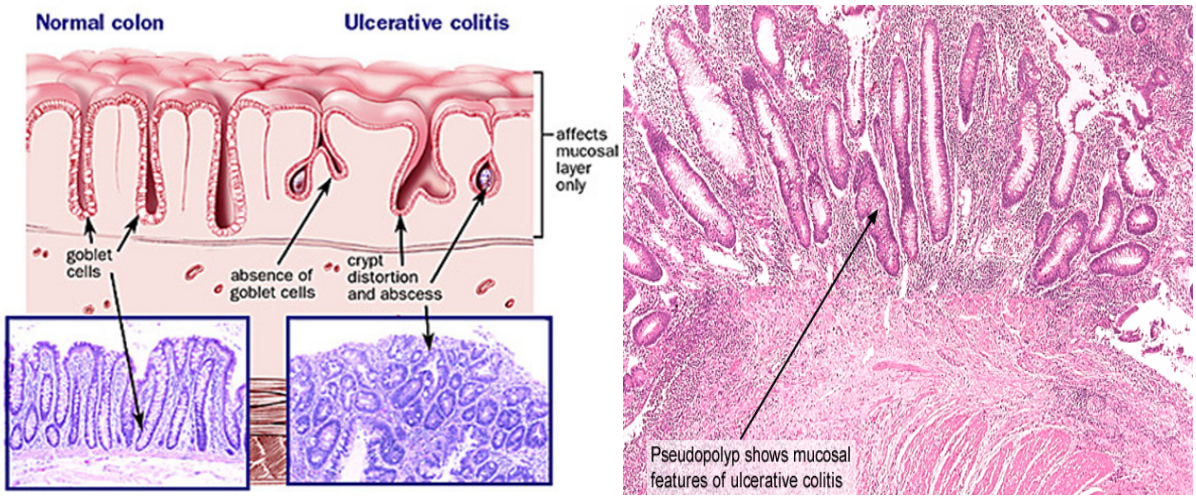


Figure4. Histological Samples - UC: mucosa with acute and chronic inflammation, crypt abscess formation, cryptitis and development of pseudopolyps. Artwork is reproduced from the Courtesy of Cellular Pathology Dept, St George's Univertiy of London. www.elu.sgul.ac.uk. copyright 2009.

The microscopic features of this disease are an inflammatory process limited to the mucosa and superficial submucosa, deformation of crypt architecture and reduction of crypt

number, edema and huge cell infiltrates consisting of B/T-lymphocytes, neutrophils and macrophages that invade the epithelium and cause crypt abscesses [31].

1.1.5 Treatment Options in IBD - Medication and Surgery

Treatments for IBD are either medical or surgical options. Nowadays there are many different drugs on the market especially aminosalicylates, anti-TNF agents, corticosteroids and of course antibiotics which helps to inhibit the overwhelming inflammatory response [2], [32], [33]. These medications allow the intestinal wall to recover, thereby decreasing symptoms like abdominal pain etc. and further other treatments are used to decrease flare-ups and maintaining remission [34], [35]. Surgery only comes into play after all medical options have failed.

The first option for patients with an inflammation of the digestive tract (CD) or mild to moderate UC is 5-aminosalicylic acid (5-ASA) [36], [37], an anti-inflammatory drug, followed by oral and intra-venous application of corticosteroids [38]. Azathioprine [39], [40], mercaptopurine [40], [41] and infliximab [2], [42], [43] can be used in refractory cases, cyclosporine [44], tacrolimus [45] and infliximab for severely active disease unresponsive to the application of corticosteroids. There are studies that people with IBD produce a higher amount of TNF-α, and Infliximab, a monoclonal antibody, prevents it from binding to the cell receptor [46].

Surgical treatment in patients with IBD varies, depending upon the disease. In about 30% of persons with UC, medical treatment is not 100% successful and in such patients, surgery can be considered [47]. UC is a surgically curable disease because the disease is limited to the colon and so it can be cured after colectomy [34], [48]. Emergency surgery is indicated in case of life-threatening complications such as perforation, refractory rectal bleeding requiring blood-transfusions and toxic megacolon refractory to medical management [49]. Dysplasia or cancer as well as UC refractory to medical therapy and intolerance to long-term immunosuppression can necessitate elective surgery [48]. The surgical technique that is used in most cases of UC is the curative total colectomy with ileal J-pouch anastomosis [50]. Although surgery is not curative in patients with CD, approximately 70% of persons will require surgery at some point of time.

The most common surgery for CD is segmental resection, where a segment of intestine with active disease is removed and the remaining bowel is reanastomosed (ends of healthy bowel are joined together) [35], [51].

1.2 Colorectal Cancer (CRC)

1.2.1 Epidemiology of CRC

CRC, commonly known as bowel cancer, is a serious malignant disease. It often begins in the large intestine and depending on the first appearance; CRC can be termed as colon cancer or rectal cancer. Two-third of bowel cancer starts in the colon, while one-third develops in the rectum [52].

CRC is the third most frequent cancer diagnosed in men and women, and the second most malignant tumour in Europe. Each year more than 450,000 Europeans are newly diagnosed and 230,000 of them die [53], [54]. Since the 80's, the CRC mortality rate is trending downwards, a fact which is most likely due to the introduction of general screening and improvements in therapy. Finding polyps and tumors in the earlier stages, CRC becomes easier to treat and also the improvement of medication options have contributed to an increase in survival rates [53]. In Austria CRC is the third most common cancer in men and 2nd in women. In 2009 13.4 % of all cancers in males, and 13.7 % of all cancers in females were linked to CRC [55], [56]. The lifetime risk of developing CRC in the average risk person is around 6 % and common ages affected by colon cancer are those over 65 years [57], [58].

There is a wide geographical variation in incidence and mortality across the world due to differences in diet (especially consumption of red meat), alcohol, as well as obesity and a lack of physical exercise [59], [60], [61], [62], [63], [64]. The CRC incidence rates worldwide are higher for men than women for both colon and rectal cancers (rate ratio 1.4:1.0). There are 10-fold differences in both sexes in incidence and a six-fold variation in mortality between the different regions of the world.

In 2008, mortality rates were found to be highest in Central/Eastern Europe (20.1 and 12.2 per 100,000 in males and females), but lowest in Middle Africa and South-Central Asia (3-4 per 100,000 in both sexes) **[Figure5]** [54].

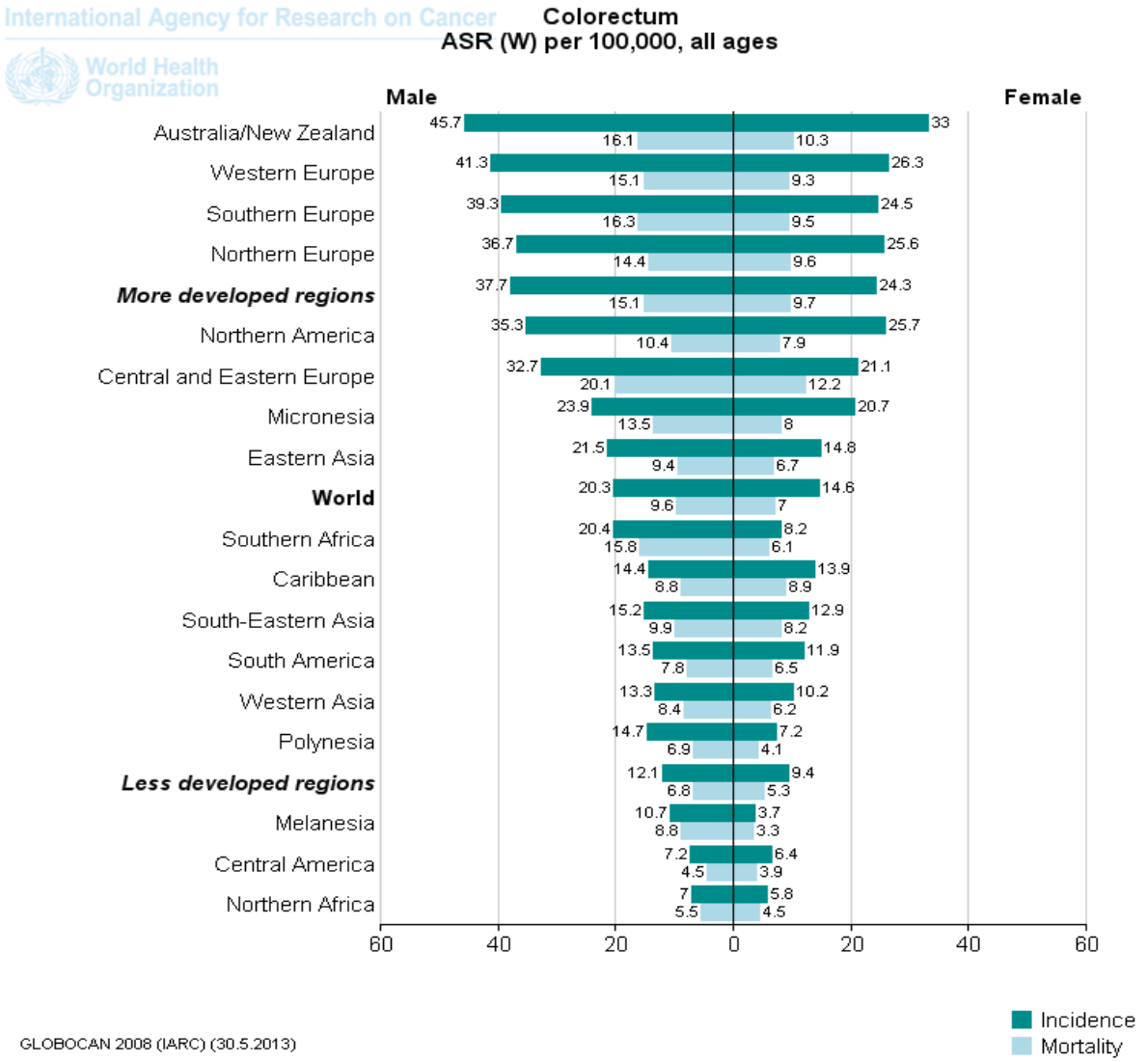


Figure5. GLOBOCAN 2008: CRC incidence and mortality worldwide [54].

Many studies revealed the complexity of the pathogenesis of CRC, which includes environmental as well as hereditary factors. CRC can be divided roughly into sporadic, familial and hereditary cases as well as IBD associated CRC [31], [65]. Estimated 75% of all

CRC cases are sporadic and not caused by genetic predispositions most frequently as a result of alterations in the Wnt-APC-β-catenin signaling pathway that intrinsically increases signaling activity. Mutations in the APC gene are seen in 70 to 80% of sporadic tumours, and often occur early in the development of CRC [65], [66].

The remaining 25 % are either familial like familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) or IBD related **[Figure6]** [67].

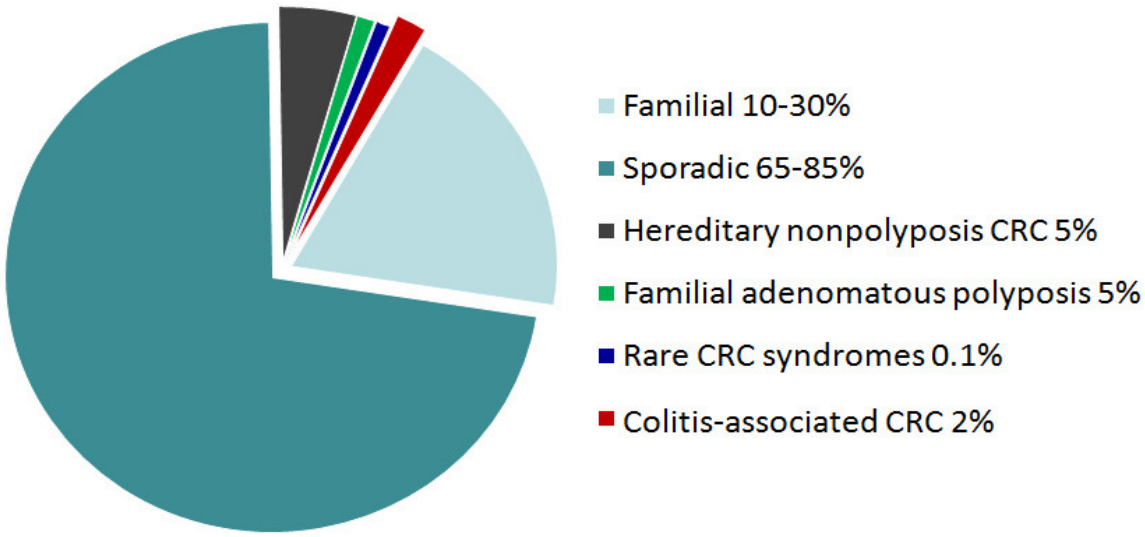


Figure6. Colorectal Cancer Subgroups and percentage of distribution: Familial 20%, Sporadic 75%, HNPCC 5%, FAP 5%, Rare CRC syndromes 0.1% and Colitis-associated CRC 2% [67].

CRC should be preventable, through increased surveillance, chemopreventive agents and a healthy and balanced diet. There is tentative evidence that high calcium supplementation [68] and vitamin D intake [69], [70], as well as the chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) [71] and aspirin [72] are associated with a risk reduction in such groups [73]. Experimental evidence suggests a chemopreventive effect of mesalazine (5-ASA) to reduce CRC risk by approximately 50% [74].

As previously mentioned CRC is often caused by accumulation of alterations in oncogenes and tumor suppressor genes which can lead to aberrant regulation of β-catenin signaling [75]. Mutations of adenomatous polyposis coli (APC), β-catenin, but also other components

of the well known Wnt-pathway promote the transition of single pre-neoplastic cells to aberrant crypt foci (ACF) and then to adenoma and colorectal carcinoma **[Figure7]** [76].

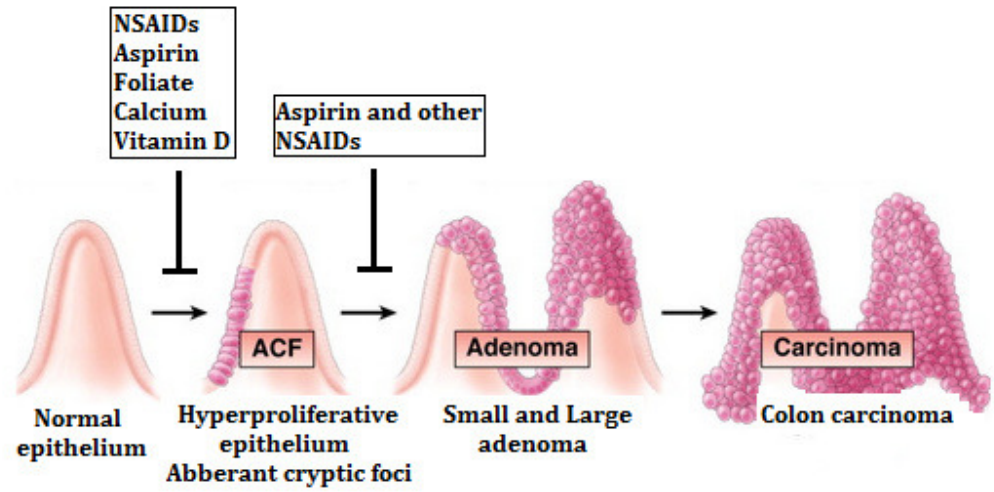


Figure7. The effect of chemopreventive agents like NSAIDs, Aspirin, Fliate, Calcium and Vitamin D at different stages of colon carcinogenesis [76].

Approximately 80% of CRCs develop from adenomatous polyps which offers the opportunity to screen and this has the potential to reduce colorectal cancer deaths by 60% [77]. The most common screening tests are fecal occult blood testing, flexible sigmoidoscopy and colonoscopy [65]. In 2007, the enzyme M2-PK has been identified as an important biomarker to test for CRC and polyps [78].

1.2.2 Sporadic Colorectal Cancer (SCC)

The majority of CRC cases are sporadic which means they are not related to family history or genetics. SSC develops from dysplasia in 1 or 2 foci of the colon and occurs as a result of genomic instability which refers to a high frequency of mutations within the genome of a cell [67]. The two main types of genomic instability are chromosomal instability (CIN) and microsatellite instability (MSI), counting 85% and 15% of SCC [31], [79]. CIN indicates an increased rate of chromosome missegregation and results in abnormal DNA content

(aneuploidy). Loss of chromosomal material (loss of heterozygosity) is a common cause of genetic disorders and important genes like APC and p53 can be rendered nonfunctional by such mutations [80].

In 1990, Fearon and Vogelstein described a multi-gene and selection model for CRC which points out the importance of several key genes especially APC and p53 in colon carcinogenesis, while further studies confirmed other players like k-ras oncogene, the tumor suppressor gene deletion in colon cancer (DCC), as wells as the enzyme cyclooxygenase-*2* (COX-2), which are also strongly involved in the development of CRC **[Figure8]** [81], [82], [83]. Colon cancer is a good example for the multi-hit model of cancer induction:

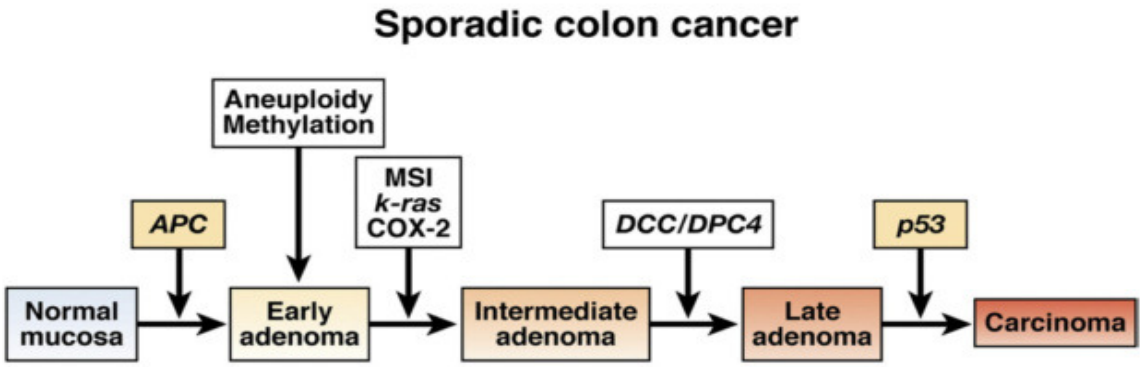


Figure8. Molecular pathogenesis and alterations in sporadic colon cancer (SCC) [31].

The APC gene is located on chromosome 5q21-q22 and has its key role in destabilizing free β-catenin [84]. It's considered as a tumor suppressor gene which helps to prevent uncontrolled growth of cells that result in cancerous tumors and is also known as the "gatekeeper" of the colon. Mutations and thereby loss of function occur in approximately 85% of all sporadic and inherited colorectal tumors and is typically an early event in SCC pathogenesis [83]. These mutations render β-catenin refractory to phosphorylation by glycogen synthase kinase-3β (GSK-3β) [85], leading to an accumulation of stabilized free β-catenin which is an early and perhaps the initiating event in colon carcinogenesis [79]. Once a sporadic adenoma forms, other alterations in genetic regulation arises, such as induction of k-ras oncogene and loss of function of tumor suppressor genes DCC.

Inactivation of p53 gene occurs late which often coincidences with the transition from adenoma to carcinoma [31], [79].

Epigenetic changes like aberrant DNA methylation can also contribute to altered gene expression in tumorigenesis [86]. Hypermethylation of CpG islands can lead to a block in transcription of tumor suppressor genes. Mismatch repair gene MLH1 inactivation due to promoter methylation is a trigger for MSI in sporadic microsatellite unstable colon cancers [87].

1.2.3 Colitis-associated Colon Cancer (CAC)

Patients with UC and CD are at increased risk for developing CRC and the longer a person has had the disease, the more prone he or she will be for cancer [88]. The presence of other inflammatory disorders (such as primary sclerosing cholangitis) also increases the risk of CRC, whereas it decreases when patients get medications to reduce inflammation (mesalamine and/or steroids). People with an inflammation of the digestive tract (CD) have a 2% chance to get colorectal cancer after 10 years, 8% after 20 years and 18% after 30 years [89]. Previous studies revealed that the prevalence of CRC in UC is 3.7 % overall and 5.4 % for patients with pancolitis [90], while family history of CRC increases the cancer-risk in IBD patients at least 2-fold [91]. CAC arises at younger age and progresses to invasive carcinoma from flat and nonpolypoid dysplasia more frequently than SCC [79].

The molecular and genetic features that lead to SSC (genomic instability and DNA hypermethylation), also appear in CAC but unlike the normal colonic mucosa, cells of the inflamed mucosa have these genetic alterations before there is any histological evidence of dysplasia or cancer [31] and there is tentative evidence that oxidative stress (ROS/NOS) is likely be involved [92]. Inflammatory cells such as neutrophils and macrophages are known to produce reactive oxygen and nitrogen species which can lead to DNA damage or alter the regulation of protective genes against carcinogenesis (such as p53, DNA MMR proteins and DNA base excision-repair (BER) proteins), transcription factors (such as nuclear factor–B), or signaling proteins (such as cyclooxygenase-2) **[Figure9]** [79].

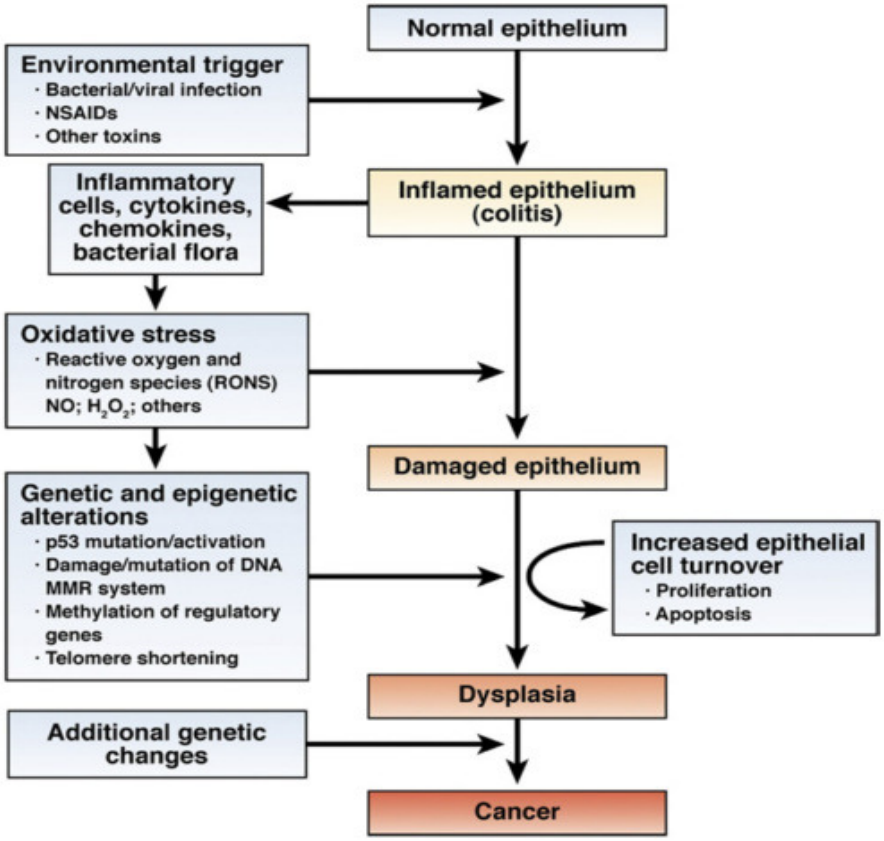


Figure9. Molecular and genetic events from inflammation to cancer. Enviromental triggers like infections or toxins can lead to inflamed tissue while inflammatory cells, oxidative stress, genetic alterations and other factors can accelerate the transition from inflamed/damaged epithelium to cancer [31].

CAC related chronic inflammation is also characterized by the overproduction of pro-inflammatory cytokines such as IL-6 and TNFα, which lead to mutations in tumor suppressor genes (APC, p53, k-ras) but also oncogenes and genomic instability via several mechanisms [93]. The neoplastic transformation in CAC is similar to the adenoma-carcinoma sequence in sporadic CRC, but some alterations occur at different time points [79]. In CAC, p53 mutations occur early and can even be detected in non-dysplastic mucosa, while APC is the driving factor for the transition from adenoma to colon carcinoma **[Figure10]** [31]**,** [67], [79], [88].

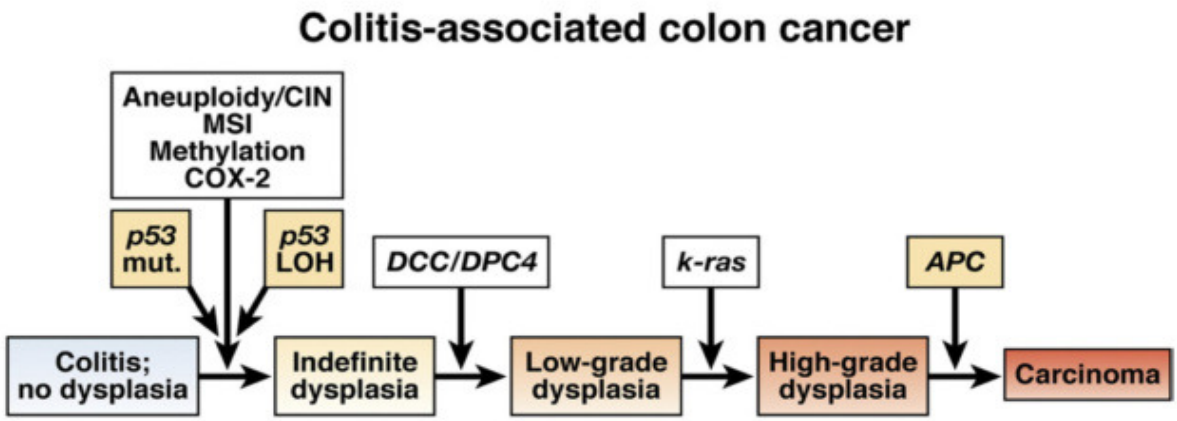


Figure10. Molecular alterations in colitis-associated CRC. APC mutations are late events while p53 arises very early [31].

As previously mentioned, epigenetic alterations also contribute to progression of CAC, where CpG island methylation precedes dysplasia and can be localized throughout the mucosa of patients with UC [94]. CpG islands are regions all over the human genome, often associated with promoter regions, which consists of a higher CpG site concentration where stable methylation patterns with approximately 70% of all CpG-dinucleotides being methylated can be found [95]. Methylation of these sites result in transcriptional repression and occur especially increased in a subset of CRCs, known as CpG-island methylator phenotype (CIMP) cancers [96].

Among neoplastic samples from persons with UC, hMLH1 hypermethylation was observed in 6 of 13 (46%) with high levels of MSI, 1 of 6 (16%) with low levels of MSI, and 4 of 27 (15%) without MSI [97].

1.3 Neutrophils and the Inflammatory Process

1.3.1 Polymorphonuclear neutrophils (PMNs)

Neutrophils are very important immune cells of the innate response during infection and have a key role in maintaining intestinal homeostasis [98]. They belong to the polymorphonuclear cell family (together with basophils and eosinophils) which can be

distinguished by their different staining properties (Wright stain and May Grünwald stain). PMNs are the most common type of white blood cells with an average diameter of 8–9 µm and can be characterized by the presence of cytoplasmic granules and a nucleus divided into 2–5 lobes **[Figure11A]** [99].

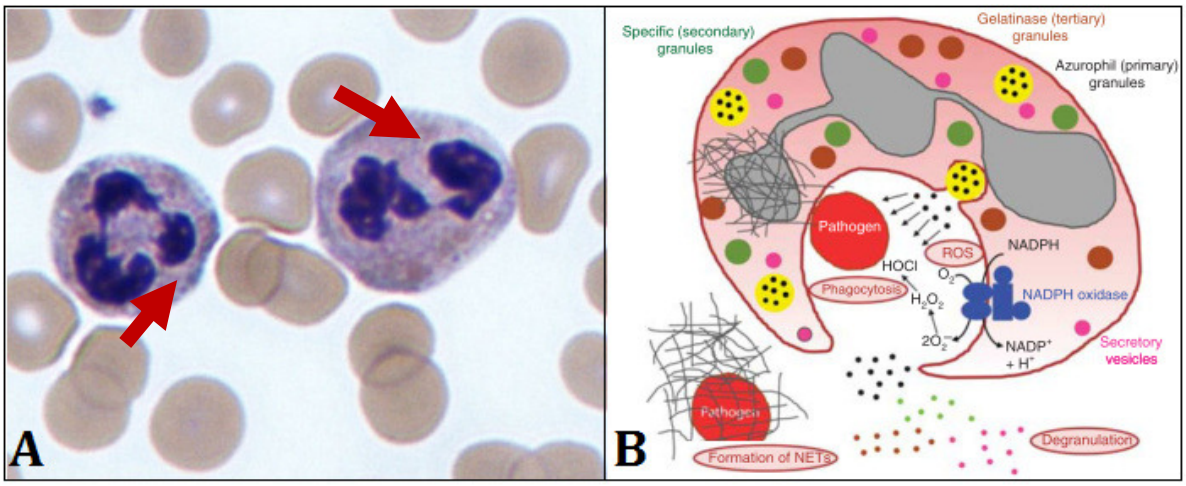


Figure11. A: Giemsa staining of segmented neutrophils surrounded by erythrocytes. Red arrows indicate granules in the cytoplasm [100]. **B**: The multifunctional ability of PMNs: They are able to produce ROS, release three types of granules by a process called degranulation, form NETs and engulf pathogens via phagocytosis [98].

They are produced via granulopoeisis within the bone marrow (approximately 10^{11} PMNs daily and around 10^{12} per day during acute inflammation) and represent 50 to 65% (25×10^9 cells) of the total circulating leukocytes which are known as the ''first defense line'' against pathogens or infectious agents that penetrate the body's physical barriers [30], [101]. Granulocyte colony-stimulating factor (G-CSF) is a growth factor and cytokine produced by a number of different tissues and is the main regulator of granulopoiesis which affects the commitment of progenitor cells to the myeloid lineage, proliferation of precursors, as well as stimulating the release of mature cells into blood stream [102]. Upon release into circulation, the average lifespan of PMNs is only 4 to 10 h before they marginate (position themselves to the blood vessel endothelium), following a selectin-

dependent capture and integrin-dependent adhesion, after which they enter tissue pools where they survive up to 2 days [103], [104].

PMNs have several specific cell surface receptors, including receptors for detection and adhesion to endothelium, cytokine receptors, as well as chemokine receptors like the well known CXC chemokine receptor 2 (CXCR2), regulating neutrophil functions in inflammatory diseases [105]. It allows the detection of chemical gradients such as interleukin-8 (IL-8), one of the major mediators of inflammatory response, which acts as a chemotactic signal attracting PMNs to the site of inflammation [106]. Within the inflammatory process, PMNs contribute to the recruitment of other immune cells e.g. macrophages and promote mucosal healing by releasing mediators necessary for the resolution of inflammation [98]. Although the role of neutrophils is clearly beneficial in many cases, accumulation of activated PMNs within colonic crypts like in IBD is associated with severe disease symptoms and mucosal injury [98], [107], [108]. The mucosal injury of UC is characterized by increased transepithelial migration of PMNs forming crypt abscesses [29], [98].

1.3.2 Oxidative stress

As mentioned previously, chronic inflammation can promote colon cancer due to chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylation (CIMP), but also oxidative stress can be linked to the development of CRC [31]. IBD has been considered to be an "oxyradical overload" disease in which stress induced by degranulating neutrophils can lead to cellular damage that contributes to pathogenesis of colitis itself and is suggested to be one of the major factors driving colon carcinogenesis [31], [88], [98], [103]. Oxidative stress which occurs when reactive oxygen is released and the human body is not capable to detoxify the reactive intermediates, can alter cellular components including proteins, mRNAs and DNA [109]. It is however unclear, whether oxidative stress directly or alone may cause mutations in cells. Many previous studies confirmed that activated PMNs do not only release reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) or hypochlorous acid (HOCl), but also reactive nitrogen species (RNS), antimicrobial peptides (α-defensins), myeloperoxidase, hydrolytic

enzymes (lysozyme, collagenase), proteases (cathepsin G, elastase), metal chelators (lactoferrin) and several cytokines which are able to trigger genomic instability [93], [98]. In recent years, neutrophil extracellular traps (NETs) became a very hot topic and is often described as a critical component for PMNs to trap and kill invading microorganisms **[Figure11B]** [110].

The generation of ROS is attained by the activation of a membrane-bound multisubunit complex, the so called nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase - Nox). It is composed of five phox subunits: p47phox, gp91phox (contains heme), p22phox, p67phox, p40phox and a Rho guanosine triphosphatase (GTPase), usually Rac1 or Rac2 [111]. Phosphorylation of p47phox induces the activation of the complex by initiating the assembly of its subunits at the cell membrane **[Figure12]** [112]:

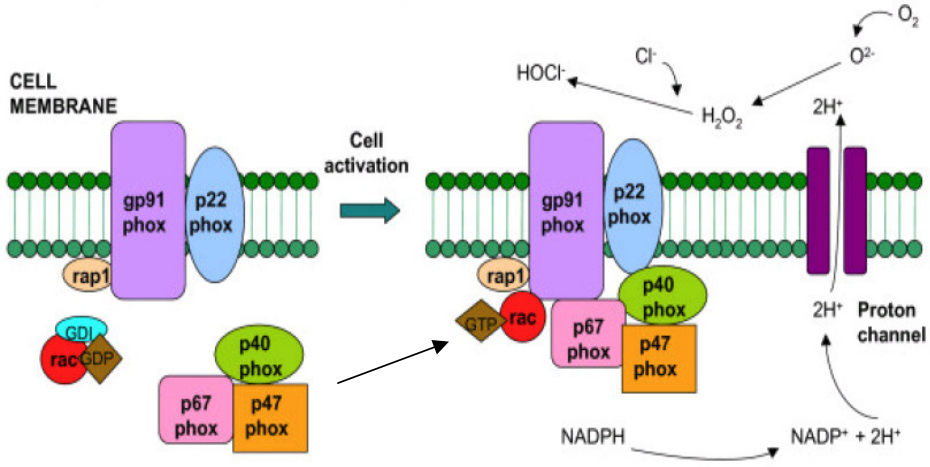


Figure12. Nox complex assembles at the cell membrane and generates superoxide anion by transferring electrons across membrane and coupling these to oxygen [113].

Nox is important for pathogen killing upon phagocytosis and for regulating proinflammatory signaling within phagocytic cells [114]. It plays also a critical role in pathology e.g. atherosclerosis (atheroma), where ROS activates an enzyme that makes the macrophages (containing cholesterol) adhere to the artery wall [115]. Nox deficiency (e.g. chronic granulomatous disease) can lead to severe, recurrent bacterial and fungal

infections caused by defective respiratory burst function and patients often die as a result of regular microbial infections [113], [116].

1.4 Microsatellite Instability (MSI) in Colorectal Cancer

MSI is a hypermutable phenotype caused by an impaired DNA mismatch repair (MMR) system (see below) [117]. It is the phenotypic evidence that the MMR system doesn't work properly any more, causing frame-shift mutations of short tandem repetitive sequences throughout the genome which are composed of one to six nucleotide bases [118]. MSI represents a hallmark of MMR deficiency and is detected in 15% of all CRCs; 3% of them are associated with Lynch syndrome (HNPCC) due to point mutations in MMR-genes and the other 12% are caused by sporadic aberrant CpG-island methylation of the MLH1 promotor which occurs in tumors with the CpG island methylator phenotype **[Figure13]** [118], [119].

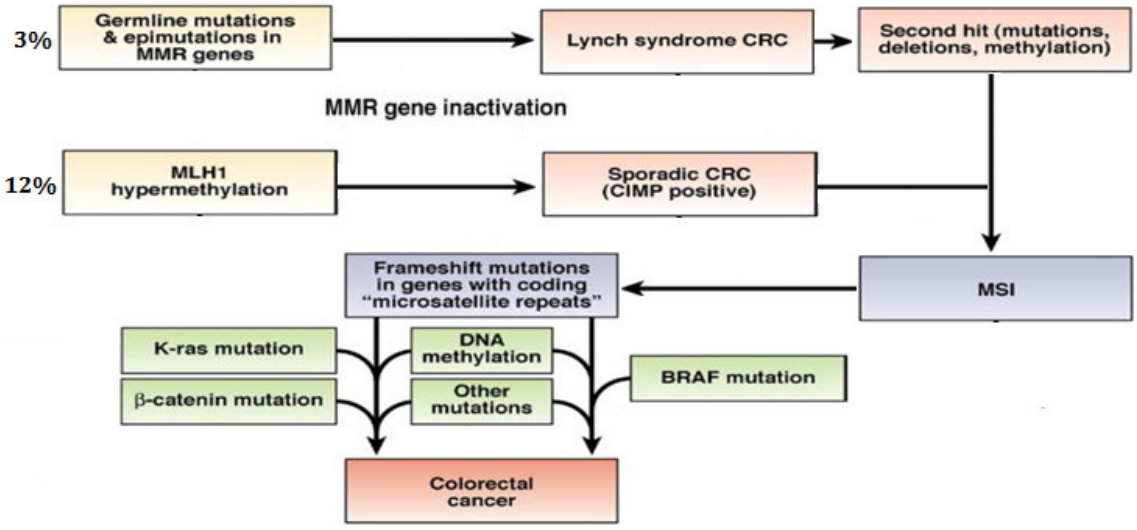


Figure13. Two molecular pathways can lead to the development of CRC with MSI; 3% Lynch syndrome and 12% Sporadic CRC (CIMP positive)[118].

The most common microsatellites in humans are the (A)n and the (CA)n repeat, which are spread all over the human genome about 10^5 times and can appear in coding regions, especially in promoters of several cancer-related genes [120], [121]. Mutations within the

10-bp polyadenine region (A10) of the transforming growth factor β receptor type II (TGFβRII) are found in approximately 90% of all colorectal cancers with MSI [122].

Further studies identified several other key players affected by MSI which are regulators of cell proliferation (GRB1, TCF-4, WISP3, insulin-like growth factor-2 receptor, axin-2, and CDX), cell cycle or apoptosis (BAX, caspase-5, BCL-10, PTEN and FAS etc.), as well as the DNA repair system (PMS1, PMS2, MBD-4, BLM, CHK1, MLH3, RAD50, MSH2, MSH3, and MSH6) [123], [124].

Microsatellites are abundant throughout the genome and the lengths of the repeated sequences are very polymorphic among individuals but are unique and uniform in every tissue in each person. They are also stably inherited and valuable for linkage mapping and genomic analysis [125], [126]. There are two forms of MSI which can be distinguished according to the number of mutated microsatellites. The tumor is classified as MSI-high (MSI-H) when two or more (typically both mono- and dinucleotide repeats) of the five microsatellite sequences recommended by the National Cancer Institute (NCI) are mutated [125]. If only one of the microsatellite sequences is mutated (typically a dinucleotide repeat), the tumor is termed MSI-low (MSI-L) and microsatellite stable (MSS) if there is no mutation in the microsatellite panel [127], [125].

In UC, there is experimental evidence showing that MSI was not only found in dysplastic and cancerous tissue but also in inflamed non-dysplastic mucosa [30]. The group of Suzuki et al used a panel of 5 dinucleotide repeat markers and found out that 21% of 63 colitis-associated tumors showed at least 1 mutated microsatellite marker [128]. Brentnall et al described instability in at least 1 of 7 dinucleotide repeat markers in 50% of non-neoplastic mucosa samples from patients with chronic UC [129]. As mentioned, patients with IBD have an increased risk for CRC but it isn't completely clear how chronic inflammation can lead to tumorigenesis. The inflammatory process can increase mutagenesis due to oxidative stress and the formation of free radicals and this can be linked to an impaired DNA MMR system [130]. The current hypothesis is that high doses of ROS and RNS released by inflammatory cells like PMNs can overwhelm DNA repair pathways leading to an accumulation of DNA lesions which can turn into mutations [131]. So it can be said that the mechanism for inflammation-associated mutagenesis is a combination of mutagen accumulation together with an inactivation of the DNA repair system by oxidative stress

[130] or by promoter hypermethylation [97]. Similarities reported for base excision repair (BER) enzymes, may also result in MSI [132].

1.5 The Mismatch Repair System (MMR)

DNA mismatch repair is a process that takes place in almost every cell of a living organism (prokaryotic and eukaryotic) which helps to deal with spontaneous or acquired DNA damage. The MMR system is evolutionally conserved and it serves as a control mechanism to detect and avoid mismatch errors during DNA replication which can lead to a complete loss or aberrant protein function [133].

The DNA replication itself is not a fully error-free process. Polymerases induce frame-shift errors due to a process called polymerase slippage, where the DNA polymerase slips during the replication from the DNA template strand at the repeat region and reattaches at a more distant site, which can cause an expand section of DNA [134]. DNA MMR is always present to eliminate such multiple base insertion-deletion loops (IDLs) as well as single base pair mismatches. IDLs affect repetitive DNA sequences and can lead to gains or losses of short repeat units (e.g. CA) within microsatellites which is referred to as microsatellite instability **[Figure14]** [135].

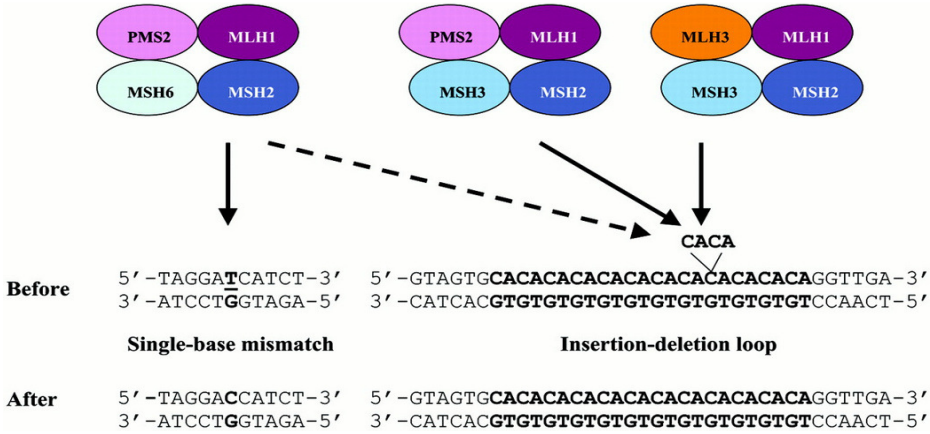


Figure14. MMR protein complexes and different types of mismatches [135].

The MMR system includes homologues of bacterial MutS and MutL, heterodimers which comprises of 6 different proteins hMSH1-hMSH6 and hMLH1-hMLH3, as well as post

meiotic segregation increased (hPMS1 and hPMS2) [133]. hMSH2 is the main mismatch recognition component which forms a heterodimer with MSH6 or MSH3 (complexes are called hMutSα and hMutSβ) depending on whether single base pair mismatches or IDLs have to be repaired [136]. Subsequently hMutSα or hMutSβ recruit hMutLα (heterodimer of hMLH1 and hPMS2) which coordinates the interplay between the mismatch recognition complex and several other enzymes, such as DNA endonuclease, helicase, polymerases δ and ε, ligase, etc. which are required to complete the repair process. Hydrolysis of ATP is thought to activate hMutSα which binds via the "sliding" clamp to the DNA **[Figure15]** [119], [137], [138].

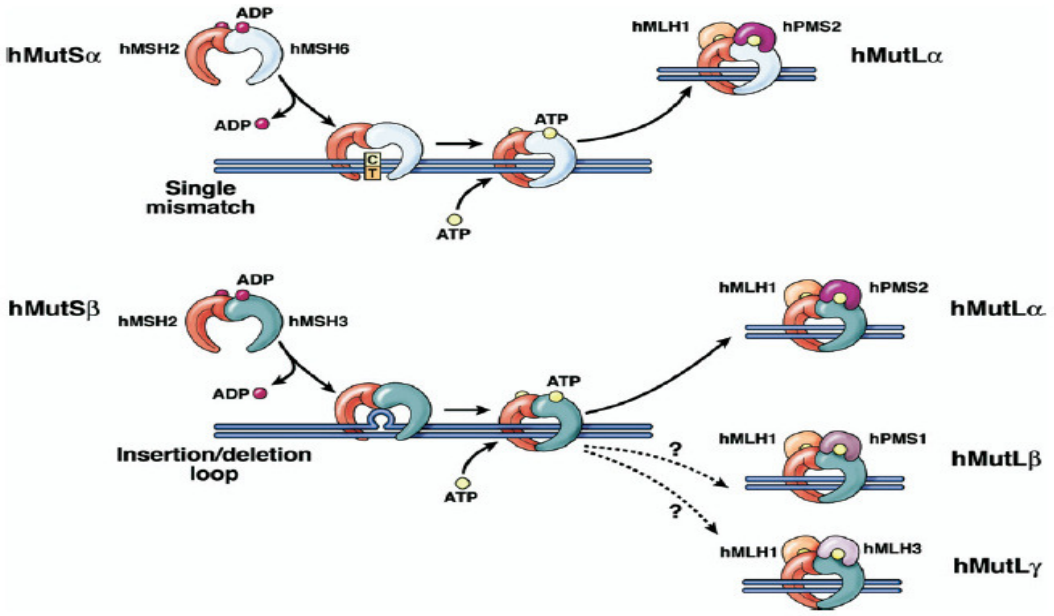


Figure15. hMutSα and hMutSβ during MMR. Single mismatches are recognized by hMutSα while larger insertion-deletion loops are recognized by hMutSβ. Subsequently both heterodimers bind to hMutLα which coordinates the further repair process [119].

MLH1 heterodimerize with PMS2 but also with two additional proteins, MLH3 and PMS1. The MLH1–MLH3 heterodimer as well as the MLH1–PMS2 complex primarily functions in the repair of IDLs [119].

In CRC a genetic instability in the DNA MMR system is often found [139]. For example HNPCC is caused by the inactivation of the MMR and ends up in microsatellite instability

(MSI, see above). Of the HNPCC tumors that show MSI, over 50% contain mutations in the human MSH2 gene on chromosome 2 and about 20-30% in the hMLH1 gene on chromosome 3 [140] and there is also a known link between MLH1 and CD/UC [141], [142].

1.6 Cytokines and their role in tumorigenesis

We already discussed the role of chronic inflammation which can promote cancer development, but many molecular mechanisms behind are still under investigation and can differ between colitis-associated and other forms of CRC [143]. Previous work has elucidated that the release of free radicals (ROS and RNS) during inflammation might induce accumulation of genetic alterations within the genome thus leading to the onset of tumor progression but recent studies give the concept that also the large amount of cytokines and chemokines released during the inflammatory process by immune and non immune cells may can influence carcinogenesis, including initiation, promotion, progression, and metastasis [144]. Around 20% of all cancers arise in association with chronic inflammation and most solid tumours contain inflammatory infiltrates where cytokines and chemokines may promote growth of tumor cells, perturb their differentiation and support their survival [143], [145].

These infiltrating immune cells and the production of cytokines and chemokines create a pro-tumorigenic microenvironment which contributes to tumor initiation and progression. Up to now there a many cytokines investigated which are implicated in UC and CAC development. Some of them promote all stages from initiation to invasion and metastasis, whereas others are stage specific. For example increased levels of pro-inflammatory TNFα, IL-10, IL-23, IFNγ, IL-6 and IL-8 are related to tumorigenesis while several, such as TNFα, IL-1, IL-6, IL-11, IL-22 and IL-23 are involved in tumor promotion. IL-6 and TNFα also play a role in tumor invasion and metastasis [146].

[Table-2] represent a summarization of all cytokines which can be linked to inflammatory bowel disease (IBD), ulcerative colitis (UC), colorectal cancer (CRC) and colitis-associated cancer (CAC) [146]:

Cytokine	Function	Role in disease
IL-1β	Pro-inflammatory	Primary response to IEC bacterial infiltration
		Protective against tumor growth in a mouse model
		Associated with advanced adenoma
		Associated with UC, CD and CRC
IL-2	T cell response	T cells contribute to chronic IBD
	Th2 response	Decreased levels associated with adenoma recurrence
		Associated with CD
IL-4	Th2 response	Associated with UC
		Promotes tumor growth in mouse model of CRC
IL-5	Th2 response	Characteristic of UC
IL-6	Pro-inflammatory	Associated with increased CRC occurrence, and mortality
		Associated with UC and CD
IL-8 (CXCL8)	Neutrophil chemoattractant	Associated with UC, CD and CRC
IL-10	Anti-inflammatory	Lower levels associated with CRC and higher levels with UC
	T reg response	Reduced levels increase tumor growth in mice
		Increased levels associated with decreased risk of advanced adenoma
IL-11	Th1 response	Survival and growth
IL-12 (p35/p40)	Pro-inflammatory	Characteristic of UC
	Th17 response	Predictive for chronic colitis in comparison to acute colitis
		Increased in Stage I CRC patients
IL-17	Th17 response	Predictive for chronic colitis in comparison to acute colitis
	Pro-inflammatory	Associated with murine colitis
		Elevated in CD patients
		Increased level in CRC patients
IL-18	Pro-inflammatory	Primary response to bacterial infiltration in IBD
		Protective against tumor growth in mouse model
IL-22	Innate immunity	Survival
IL-23 (p40/p19)	Pro-inflammatory	Involved in murine colitis and CAC
		Associated with increased risk of CRC
TNFα	Pro-inflammatory	Chronic expression present in IBD
		Higher concentration associated with adenoma
		Involved in murine colitis and CRC
		Associated with increased CRC occurrence
IFNγ	Th1 response	Associated with improved prognosis of CRC
		Characteristic of CD
KC (CXCLI)	Neutrophil migration	Predictive for chronic colitis in comparison to acute colitis
G-CSF	Neutrophil response	Predictive for chronic colitis in comparison to acute colitis
TGFβ	Anti-inflammatory	Characteristic of UC
	Th2 response	EMT, CRC promotion and invasion
CRP	Pro-inflammatory	Associated with adenoma and CRC

Table-2. Cytokines involved in IBD and CRC. They regulate initiation of the innate immune response, stimulation of cell-mediated adaptive immunity, homeostasis, cell migration, survival and anti-inflammatory responses [146].

Murine models like the sulfate sodium/ azoxymethane (DSS/AOM) mouse model are commonly used to investigate changes in cytokine expression and alteration in known inflammatory pathways during UC and CAC [147]. The AOM/DSS model mimics human UC disease by increasing intestinal epithelial permeability, thereby facilitating transmucosal infiltration of intestinal bacteria and carcinogens which leads to immune cell infiltration and an inflammatory response [148]. Yutao et al demonstrated that DSS treatment reversibly induce the expression of various cytokines including IL-1β, TNFα, IFNγ, IL-6, IL-10, IL-12, and chemokines MIP-2 [149].

Experimental evidence suggested that cytokines from immune cells can increase intracellular ROS and RNI in pre-malignant cells, leading to DNA damage, epigenetic changes that silence tumor suppressors and promote tumor initiation **[Figure16A]** [76]. Different factors like tissue injury, commensal/pathogenic microbiota, and necrotic products can stimulate cytokine production by immune cells within the tumor microenvironment. This crucial mechanism depends on nuclear factor-κB (NF-κB) activation, whereas signal transducer and activator of transcription 3 (STAT3) has anti- and pro-inflammatory roles in immune cells [150].

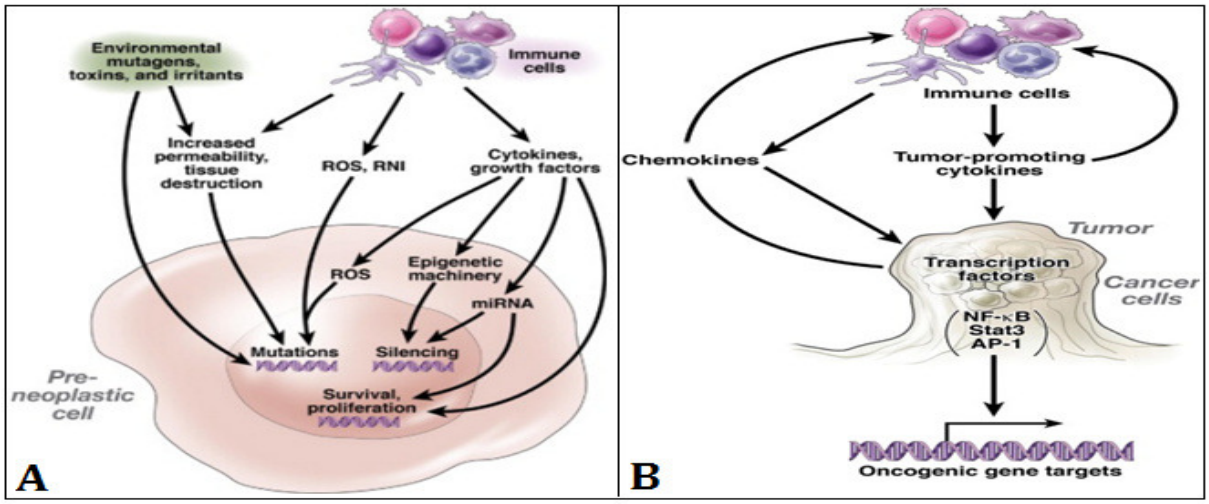


Figure16. The role of cytokines in tumor initiation and progression [76].

Such soluble factors in the tumor microenvironment activate transcription factors such as NF-κB, STAT3, and AP-1, which regulate expression of several genes important for

processes such as cell proliferation, tumor growth, resistance to cell death, angiogenesis, and tumor progression, invasion and metastasis [76]. NF-κB, STAT3, and AP-1 activation induces positive signaling loops that increase production of cytokines and chemokines and recruit and activate inflammatory cells at the side of infiltration **[Figure16B]**.

IL-6, IL-8 and TNFα are key players in the initiation and maintenance of colon inflammation during IBDs and show influence in the development of CAC [144]. The serum levels especially of these cytokines are elevated in colorectal carcinoma patients, and some studies have suggested that increased plasma levels may have a prognostic value and so they are emerging as potential targets for anticancer therapy [151]. Cancer cells can stimulate neutrophils and macrophages to produce IL-6 which activates STAT3 in tumor cells [152]. Serum levels of IL-6 are increased in IBD patients and correlates with tumor stage, size, metastasis [151]. In vivo experiments with IL-6 $^{-/-}$ murine models showed a reduced tumor number upon AOM/DSS treatment compared to the WT mice but treatment with recombinant IL-6 increased both tumor multiplicity and tumor size [153].

Szkaradkiewicz et al. detected increased levels of IL-8 in the serum of UC, CD and CRC patients and demonstrated that IL-8 and its receptor CXCR2 are two common significantly upregulated chemokines in colon cancer. It has been shown that IL-8 through binding to CXCR2 can act not only in inflammatory processes and infectious diseases, it also can influence cancer cells via several receptors to promote migration, invasion and proliferation [154].

TNFα is a pro-inflammatory cytokine and activates oncogenic signaling pathways in epithelial cells, including Wnt and NF-κB regulating their growth and survival [155]. Recent studies for UC have indicated linkage to chromosome 6 in the TNF-alpha gene region where many single nucleotide polymorphisms (SNP) within promoter region shows correlation with either increased or decreased cytokine production [156]. The ability of TNF to induce DNA damage can may linked to the production of ROS and several studies demonstrated that treatment of cells with TNFα increases chromosomal instability, gene mutations and gene amplifications, suggesting a direct mechanism which leads to cancer development [157]. Pharmacological inhibition of TNFα with antibodies is very efficient in the treatment

of IBD patients and several inhibitors have been tested as potential medication against colon cancer [158].

Further research of different cytokine expression pattern during colon cancer may lead to the development of new diagnostic and prognostic tools. Analyzing biomarkers in various stages of IBD and CAC progression should help to identify differential serum profiles for chronic inflammation or specific CAC stage. Nowadays there are several CRC tests available and the addition of serum analysis could offer a diagnostic and prognostic feature with reduced invasiveness and increased patient comfort [146].

1.7 Identification of frame-shift mutations in colon cells

As mentioned previously MSI represents a hallmark of MMR deficiency in human cancers and since MSI is frequently detected in colitis tissue without dysplasia, inactivation of the MMR system is suggested to be an early event in UC-associated colon carcinogenesis [118]. 1993 Parsons et al discovered that the HCT116 cell line had defects in MMR activity and MSI that were associated with biallelic mutations in the MLH1 gene. He created a new model by transferring the human chromosome 3 into HCT116 (HCT116+chr3 cells) and thereby restored the MLH1 activity [159]. Up to now this model is used in many labs worldwide and helps to explore the role of MMR for regulation of cell cycle and DNA damage [160]. 2003 Gasche et al took over the idea of this human colon adenocarcinoma model and developed a flow cytometry-based reporter assay for the quantitation of frameshift mutations within a (CA)13 repeat in human mismatch repair-deficient (HCT116, hMLH1-mutated) and mismatch repair-proficient (HCT116+chr3, hMLH1 wt) cells [161]. In the following years C. Campregher, a senior postdoc from the Gasche lab, established a panel of novel EGFP-based frameshift reporter cell lines for the comparison of spontaneous mutation rates within mono-, di- and tetranucleotide repeats [162].

The pIREShyg2-EGFP/MS plasmid harboring A10, G10, G16, (CA)13, (CA)26 and (AAAG)17 repeats immediately after the translation start codon of the EGFP gene was cloned and

stably transfected into HCT116 and HCT116+chr3 colon cancer cells but also into primary human colon epithelial cells (HCEC) **[Figure17A]** [161], [162].

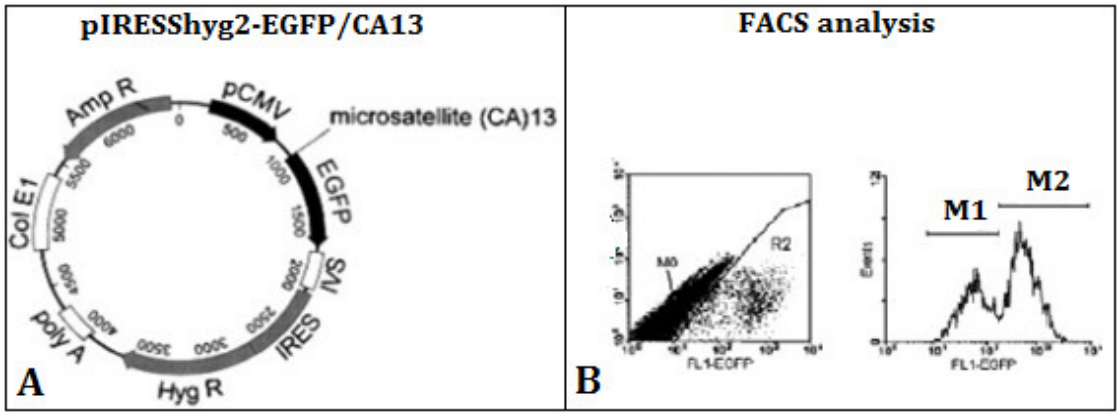


Figure17. A: pIREShyg2-EGFP/CA13 plasmid and FACS analysis. The CA13 repeat was introduced immediately after the EGFP initiation codon. **B:** Fluorescent cells can be detected and analyzed by FACS [161].

The insertion of such microsatellites shifts the EGFP coding sequence out of its reading frame thereby leading to a premature stop codon and a non-functional truncated protein without fluorescence [161], [162]. If the EGFP reading frame gets restored after a frame-shift mutation event (either insertion or deletion) EGFP-expressing cells can be detected by flow cytometry and mutation rates can be calculated. This system gives the possibility to identify EGFP-positive population with low fluorescence intensity which was considered as the "transiently mutated fraction" (M1), and with high fluorescence intensity as the "permanently mutated fraction" (M2) [163] **[Figure17B]** [→more details in the material and methods part].

With the advantages of this reporter assay Campregher et al established an *in vitro* co-culture system using PMNs as effector cells and various colon epithelial cell lines (the above mentioned reporter clones) as targets. This system mimics the carcinogenic environment in UC and is used to answer questions like if and especially how PMNs are able to induce frameshift mutations within colon epithelial cells but it should also give a more detailed insight into molecular pathways which are activated upon PMN induced stress. Some of these questions have already been answered [163].

2. Hypothesis and Aim of this study

Here we hypothesize that exposure of intestinal epithelial cells to secreted products from degranulating PMNs, the major inflammatory cell type in UC, leads to replication errors and frameshift mutations within mono-, di- and tetranucleotide sequences, an inflammation-linked process which may drive colon carcinogenesis. These events are likely triggered by specific factors released by activated PMNs. Therefore we aim to identify specific PMN-released molecules which cause replication errors and frameshift mutations in colon epithelial cells in order to develop specific inhibitors of colitis-associated CRC development.

3. Results

3.1 Activated PMNs induce frameshift mutations in colon epithelial cells

3.1.1 Adaptation of a previously established co-culture system

In a previous study a co-culture system for the simulation of PMN-driven mutagenesis was established [163]. Here we adapted this co-culture system with PMNs as effectors and the above mentioned frameshift-reporter cells as targets in order to check the stability of microsatellite sequences during PMN-induced stress and to identify the factor(s) which is released from activated PMNs that drives MSI **[Figure18]**. The system was improved to a kind of semi high throughput (HTS) method to make this assay more convenient for screening several potential antagonizing agents including ROS/RNS scavengers, neutralizing antibodies and small molecule inhibitors **[Table-8]**.

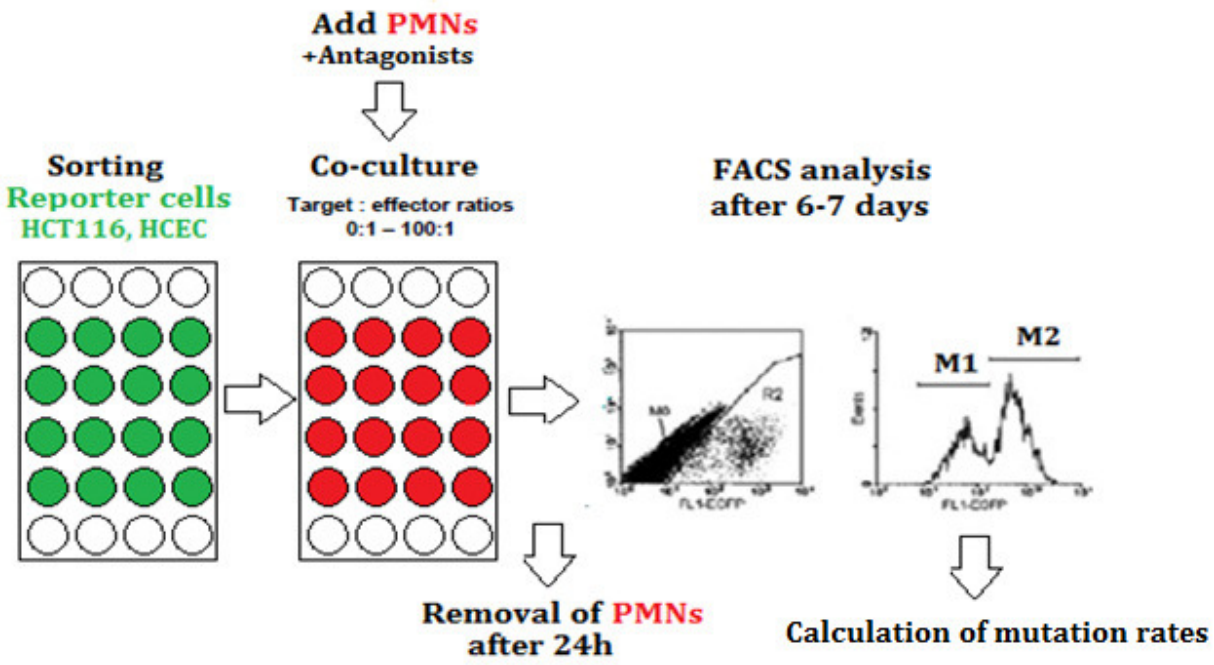


Figure18. Working procedure: Target cells were sorted into 24well plates and treated with effector cells and with or w/o antagonist for 24h. After 6-7 days reporter cells were analyzed by FACS for MSI [161].

As mentioned the main purpose of this new semi-HTS method was to make the pre-established co-culture system easier to screen for a larger number of antagonizing factors in a shorter time and under more physiological conditions. Therefore one important part was to switch from 6-well to a 24-well system which gives the possibility to screen more samples at once and of course less blood (PMNs) is required.
The older protocol instructs to activate PMNs 30' with 75nM Phorbol 12-myristate 13-acetate (PMA) and then 2 PBS washing steps should remove the remaining PMA. In order to avoid the loss of important factors or free radicals which have a rather short half life through these two washing steps but also to facilitate the handling of this system, a direct co-culture method was established where freshly isolated PMNs are activated with PMA directly within the well.

The development of this direct co-culture system in 24-well format led to following 2 problems:

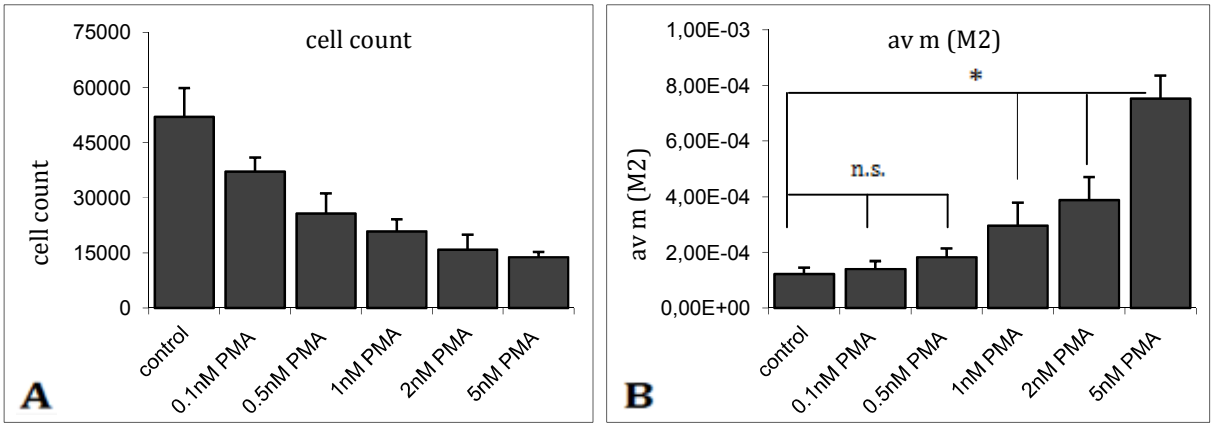
→PMA alone influences the mutation rate in this system
→A new effector:target ratio which is compatible for this 24-well system had to be found

3.1.1.1 PMA induces MSI in colon epithelial cells

PMA is a specific activator of the signal transduction enzyme Protein Kinase C (PKC) and the most common used phorbol ester in biomedical research of carcinogenesis. It is known to be used at nanomolar concentrations as a strong PMN activator but has also several other effects in cells and tissues where it can act as a potent tumor promoter [164].

We tested the effect of PMA alone on one of our reporter clones (HCT116+chr3 G16.1) **[Figure19]**. Therefore $1x10^3$ cells were sorted (FACSaria), cultured and on the next day treated with different concentrations of PMA (0.1-5nM) for 24 hours.

After this treatment period PMA-containing medium was exchanged with fresh IMDM. Cells were analyzed on day 7 by FACS and proliferation and mutation rate calculated:



sample	av total cells	± SD (σ)	*p* -value
control	5,20E+04	7,86E+03	-
0.1nM PMA	3,72E+04	3,75E+03	0.014
0.5nM PMA	2,57E+04	5,50E+03	0.0016
1nM PMA	2,09E+04	3,28E+03	0.0003
2nM PMA	1,59E+04	4,08E+03	0.0002
5nM PMA	1,38E+04	1,44E+03	0.00008

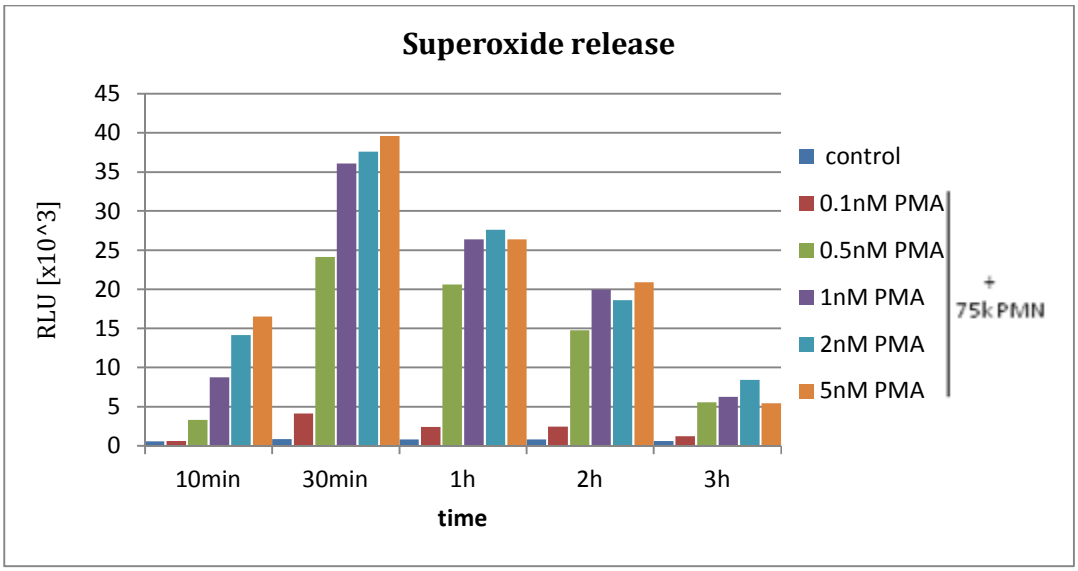
sample	mutation rate	± SD (σ)	*p* -value
control	1,22E-04	1,36E-05	-
0.1nM PMA	1,40E-04	1,92E-05	n.s.
0.5nM PMA	1,82E-04	2,04E-05	n.s.
1nM PMA	2,95E-04	7,56E-05	0.008
2nM PMA	3,87E-04	8,16E-05	0.001
5nM PMA	7,52E-04	2,60E-04	0.002

Figure19 and Table-3: Results from PMA treated HCT116+chr3 G16.1 clone after 7 days. Left: Dose depended decrease in cell proliferation upon PMA treatment. Right: The higher the PMA dose, the greater the increase in mutation rate, *p<0.05, n.s.: not significant.

In this clone a dose dependent decrease in cell proliferation could be observed and even 0.5nM PMA treatment for 24h resulted in a reduction of 51% in cell count **[Figure19A]**. Interestingly not only proliferation rate was affected by PMA, also the mutation rate increased the more of the reagent was added **[Figure19B]** leading to the suggestion that above a certain dose PMA induces MSI in colon epithelial cells.

Although 0.1 and 0.5nM PMA indicated a decrease in cell proliferation, there was no significant increase in mutation rate, so we had to figure out which of these two concentrations could be used for the following direct co-culture experiments.

Therefore a highly sensitive Lucigenin-amplified chemiluminescence assay was used for specific analysis of superoxide release to check for the activity of PMA activated PMNs.
75x10^3 PMNs treated with the above mentioned PMA concentrations were incubated together with 1mM Lucigenin (10minutes-3hours) to amplify the chemiluminescence signals **[Figure20]**:



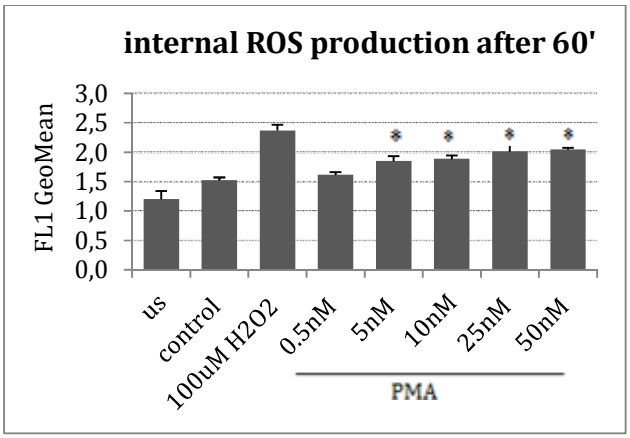
RLU	control	0.1nM PMA	0.5nM PMA	1nM PMA	2nM PMA	5nM PMA
10min	5,88E+02	6,12E+02	3,30E+03	8,73E+03	1,42E+04	1,65E+04
30min	8,52E+02	4,11E+03	2,41E+04	3,61E+04	3,76E+04	3,96E+04
1h	8,37E+02	2,42E+03	2,06E+04	2,64E+04	2,76E+04	2,64E+04
2h	7,99E+02	2,43E+03	1,47E+04	2,00E+04	1,86E+04	2,09E+04
3h	6,10E+02	1,22E+03	5,54E+03	6,23E+03	8,44E+03	5,45E+03

Figure20 and Table-4: Oxidative response of PMA stimulated PMNs determined by lucigenin-amplified chemiluminescence, control group: 75x10^3 PMNs untreated. The maximum peak of superoxide release was measured after 30minutes for all PMA concentrations.

With the help of this experiment we found out that 0.1nM PMA (red) was not sufficient enough to activate PMNs in a proper way. All PMA concentrations showed their maximum peak of PMN activation and superoxide release after 30 minutes. In comparison to 0.1nM, 0.5nM PMA (green) led to a strong PMN activity after 30' and afterwards to a time dependent decrease in superoxide release. Finally we suggested that 0.5nM PMA has to be

the optimal concentration for co-culture experiments because no significant increase in mutation rate could be detected but it sufficiently activated PMNs.

To answer the question how PMA induces MSI in this system, another experiment was performed where internal ROS production was measured within the HCT116 cell line upon PMA treatment. Cells were incubated with the cell permeant reagent 2',7' – dichlorofluorescein diacetate (DCFDA) which can form intracellular and in the presence of ROS the highly fluorescent compound DCF which can be measured by FACS (FL1 GeoMean). Internal ROS production was determined after 30 minutes (data not shown) and 1 hour with PMA concentrations ranging from 0.5nM – 50nM **[Figure21]**:



sample	FL1 GeoMean	± SD (σ)	*p* -value
us	1,2	1,41E-01	-
control	1,5	4,95E-02	-
H2O2	2,4	9,90E-02	0,04
0.5nM	1,6	4,95E-02	0,02
5nM	1,9	8,49E-02	0,011
10nM	1,9	6,36E-02	0,03
25nM	2,0	1,20E-01	0,0820
50nM	2,0	3,54E-02	0,0070

Figure21 and Table-5: Internal ROS measurement upon PMA treatment by FACS. FL1 GeoMean was directly calculated with the FACS software. Dose dependent increase of internal ROS production within HCT116 cells. us: unstained and H2O2 as positive control. *p<0.05.

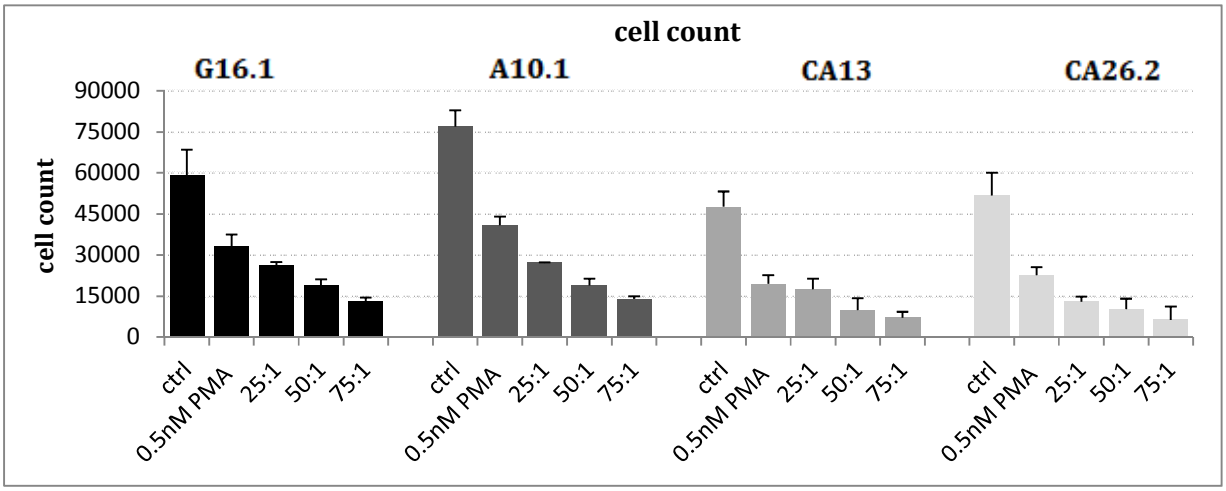
It is already known that oxidative stress is a potential mutagen which can lead to accumulation of frameshift mutations and may contribute to MSI [165]. In this experiment we observed a PMA dose depended increase in the production of internal ROS within HCT116 cells leading to the hypothesis that PMA may induce DNA damage and MSI by promoting cellular stress due to the accumulation of free radicals within the cell.

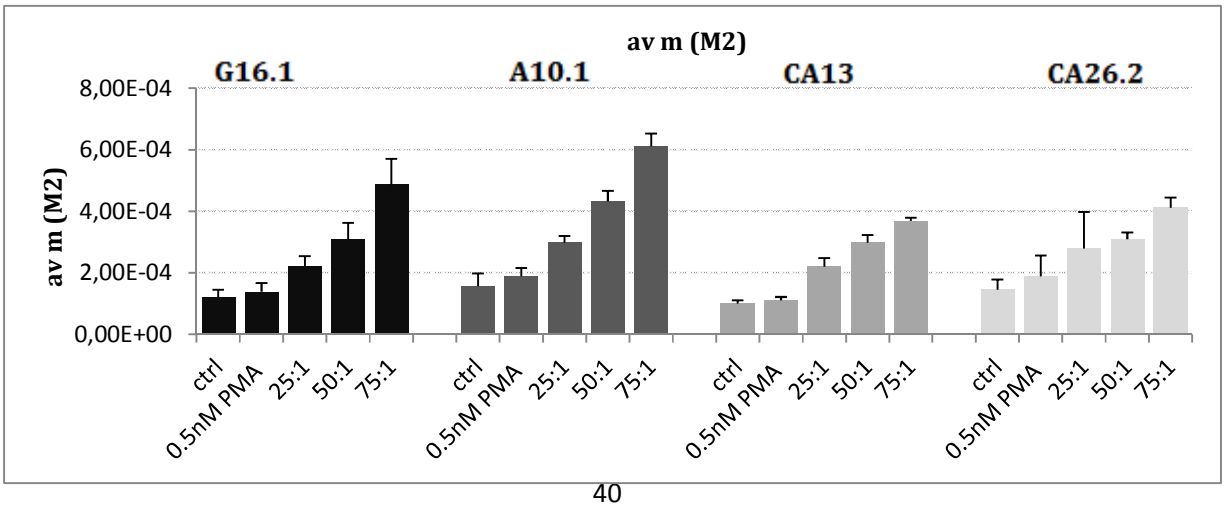
3.1.1.2 Find new effector:target ratio and selection of most sensitive reporter clones

By switching from 6-well to 24-well format and changing to a lower PMA concentration we considered that also the effector:target ratio in our system had to be new adjusted. From all pre-established EGFP-based frameshift reporter clones with G16, A10, CA13, CA26 and AAAG17 repeats within HCT116+chr3 **[Figure22]** and HCEC lines **[Figure23]** we wanted to select the most sensitive mono- and dinucleotide repeat for further co-culture experiments.

To find the new effector:target ratio and to compare all different repeats at once for their sensitivity we combined these two questions into one big experiment where we tested the whole panel of clones with different PMN:Reporter ratios to find out which of them perfectly fits into the new system:

<u>HCT116+chr3 reporter clones</u> - total cell count and mutation rate (M2):





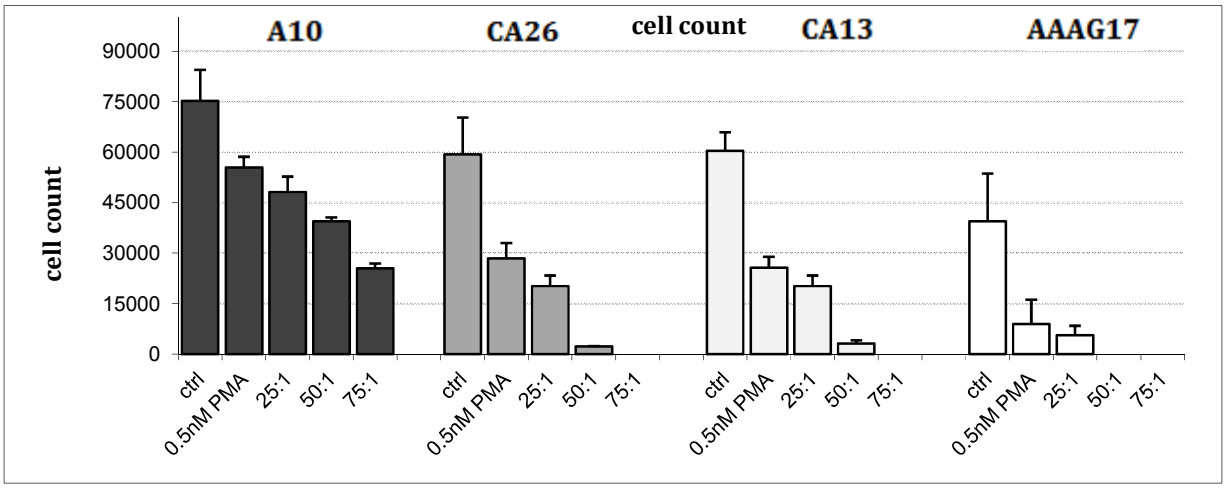
Microsatellite	effector:target ratio	av total cells	± SD (σ)	*p* -value
G16.1	control	5,92E+04	9,35E+03	-
	25:1	2,63E+04	1,23E+03	0.012
	50:1	1,90E+04	2,23E+03	0.041
	75:1	1,30E+04	1,49E+03	0.003
A10.1	control	7,70E+04	5,96E+03	-
	25:1	2,63E+04	1,23E+03	0.043
	50:1	1,90E+04	2,23E+03	0.032
	75:1	1,30E+04	1,49E+03	0.007
CA13	control	4,77E+04	5,61E+03	-
	25:1	2,63E+04	1,23E+03	0.039
	50:1	1,90E+04	2,23E+03	0.003
	75:1	1,30E+04	1,49E+03	0.0065
CA26	control	5,19E+04	8,27E+03	-
	25:1	2,63E+04	1,23E+03	0.05
	50:1	1,90E+04	2,23E+03	0.002
	75:1	1,30E+04	1,49E+03	0.043

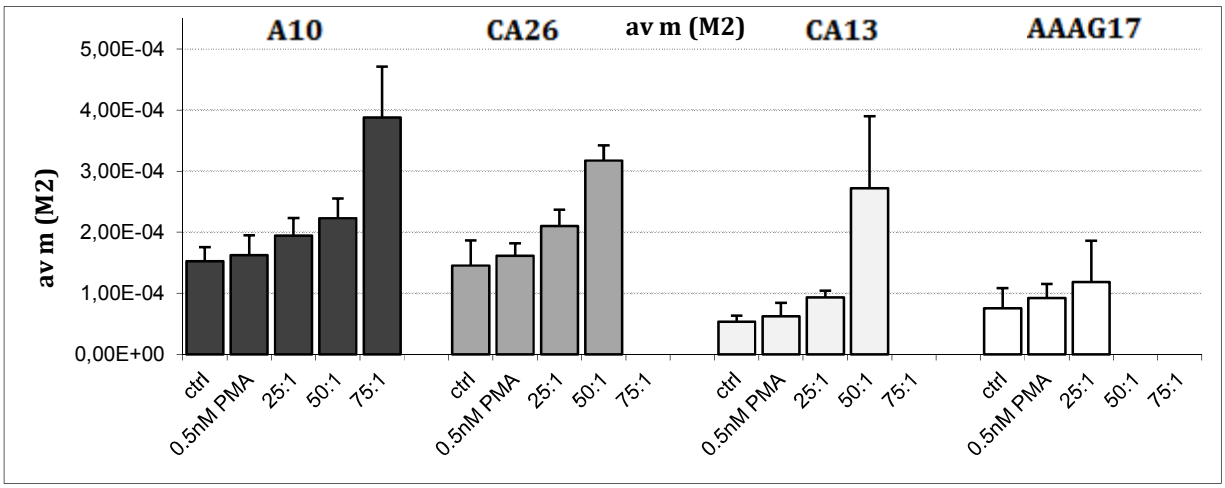
Microsatellite	effector:target ratio	mutation rate (M2)	± SD (σ)	*p* -value
G16.1	control	1,22E-04	2,36E-05	-
	25:1	2,22E-04	3,26E-05	0.043
	50:1	3,10E-04	5,32E-05	0.05
	75:1	4,88E-04	8,35E-05	0.0010
A10.1	control	1,57E-04	4,17E-05	-
	25:1	2,99E-04	2,18E-05	0.013
	50:1	4,33E-04	3,40E-05	0.004
	75:1	6,12E-04	4,17E-05	0.0021
CA13	control	1,01E-04	1,03E-05	-
	25:1	2,21E-04	2,72E-05	0.031
	50:1	2,99E-04	2,45E-05	0.045
	75:1	3,69E-04	1,12E-05	0.003
CA26	control	1,46E-04	3,30E-05	n.s.
	25:1	2,80E-04	1,18E-04	0.021
	50:1	3,10E-04	2,23E-05	0.03
	75:1	4,12E-04	3,30E-05	0.004

Figure22 and Table-6: HCT116+chr3 clones – proliferation rate and calculated mutation rate. Reporter clones (G16.1, A10.1, CA13, CA26.2) were treated with different PMN ratios (25:1, 50:1, 75:1) activated with 0.5 nM PMA. Control: only reporter cells without PMN treatment. PMA control: 0.5nM PMA w/o PMNs.

Reporter clones were treated with different PMN ratios in order to find the right effector:target ratio for the direct 24-well co-culture system. Therefore PMNs were isolated from fresh blood (protocol in the Material&Methods part) and pre-sorted (1x10^3/well) reporter clones were treated with different PMN ratios ranging from 25:1 -100:1 activated with 0.5nM PMA directly within the well. With a ratio of 100:1 (data not shown), most of the reporter clones died leading to the suggestion that this hit was too harmful. All other ratios worked quite well and by comparing all 4 clones we observed that the A10.1 for mono- and CA26.2 for dinulceotide repeat demonstrated the best cell survival and both were more sensitive for framshift errors than the G16.1 and CA13 repeat. Mutant fraction (M1) (data not shown) presented almost same results. For the two selected HCT116+chr3 clones (A10.1 and CA26.2), 75:1 ratio seemed to be a good choice for further experiments.

<u>HCEC reporter clones</u> - total cell count and mutation rate (M2):





Microsatellite	effector:target ratio	av total cells	± SD (σ)	*p* -value
A10.1	control	7,52E+04	9,35E+03	-
	25:1	4,82E+04	4,57E+03	0,042
	50:1	3,94E+04	1,23E+03	0,021
	75:1	2,54E+04	1,49E+03	0,003
CA26	control	5,94E+04	1,10E+04	-
	25:1	2,02E+04	3,13E+03	0,0054
	50:1	2,17E+03	1,87E+02	0,001
	75:1	-	-	-
CA13	control	6,04E+04	5,61E+03	-
	25:1	2,02E+04	3,13E+03	0,0047
	50:1	3,16E+03	9,27E+02	0,0013
	75:1	-	-	-
AAAG17	control	3,94E+04	1,43E+04	-
	25:1	5,56E+03	2,84E+03	0,02
	50:1	-	-	-
	75:1	-	-	-

Microsatellite	effector:target ratio	mutation rate (M2)	± SD (σ)	*p* -value
A10.1	control	1,52E-04	2,36E-05	-
	25:1	1,94E-04	2,89E-05	n.s.
	50:1	2,23E-04	3,26E-05	n.s.
	75:1	3,88E-04	8,35E-05	0,0047
CA26	control	1,45E-04	4,17E-05	-
	25:1	2,10E-04	2,72E-05	n.s.
	50:1	3,18E-04	2,50E-05	0,01
	75:1	-	-	-
CA13	control	5,34E-05	1,03E-05	-
	25:1	9,33E-05	1,12E-05	0,02
	50:1	2,72E-04	1,18E-04	0,03
	75:1	-	-	-
AAAG17	control	7,56E-05	3,30E-05	-
	25:1	1,18E-04	6,82E-05	n.s.
	50:1	-	-	-
	75:1	-	-	-

Figure23 and Table-7: HCEC clones – calculated proliferation and mutation rate. Reporter clones (A10, CA13, CA26, AAAG17) treated with different PMN ratios (25:1, 50:1, 75:1). Control: only reporter cells without PMN treatment. PMA control: 0.5nM PMA w/o PMNs.

Here we took advantage of a recently established primary human colorectal epithelial cell line (HCEC 1CT - Roig et al., 2010) to better understand the early events in colitis-associated tumorigenesis. The data from HCT116+chr3 served as comparator. HCEC 1CT cells were provided from Jerry Shay lab (UTSW, Dallas, TX) and previously transfected with pIREShyg2-EGFP-A10, -CA26 , -CA13 and -AAAG17 plasmids. Primary cells are difficult to culture and transfect thus represented a methodological challenge in this area of research. HCEC clones were treated same as HCT116+chr3 with 0.5nM PMA activated PMNs in ratios ranging from 25:1-100:1. Data gained from these new reporter cells differed from previous established ones since in HCT116+chr3 cells the MMR-protein hMSH3 (and possibly also other genes) is not expressed.

HCEC clones were much more sensitive to PMN treatment than the HCT116+chr3 clones. Interestingly only the mononucleotide repeat A10 was capable to deal with a ratio of 75:1. The two dinucleotides CA26 and CA13 were able to survive a hit of 50:1 and the maximum tolerable ratio for the tetranucleotide repeat was 25:1. These results lead to the suggestion that for HCEC clones also the A10 (max 75:1) and CA26 (max 50:1) repeat serves as a good model for mutation analyses to check the stability of microsatellite sequences during PMN-induced stress and for testing potential factors driving MSI.

3.2 Identification of PMN-released factors driving MSI

As mentioned the most important goal of this thesis was to identify the factor(s) which is released from activated PMNs that drives MSI. We used a variety of known antagonists in our co-culture experiments in order to block the genotoxic effect of PMNs. Several agents were appropriated for this study, including ROS/RNS scavengers, neutralizing antibodies and small molecule inhibitors **[Table-8]**:

PMN-factor	**Antagonist(s)**
Free radicals/Non-radicals	
$O\bullet^{2-}$ superoxide	superoxide dismutase
•OH hydroxyl	taurine, ethyl alcohol
$RO\bullet_2$ peroxyl	phycocyanin
RO• alkoxyl	α-tocopherol
NO• nitric oxide	carboxy-PTIO
$NO\bullet_2$ nitrogen dioxide	curcumin
HOCl hypochlorous acid	terbutaline, taurine, lycopene
H_2O_2 hydrogen peroxide	catalase
ROOH hydroperoxide	hydroperoxide reductase
$ONOO^-$ peroxynitrite	uric acid
Interleukins/Cytokines/Matrix Proteins	
myeloperoxidase	anti-myeloperoxidase
IL-6	Jak-3 inhibitor
IL-8	anti-interleukin 8, Reparixin
G-CSF	anti-G-CSF, G-CSF siRNA
TNFα	anti-TNFα, SPD304
IP-10	anti-IP-10
MIP-1α	anti-MIP-1α
VEGF	anti-VEGF
lyxozyme	anti-lyxozyme
lactoferrin	anti-lactoferrin
haptoglobin	anti-haptoglobin
petnraxin 3	anti-petnraxin 3
prodefensin	anti-prodefensin
Intracellular Proteins	
NF-kappaB	tepoxalin
PKC	Ro 31-8220
NADPH	apocynin, sphingoids

Table-8: Known PMN-released and intracellular factors and their antagonists. Agents used in this thesis are marked in green.

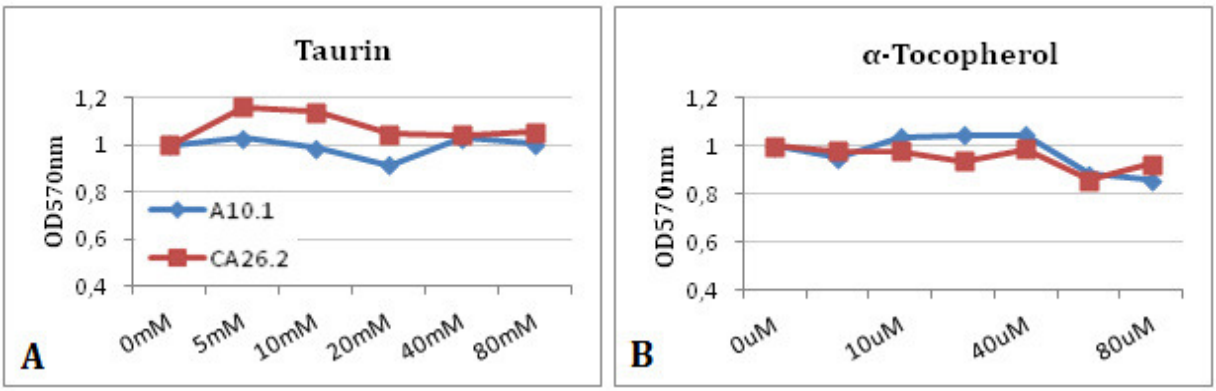
3.2.1 Effect of ROS antagonists on MSI

Reactive oxygen and nitrogen species are normal products of cell metabolism and required for many regulatory pathways but are also known to play a dual beneficial/detrimental role depending on local concentration and mode of generation [166]. Under normal conditions cells are able to control the balance of ROS generation and elimination by possessing some anti-oxidant defense mechanisms. These mechanisms include small molecules and specific enzymes which are also promising pharmaceutical agents with free radical scavenging properties to counteract excessive ROS generation in biological systems and to prevent DNA damage [109].

The aim was not only to find the key player(s) in PMN-induced carcinogenesis which increase the mutation rate and drive the transition from normal to cancer cells, but also to explore specific antagonizing agents/molecules that may interfere with such events. Those findings may provide further insight and stimulate the development of chemopreventive drugs which helps to counteract colitis-associated cancer.

We used the established direct co-culture system where pre-sorted reporter clones were treated with PMNs in the presence or absence of known ROS/RNS antagonist to verify which of them have the ability to counteract or scavenge PMN-induced mutagenesis *in vitro*. First we determined the cytotoxic effect of the antagonizing reagents by monitoring the mitochondrial activity which is related to cell viability.

Therefore a MTT-assay was performed where different doses of several compounds were tested on HCT116+chr3 A10.1 (blue) and CA26.2 (red) clones **[Figure24A-H]**:



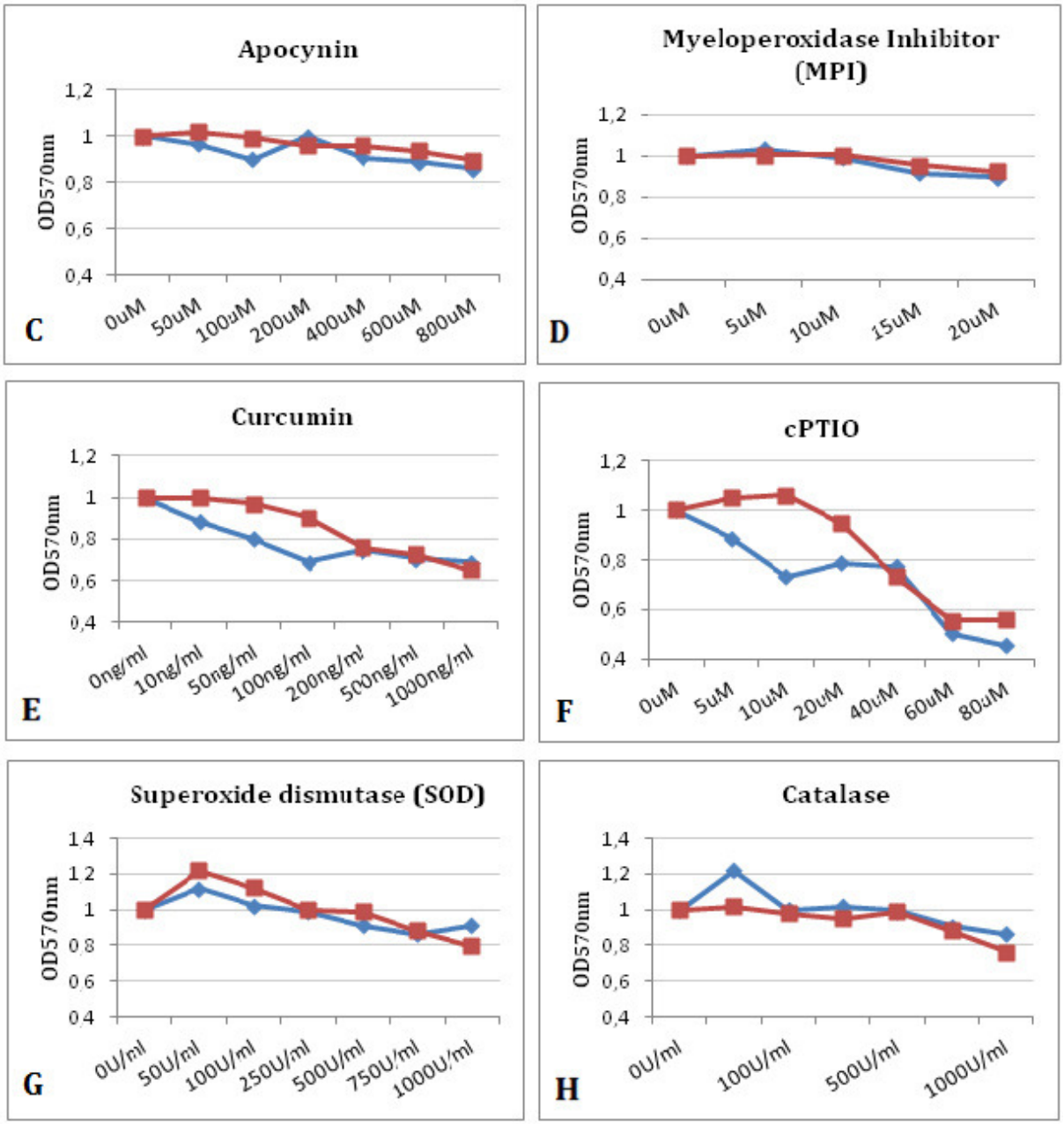
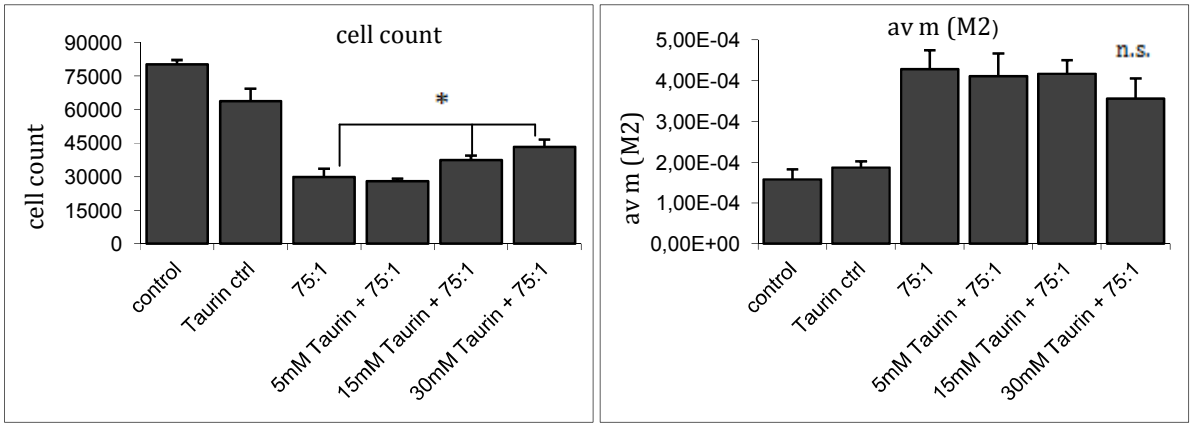


Figure24. MTT assay of ROS antagonists: HCT116+chr3 A10.1 and CA26.2 clones were treated with different concentration of antagonizing reagents. Curcumin (E) at 200-500ng/ml and carboxy-PTIO (F) at 20-40μM demonstrated the strongest cytotoxic effects while the other compounds (Taurin (A), Tocopherol (B), Apocynin (C), MPI (D), SOD (G) and Catalase (H)) with selected working concentrations indicated good cell viability.

In order to find the right dose for each reagent, we tested a specific range of concentration which we decided after doing literature search. Except for Curcumin and carboxy-PTIO all compounds showed good cell viability due to their selected concentrations. Both clones (A10.1 and CA26.2) demonstrated a dose dependent decrease above 200ng/ml Curcumin (E). Interestingly for cPTIO (F) the CA26.2 clone (red) seemed to be more stable for a concentration range up to 30µM in comparison to the A10.1 clone. At a dose higher than 30µM both clones displayed a breakdown of mitochondrial activity indicating that above that concentration the cytotoxic effect of the compound is too harmful.

3.2.1.1 Taurin in co-culture system:



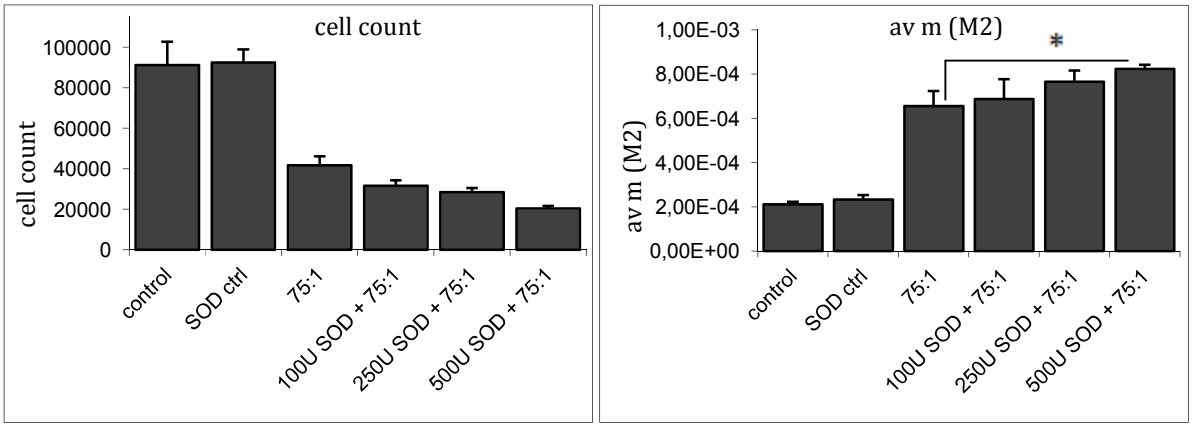
sample	av total cells	± SD (σ)	*p* -value
control	8,03E+04	2,00E+03	-
Taurin ctrl	6,37E+04	5,74E+03	0,002
75:1	2,99E+04	3,69E+03	0,001
5mM Taurin + 75:1	2,80E+04	1,24E+03	n.s.
15mM Taurin + 75:1	3,74E+04	2,14E+03	0,03
30mM Taurin + 75:1	4,33E+04	3,32E+03	0,01

sample	mutation rate	± SD (σ)	*p* -value
control	1,57E-04	2,55E-05	-
Taurin ctrl	1,87E-04	1,58E-05	n.s.
75:1	4,28E-04	4,70E-05	0,004
5mM Taurin + 75:1	4,10E-04	5,71E-05	n.s.
15mM Taurin + 75:1	4,17E-04	3,42E-05	n.s.
30mM Taurin + 75:1	3,56E-04	5,00E-05	n.s. (0,2)

Figure25 and Table-9: The antagonizing effect of Taurin in co-culture system with HCT16+chr3 A10.1 as target cells. 30mM Taurin seems to have a rescue effect in cell viability in combination with 75:1 PMN:Reporter ratio. Taurin control is 30mM Taurin w/o PMNs, *p<0.05.

Taurin is a known hypochlorous acid (HOCL) and hydroxyl (·OH) scavenger [167]. We tested Taurin in a concentration range of 5-30mM together with 75:1 PMN:Reporter ratio. The 30mM Taurin control without PMNs showed a small reduction in cell count but in combination with 75x10^3 PMNs 15mM and 30mM Taurin gave the impression to have a rescue effect for cell survival. We got no significant reduction in mutation rate (M2) and mutant fraction (M1 - data not shown) as we co-treated Taurin together with PMNs but 30mM indicated a slight decreasing trend.

2.1.1.2 Superoxide dismutase in co-culture system:



sample	av total cells	± SD (σ)	*p*-value
control	9,13E+04	1,15E+04	-
SOD ctrl	9,24E+04	6,57E+03	n.s.
75:1	4,16E+04	4,61E+03	0,002
100U SOD + 75:1	3,14E+04	2,87E+03	0,00133
250U SOD + 75:1	2,85E+04	2,09E+03	0,001
500U SOD + 75:1	2,04E+04	1,32E+03	0,003

sample	mutation rate	± SD (σ)	*p*-value
control	2,11E-04	1,23E-05	-
SOD ctrl	2,33E-04	2,11E-05	n.s.
75:1	6,55E-04	6,99E-05	0,001
100U SOD + 75:1	6,88E-04	8,95E-05	n.s.
250U SOD + 75:1	7,65E-04	5,22E-05	n.s.
500U SOD + 75:1	8,22E-04	2,11E-05	0,04

Figure26 and Table-10: The antagonizing effect of SOD in co-culture system with HCT16+chr3 A10.1 as target cells. SOD control (500U/ml) showed no change in cell viability in comparison to control but together with PMNs, 100-500U/ml SOD indicated a dose dependent decrease in cell survival. 500U/ml SOD + PMNs demonstrated a significant increase in mutation rate *p<0.05.

Superoxide dismutase catalyzes the dismutation of superoxide (O_2^-) into oxygen and hydrogen peroxide (H_2O_2). We observed a SOD dose dependent decrease in cell count at constant PMN content indicating a combinational cytotoxic effect. Also the mutant fraction

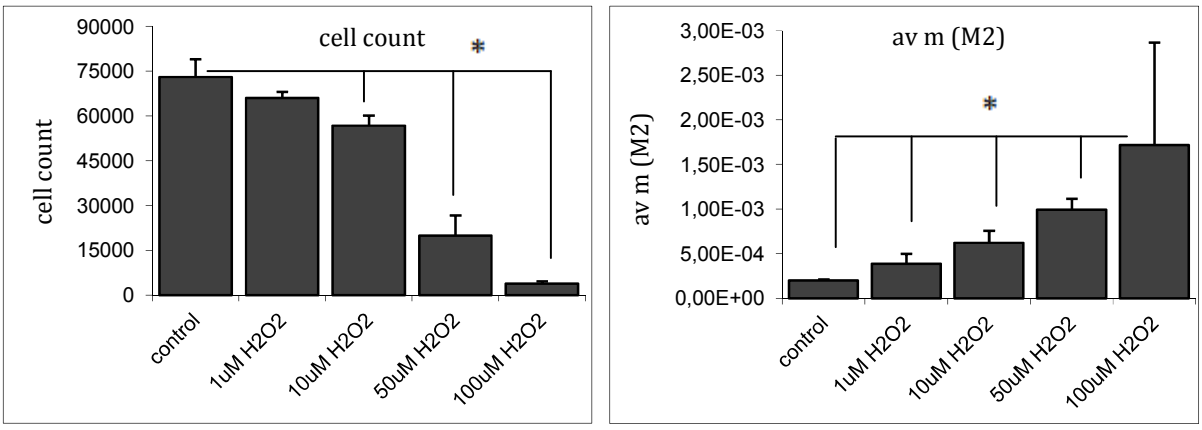
(M1 – data not shown) and the mutation rate (M2) increased the more SOD was added. A possible explanation for this phenomenon could be that one product of the SOD reaction is H_2O_2 which is a driving factor for MSI (see next chapter). The presence of excessive SOD can decrease the cellular level of O_2^- but may lead to an overwhelming accumulation of H_2O_2 in this system. Of course also other scavengers like cPTIO, α-Tocopherol, Curcumin and MPI were tested but didn't show any effect (data not shown).

3.2.2 The effect of hydrogen peroxide (H_2O_2) on colon epithelial cells

Accumulating evidence suggests that hydrogen peroxide plays a dual role in mutagenesis. Some study demonstrate H_2O_2 as a driving factor for cancer development but on the other hand it might have role in cancer chemoprevention and therapy [168].

An increase in the cellular levels of H_2O_2 can be linked to a variety of key events in cancer, including genetic instability, cell proliferation, apoptosis resistance, metastasis, angiogenesis and hypoxia-inducible factor 1 (HIF-1) activation [169]. Although it is known that H_2O_2 has important functions in cancer development, there is experimental evidence showing that increasing the cellular levels of hydrogen peroxide may be an efficient way for selectively killing cancer cells [170], [171].

First we tested the effect of hydrogen peroxide on our reporter clones. Therefore pre-sorted EGFP negative HCT116+chr3 A10.1 cells ($1x10^3$/well) were treated with different concentrations of H_2O_2 (0-100uM) for 24 hours and mutation/proliferation rate were measured on day 6:



sample	av total cells	± SD (σ)	*p* -value
control	7,30E+04	5,99E+03	-
1uM H2O2	6,60E+04	2,00E+03	n.s.
10uM H2O2	5,67E+04	3,41E+03	0,003
50uM H2O2	1,99E+04	6,75E+03	0,0004
100uM H2O2	3,84E+03	8,12E+02	0,0007

sample	mutation rate	± SD (σ)	*p* -value
control	1,99E-04	1,10E-05	-
1uM H2O2	3,87E-04	1,11E-04	0,05
10uM H2O2	6,22E-04	1,33E-04	0,031
50uM H2O2	9,92E-04	1,22E-04	0,002
100uM H2O2	1,72E-03	1,15E-03	0,01

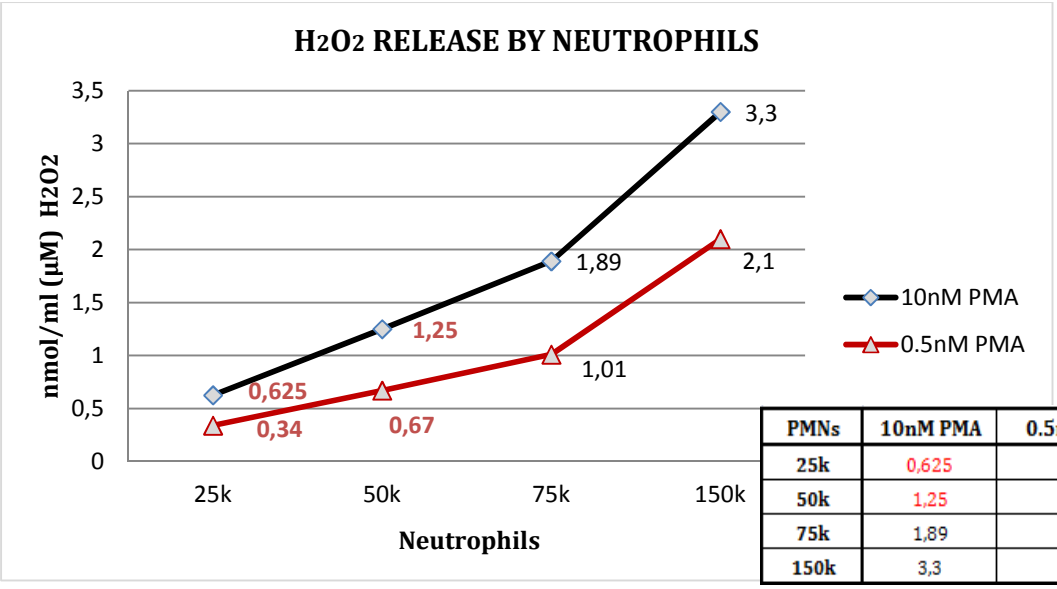
Figure27 and Table-11: H2O2 treatment on HCT116+chr3 A10.1 cells. Dose dependet decrease in cell count and significant increase in mutation rate (M2) and mutant fraction (M1) (data not shown) *p≤0.5.

H_2O_2 demonstrated a dose depended decrease in cell count and above approximately a concentration of 1µM it induced replication errors in this system.

3.2.2.1 Verification of PMN-released H2O2 as a culprit factor

Next the released H_2O_2 concentration from PMA activated PMNs was determined and we tried to clarify if this factor could be linked to PMN-induced mutagenesis.

Here we took advantage of a highly sensitive, direct and HTS-ready fluorometric assay (Abcam Hydrogen Peroxide Assay Kit) for measuring H_2O_2 in biological samples. We used freshly isolated PMNs and measured the concentration of released hydrogen peroxide from $25x10^3$, $50x10^3$, $75x10^3$ and $150x10^3$ PMA (0.5 and 10nM) activated immune cells.



PMNs	10nM PMA	0.5nM PMA	nmol/ml (µM) H2O2
25k	0,625	0,34	
50k	1,25	0,67	
75k	1,89	1,01	
150k	3,3	2,1	

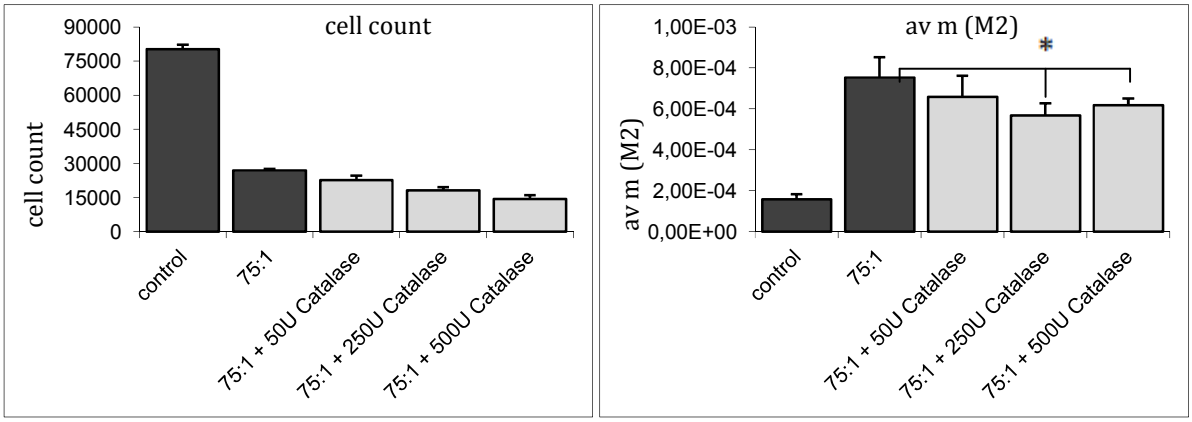
Figure28. H_2O_2 measurement of activated PMNs with the abcam hydrogen peroxide assay kit.

We were able to measure H_2O_2 release from $75x10^3$ and $150x10^3$ PMNs activated with both 0.5 and 10nM PMA. Unfortunately, the fluorescence signal from $25x10^3$ and $50x10^3$ was too low for quantitation and so we got those 4 values (red) only from calculations by using the measurable signals as template.

The assay indicated that $75x10^3$ PMNs release approximately 1nmol/ml (µM) H_2O_2 and when we combine those findings with the previous data (direct H_2O_2 treatment in cell-culture) it leads to the suggestion that the amount of released hydrogen peroxide from PMNs used in our direct co-culture experiments should be high enough to induce MSI.

To check those findings and to determine if this hypothesis can be true we tried to counteract the effect of H_2O_2 in our co-culture system by using Catalase as a scavenger. For this experiment we made use of both cell lines (HCT116+chr3 and HCEC) and compared mono- (A10) with dinucleotide (CA26) repeats.

HCT116+chr3 A10.1

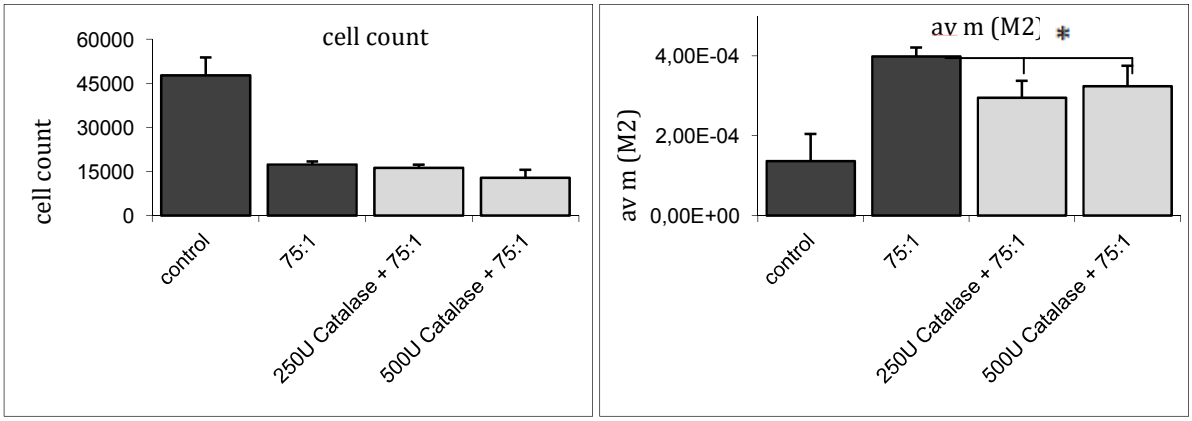


sample	av total cells	± SD (σ)	*p*-value
control	8,03E+04	2,00E+03	-
75:1	2,69E+04	6,58E+02	0,003
75:1 + 50U Cat	2,27E+04	1,92E+03	0,002
75:1 + 250U Cat	1,82E+04	1,43E+03	0,0001
75:1 + 500U Cat	1,43E+04	1,78E+03	0,0003

sample	mutation rate	± SD (σ)	*p*-value
control	1,57E-04	2,55E-05	-
75:1	7,52E-04	1,71E-04	0.0009
75:1 + 50U Cat	6,59E-04	1,02E-04	n.s.
75:1 + 250U Cat	5,68E-04	6,02E-05	0,04
75:1 + 500U Cat	6,18E-04	3,28E-05	0,05

Figure29 and Table-12: HCT116+chr3 A10.1 clone treated with $75x10^3$ PMNs. Scavenging effect of Catalase in a range of 50-1000U/ml was tested. Samples with Catalase are displayed in grey, w/o in black , *p≤0.05.

HCT116+chr3 CA26.2

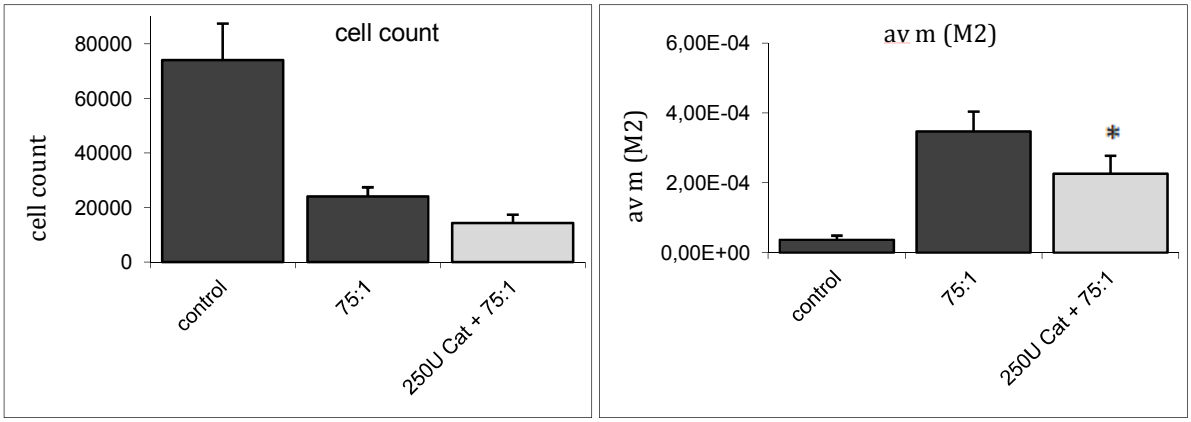


sample	av total cells	± SD (σ)	*p* -value
control	1,36E-04	6,84E-05	-
75:1	3,99E-04	2,25E-05	0,002
75:1 + 250U/ml Cat	2,95E-04	4,34E-05	0,004
75:1 + 500U/ml Cat	3,24E-04	5,18E-05	0,002

sample	mutation rate	± SD (σ)	*p* -value
control	4,77E+04	6,13E+03	-
75:1	1,74E+04	1,07E+03	0,0012
75:1 + 250U/ml Cat	1,62E+04	1,18E+03	0,003
75:1 + 500U/ml Cat	1,28E+04	2,83E+03	0,0011

Figure30 and Table-13: HCT116+chr3 CA26.2 clone treated with 75x10^3 PMNs. Scavenging effect of Catalase in a range of 250 and 500U/ml was tested, *p<0.05.

HCEC A10

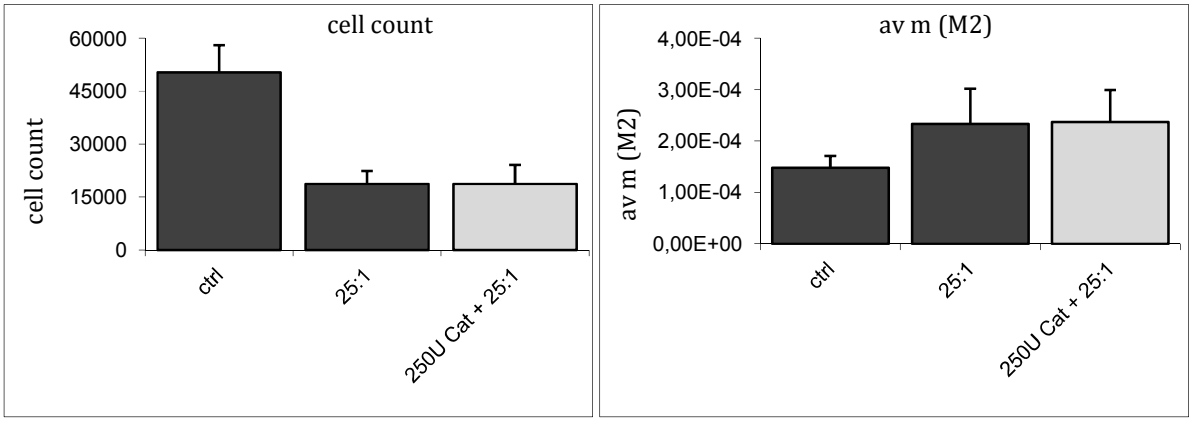


sample	av total cells	± SD (σ)	*p* -value
control	7,41E+04	1,33E+04	-
75:1	2,40E+04	3,37E+03	0,001
250U Cat + 75:1	1,42E+04	3,07E+03	0,0021

sample	mutation rate	± SD (σ)	*p* -value
control	3,63E-05	1,26E-05	-
75:1	3,47E-04	5,73E-05	0,0003
250U Cat + 75:1	2,26E-04	5,18E-05	0,01

Figure31 and Table-14: HCEC A10 clone treated with 75x10^3 PMNs. Scavenging effect of 250U/ml Catalase, *p<0.05.

HCEC CA26



sample	av total cells	± SD (σ)	*p*-value
ctrl	5,03E+04	7,86E+03	-
25:1	1,87E+04	3,76E+03	0,001
250U Cat + 25:1	1,87E+04	5,50E+03	0,0031

sample	mutation rate	± SD (σ)	*p*-value
ctrl	1,48E-04	2,36E-05	-
25:1	2,33E-04	6,89E-05	n.s.
250U Cat + 25:1	2,37E-04	6,26E-05	n.s.

Figure32 and Table-15: HCEC CA26 clone treated with 25x10^3 PMNs. Scavenging effect of 250U/ml Catalase couldn't be observed.

Catalase is a very important enzyme which protects cell from oxidative damage by catalyzing the decomposition of H_2O_2 to H_2O and O_2 [172]. The exposure of activated PMNs to our reporter clones demonstrated an increasing number in replication errors and the most suspicious factor for that is hydrogen peroxide. Here we tested a concentration range of 50-1000Units/ml Catalase to scavenge PMN released H_2O_2 molecules and to reduce upcoming mutations. As mentioned we used both cell lines (HCT116+chr3 and HCEC) and compared mono (A10) with dinucleotides (CA26). From previous results we knew that both HCT116+chr3 clones but only the HCEC A10 repeat could withstand a 75:1 treatment so we had to use a 25:1 ratio for the HCEC dinucleotide repeat. We couldn't observed any effect with the HCEC CA26 clone but monitored a reduction in replication errors of approximately 25-30% by using 250U/ml Catalase in all three other clones. It was not possible to further reduce the mutation rate by using higher concentration than 250-500U/ml. We weren't able to reduce 100% mutations leading to the suggestion that

hydrogen peroxide is just one PMN releasing culprit factor but there are also others which drives MSI.

3.2.3 Blockage of oxidative burst by Apocynin - a selective NADPH-oxidase inhibitor

Another strategy involved a complete blockage of the oxidative burst by using a known NADPH-oxidase inhibitor (Apocynin) to check whether ROS/RNS or other factors like proteins are responsible for the induction of MSI. Apocynin is a natural organic compound isolated from plants and is known for a variety of pharmacological properties. An important one is to block the translocation of the p47phox and p67phox subunit to the cell membrane, thereby inhibiting the assembly of the NADPH-oxidase [111]:

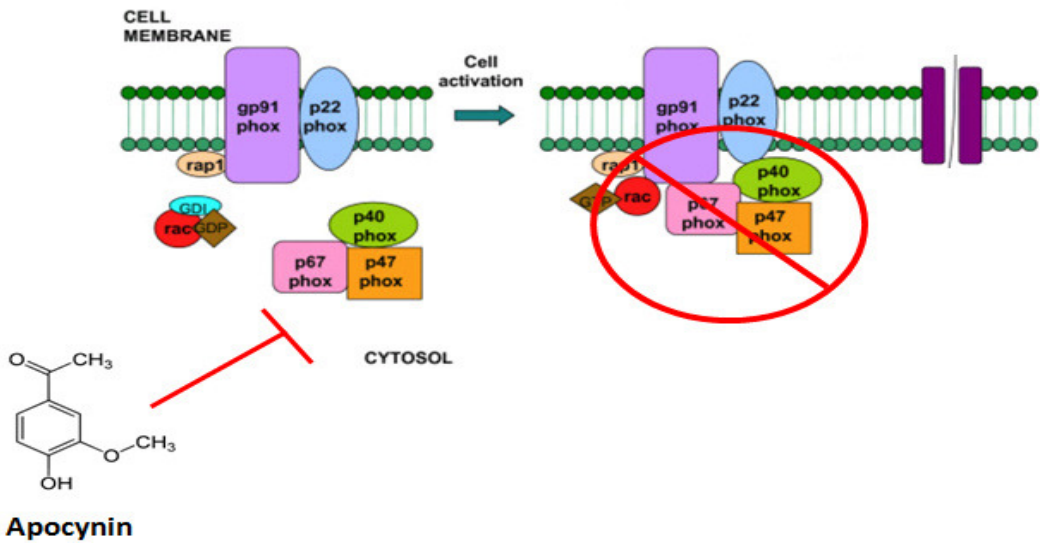
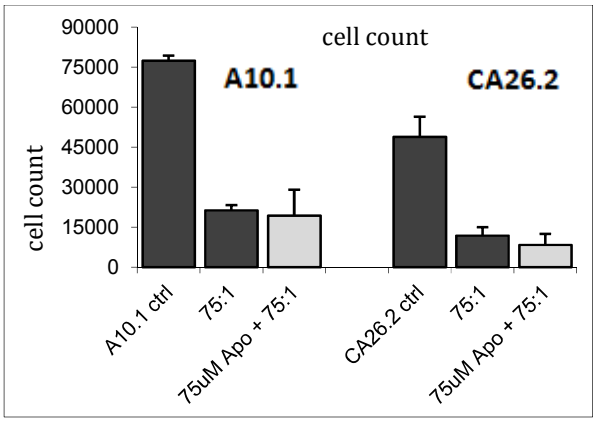


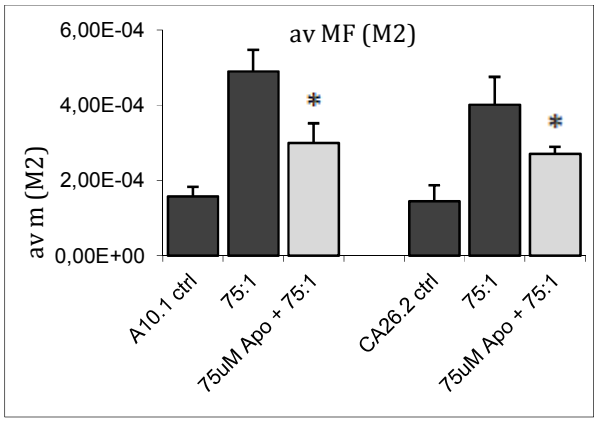
Figure33. Apocynin blocks the translocation of p47phox and p67phox to the cell membrane. Both subunits are important for the assembly of the NADPH-oxidase and removing leads to the blockage of oxidative burst by PMNs [110].

Fresh isolated PMNs were incubated for 10-15 minutes together with 75µM of the reagent, activated with 0.5nM PMA and subsequently transferred to pre-sorted HCT116+chr3 A10.1 and CA26.2 clones. After 24 hours PMNs were removed, wells washed with PBS and fresh

IMDM added. 6 days later we measured cells by FACS with the same protocol like other co-culture experiments:

HCT116+chr3 A10.1 and CA26.2 upon Apocynin treatment:





Microsatellite	sample	av total cells	± SD (σ)	*p* -value
A10.1	control	7,73E+04	2,00E+03	-
	75:1	2,13E+04	2,02E+03	0,00022
	75uM Apo + 75:1	1,93E+04	9,72E+03	n.s.
CA26.2	control	4,88E+04	7,66E+03	-
	75:1	1,18E+04	3,23E+03	0,0001
	75uM Apo + 75:1	8,33E+03	4,23E+03	n.s.

Microsatellite	sample	mutation rate	± SD (σ)	*p* -value
A10.1	control	1,57E-04	2,55E-05	-
	75:1	4,90E-04	5,77E-05	0,0003
	75uM Apo + 75:1	2,99E-04	5,33E-05	0,0048
CA26.2	control	1,44E-04	4,32E-05	-
	75:1	4,01E-04	7,48E-05	0,001
	75uM Apo + 75:1	2,70E-04	2,00E-05	0,032

Figure34 and Table-15: HCT116+chr3 A10.1 and CA26.2 clones and treatment with 75uM Apocynin blocking oxidative burst by PMNs. Grey bars indicates PMNs pre-incubated with Apocynin. Both clones showed a decrease in mutation rate (M2) of approximately 35%, *p<0.05.

We weren't able to observe a 100% reduction in mutation rate. Also higher concentrations (up to 250µM Apocynin – data not shown) and longer pre-incubation steps didn't indicate further reduction. The cell proliferation didn't change in comparison to untreated samples and we monitored a decrease of replication errors by 39% for the A10.1 repeat and 33% for CA26.2. There is experimental evidence that describe the ability of Apocynin as a selective NADPH-oxidase inhibitor and we were able to reproduce those findings. With the help of this but also previous experiments we demonstrated that ROS and RNS are not the only factors driving MSI, there have to be others like proteins/cytokines and we should pay more attention to that field.

3.3 Cytokines and their role in tumorigenesis

As mentioned in the introduction part, up to 20% of all cancers can be linked to chronic inflammation and most of solid tumors contain inflammatory infiltrates [144]. Those infiltrating immune cells can promote tumor initiation, growth and progression and many of these effects are associated with the release of proinflammatory cytokines (TNF-α, IL-6, IL-8 etc.) [143]. The mixture and concentration of cytokines produced in the tumor microenvironment is related to an impacting role in cancer pathogenesis e.g. IL-6 circulate in picomolar concentrations that can increase up to 1000-fold during inflammatory processes [173]. Under normal conditions, cytokines and chemokines are used for important signaling pathways (e.g. MAPK and NFκB) but experimental evidence supports the fact that strongly enhanced growth signaling due to an overwhelming release of cytokines correlates to the induction of DNA replication stress which leads to the formation of double strand breaks (DSB) and further to genomic instability **[Figure35]** [93], [174]:

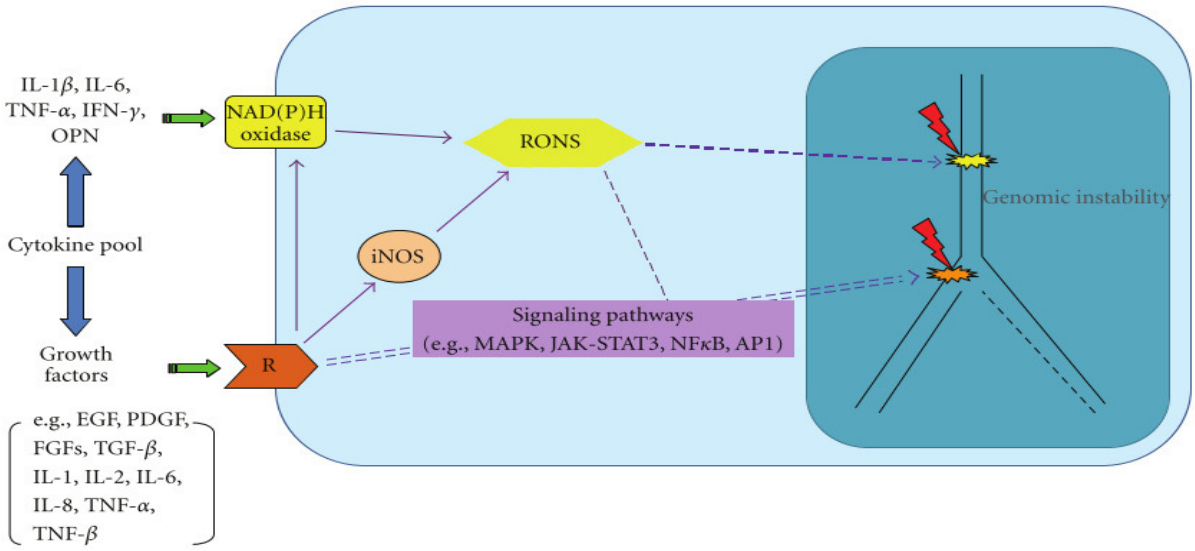


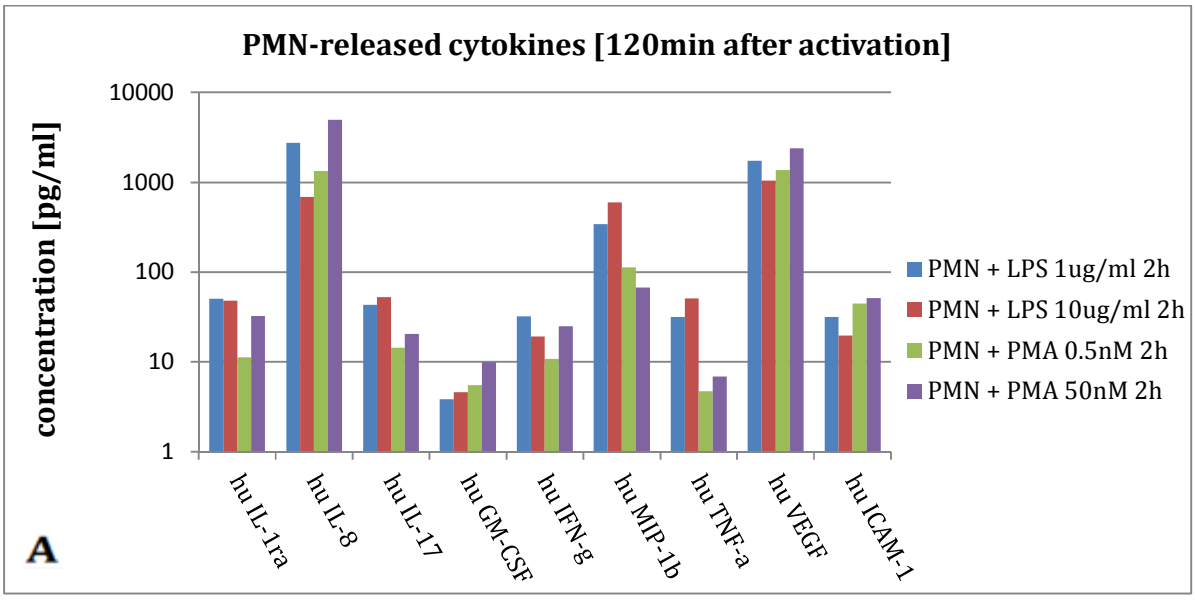
Figure35. Cytokines may lead to DNA replication stress and genomic instability through several mechanisms including upregulation of signaling pathways and internal ROS production [93].

Another interesting story is the relation between cytokines and oxidative stress because recently published reports indicates that ROS, previous described as a DNA damage

promoting factor, can act as second messenger in the NF-κB activation through proinflammatory cytokines [175]. Free radicals and cytokines can both either induce or can be induced by NF-κB which creates a positive feedback loop for their own production [176].

3.3.1 Identification of Cytokines released by activated PMNs

In previous experiments we found out that ROS is one important factor driving MSI but we were also interested to find other key players and cytokines are obviously a hot target.
First we wanted to determine the whole panel of released cytokines/chemokines from PMNs in this system and of course in which concentration they act. Therefore we used a highly sensitive multiplex bead-based assay designed to quantitate multiple cytokines and chemokines from cell supernatants in a 96-well format (Bioplex - Material part). We activated freshly isolated PMNs for different timepoints with 0.5 and 50nM PMA as well as 1 and 10µg LPS as control and measured the supernatant according to the Bioplex-assay protocol.



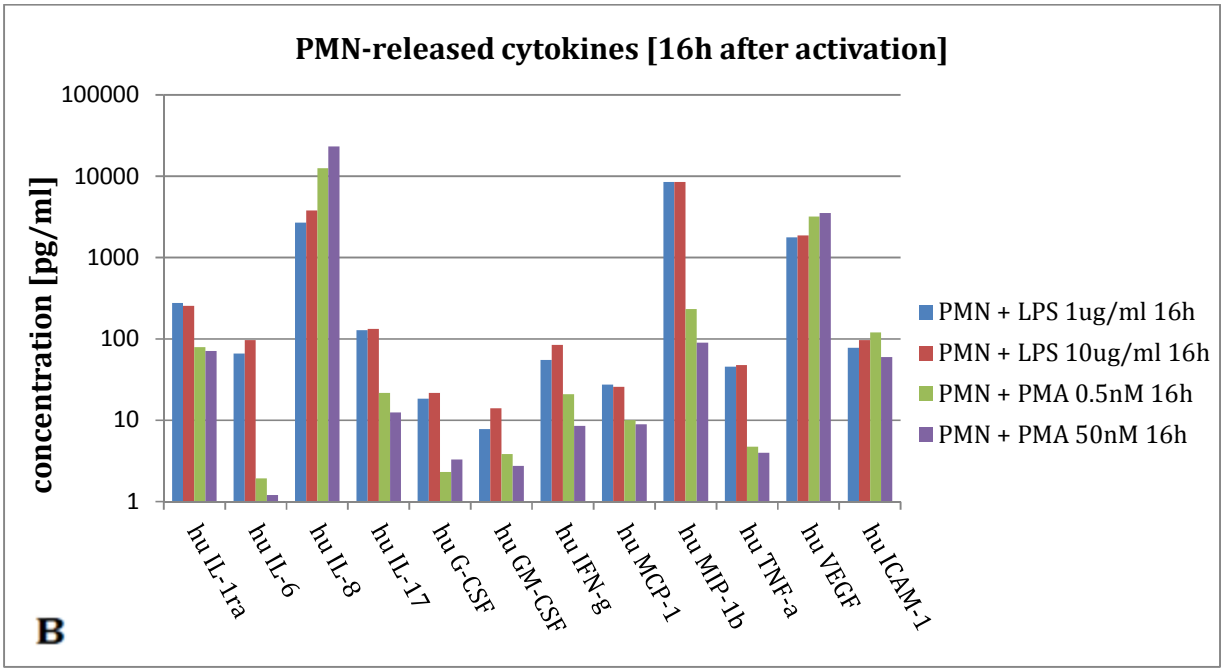


Figure36. Bioplex assay of supernatant from PMA and LPS activated PMNs after 2 **(A)** and 16h **(B)**.

Several cytokines have a well-established role in IBD such as TNF-α, IL-1a, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17 and G-CSF. Those promoting a Th2 and Th17 response are mainly associated with UC while inflammatory cytokines that initiate a Th1 response, such as IFN-γ can be linked to CD [144], [145], [146].

Here we investigated cytokines and chemokines released by activated PMNs by testing 21 of those factors (IL-1a, IL-1b, IL-1ra, ICAM-1, IL-2, IL-4, IL6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1b, TNF-α and VEGF). We monitored that IL-8 (0.5nM PMA after 2h: 1ng/ml, 16h: 10ng/ml) is the major cytokine secreted followed by VEGF (2h: 1ng/ml, 16h: 3,5ng/ml), MIP-1 (16h: 0.2ng/ml), ICAM-1 (16h: 0.1ng/ml) and IL-1 receptor antagonist (16h: 70pg/ml). IL-6 (16h: 2pg/ml) and TNF-α (16h: 5pg/ml) two important pro-inflammatory cytokines were expressed to a lesser extent.

IL-8 was originally classified as a neutrophil chemoattractant but up to now there are many publications describing this chemokine as an important factor in cancer biology [154], [177].

This experiment indicated that IL-8 is one of the key players released by activated PMNs suggesting that it may function as an important regulatory factor within our co-culture system.

Following graph summarizes main cytokines released by 0.5nM PMA activated PMNs after 16 hours:

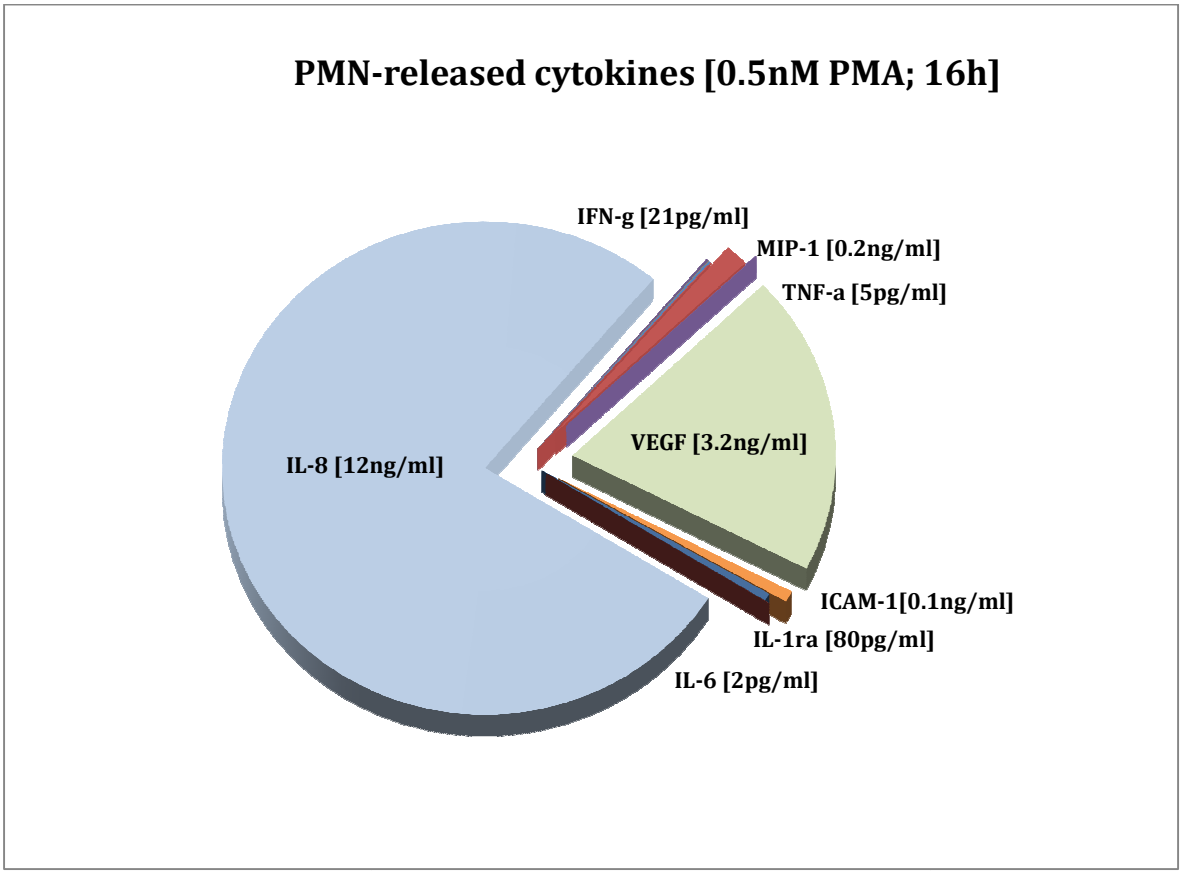


Figure37. 0.5nM activated PMNs - releasing cytokines after 16 hours with their concentrations.

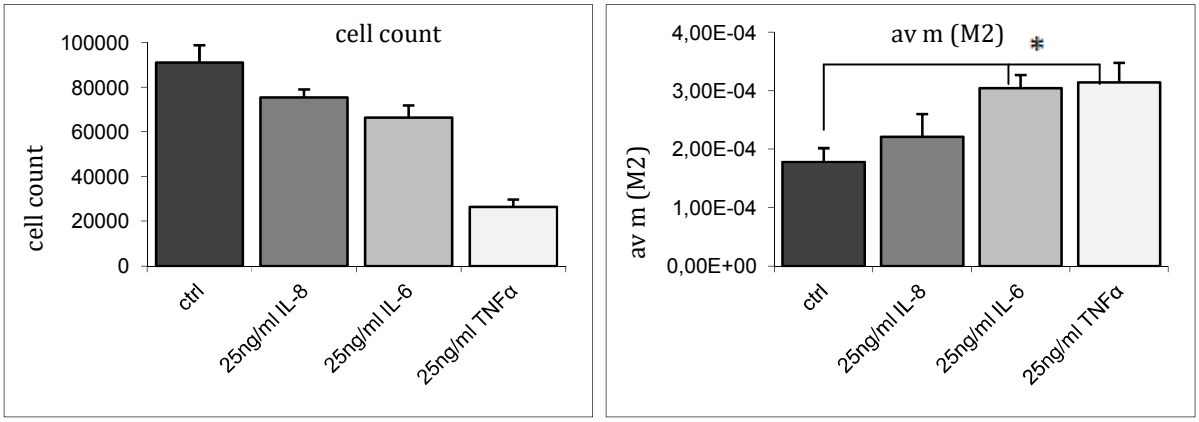
3.3.2 Effect of cytokines on MSI

Not only ROS, also an overwhelming production and the release of specific cytokines/chemokines at the site of inflammation can be associated with cellular stress. Excessive concentrations of these factors can put the whole signaling cascade out of control leading to upcoming stress and consequently to an instability of the replication machinery [93].

Here we used the HCT116+chr3 and HCEC reporter clones as targets to determine the effect of specific cytokines.

We tested three well known pro-inflammatory cytokines IL-8, IL-6 and TNF-α for their induction of MSI. First we tried those concentrations which we determined by the Bioplex assay: 10ng/ml for IL-8, 2pg/ml for IL-6 and 5pg/ml for TNF-α but we weren't able to observe any significant result (data not shown). So we decided to use same concentration (25ng/ml) for all cytokines and compared their effects.

HCT116+chr3 A10.1 + 25ng/ml IL-8, IL-6 and TNF-α



sample	av total cells	± SD (σ)	*p* -value
ctrl	9,10E+04	7,86E+03	-
25ng/ml IL-8	7,53E+04	3,75E+03	0,04
25ng/ml IL-6	6,63E+04	5,50E+03	0,01
25ng/ml TNFα	2,64E+04	3,28E+03	0,0023

sample	mutation rate	± SD (σ)	*p* -value
ctrl	1,78E-04	2,36E-05	-
25ng/ml IL-8	2,21E-04	3,89E-05	n.s.
25ng/ml IL-6	3,04E-04	2,26E-05	0,02
25ng/ml TNFα	3,14E-04	3,35E-05	0,0013

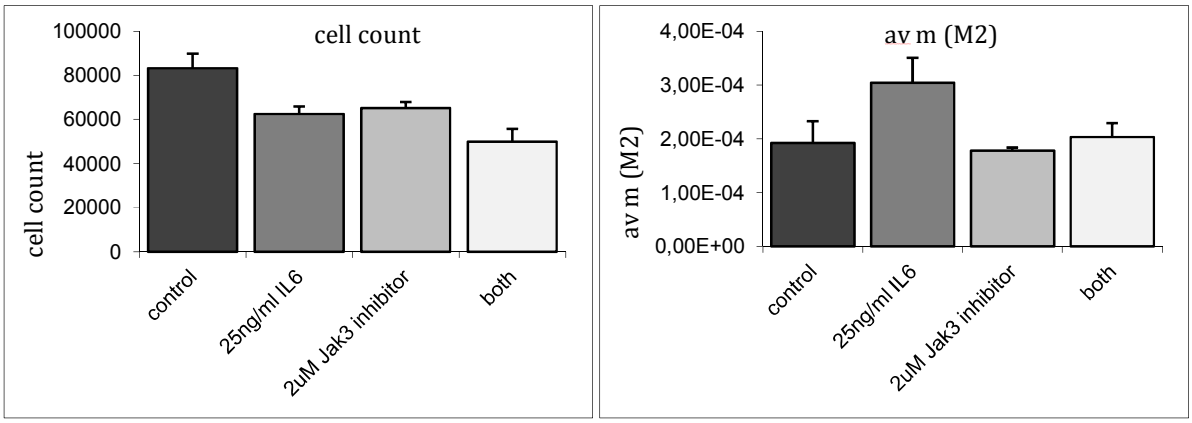
Figure38 and Table-16: Effect of 25ng/ml IL-8, IL-6 and TNF-α on HCT116+chr3 A10.1 clone. TNF-α demonstrated the strongest decrease in cell proliferation rate and together with IL-6 the highest mutation rate increase *p<0.05.

We monitored a significant decrease in cell count for all three cytokines but 25ng/ml TNF-α seemed to be the most harmful factor leading to 70% reduction in proliferation rate while IL-8 and IL-6 to 25%. Interestingly there was no significant difference in mutation rate due to 25ng/ml IL-8 but IL-6 and TNF-α treatment resulted in a 2-fold increase.

Tofacitinib - a potent Jak3 inhibitor blocks IL-6 induced mutagenesis:

Up to now, many therapeutic medications against autoimmune diseases are investigated and currently approved which should help to reduce the risk of chronic inflammatory disorders. One example is the potent janus kinase (JAK) inhibitor Tofacitinib discovered by Pfizer which is already approved for the treatment of rheumatoid arthritis (RA) but also stays in focus for the treatment of IBD [178].

Here we tested the inhibitory potential of Tofacitinib on HCT116+chr3 CA26.2 and observed a significant decrease in proliferation when co-treated 25ng/ml IL-6 together with 2µM Tofacitinib, but also a 100% reduction in mutation rate. It is already known that IL-6 signaling acts over the JAK-STAT pathway, but with this experiment we confirmed the possibility of Tofacitinib to block IL-6 induced replication errors.

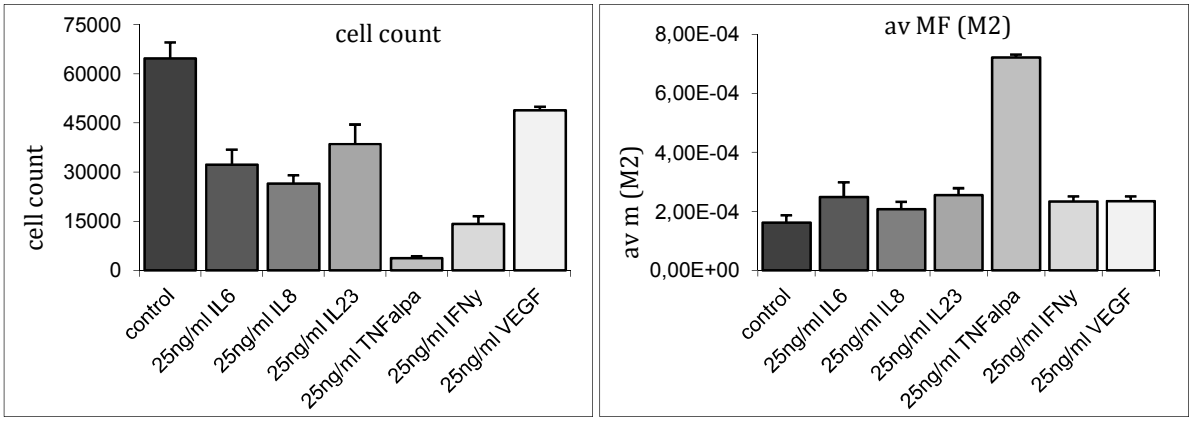


sample	av total cells	± SD (σ)	*p* -value
ctrl	8,33E+04	4,55E+03	-
25ng/ml IL-6	6,25E+04	3,75E+03	0,04
2uM Tofa	6,52E+04	4,33E+03	0,02
both	4,98E+04	2,33E+03	0,0023

sample	mutation rate	± SD (σ)	*p* -value
ctrl	1,93E-04	2,36E-05	-
25ng/ml IL-6	3,04E-04	2,89E-05	0,001
2uM Tofa	1,78E-04	1,12E-05	0,0024
both	2,03E-04	1,35E-05	0,004

Figure39 and Table-17: 25ng/ml IL-6 and the inhibitory effect of Tofacitinib on HCT116+chr3 CA26.2 clone. Tofacitinib inhibited the effect of IL-6 up to 100%, *p<0.05.

HCEC CA26 + 25ng/ml of IL-6, IL-8, IL-23, TNF-α, IFN-y, VEGF



sample	av total cells	± SD (σ)	*p* -value
ctrl	6,46E+04	4,63E+03	-
25ng/ml IL-6	3,27E+04	3,20E+03	0,00285
25ng/ml IL-8	2,66E+04	3,46E+03	0,001
25ng/ml IL-23	3,86E+04	5,59E+02	0,0032
25ng/ml TNFa	3,72E+03	2,04E+02	0,00223
25ng/ml IFNy	1,41E+04	2,13E+02	0,0012
25ng/ml VEGF	4,88E+04	1,65E+03	0,00043

sample	mutation rate	± SD (σ)	*p* -value
ctrl	1,64E-04	6,91E-05	-
25ng/ml IL-6	2,44E-04	1,63E-04	0,003
25ng/ml IL-8	2,12E-04	1,29E-05	0,0042
25ng/ml IL-23	2,54E-04	3,34E-05	0,002
25ng/ml TNFa	6,90E-04	1,19E-05	0,00012
25ng/ml IFNy	2,42E-04	1,03E-05	0,00033
25ng/ml VEGF	2,34E-04	3,35E-05	0,008

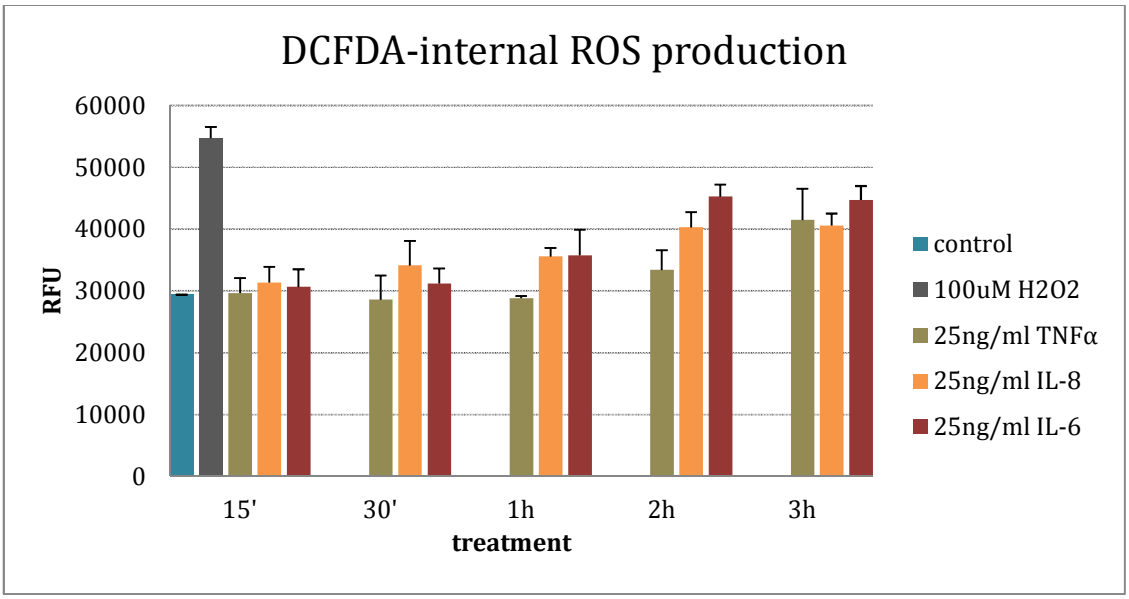
Figure40 and Table-18: Effect of 25ng/ml IL-6, IL-8, IL-23, TNFα. IFNy and VEGF on HCEC CA26 clone. All cytokines showed a decrease in Proliferation and significant increase in mutation rate. TNFα had the highest impact on this clone. *p<0.05.

We also tested the effect of IL-6, IL-8, IL23, TNFα, IFNy and VEGF (all 25ng/ml) on the new established normal human colon epithelial reporter cells (HCEC) and observed that HCEC were apparently more sensitive to the treatment of cytokines than the HCT116+chr3 cells. Not only the proliferation was dramatically affected, also all tested cytokines showed a significant increase in mutation rate. Especially TNFα indicated a decrease in proliferation of approximately 94% and a 4-fold increase in mutation rate compared to non-treated control.

3.3.3 Certain cytokines induces internal ROS production

As it was clear that cytokines are able to induce replication errors within our system, we wanted to find the link between those small molecules and DNA alterations and/or

replication failure. Therefore we took advantage of the DCFDA assay to measure internal ROS production.



Average	ctrl	H2O2	TNFa	IL8	IL6
15'	2,94E+04	5,47E+04	2,97E+04	3,14E+04	3,07E+04
30'	2,90E+04	-	2,86E+04	3,41E+04	3,12E+04
1h	2,92E+04	-	2,88E+04	3,56E+04	3,58E+04
2h	2,94E+04	-	3,34E+04	4,03E+04	4,53E+04
3h	2,99E+04	-	4,15E+04	4,06E+04	4,47E+04

Figure41 and Table-19: DCFDA assay to measure internal ROS in HCEC cells treated with 25ng/ml TNFα, IL-6 and IL-8 as well as positive control 100µM H2O2. Measurement was done in a time range between 15 minutes and 3 hours. Time dependent increase for all three cytokines could be detected.

After treatment with 25ng/ml IL-6, IL-8 and TNFα a time depended increase in internal ROS production with highest values after 3 hours for all three cytokines could be observed. Previous results indicated that IL-6 induces replication errors over the JAK/STAT signaling pathway, so maybe one way of such cascades ends up in the over-activation of specific mitochondrial enzymes, consequently to an increasing production of internal ROS which further may lead to an insufficient endogenous antioxidant system.

4. Discussion

The incidence of chronic inflammation in UC is associated with a high risk for colorectal carcinoma development [89]. One key factor in UC is the accumulation of activated PMNs which can form crypt abscesses within the mucosa of the large intestine due to their enhanced transepithelial migration [179]. Degranulating neutrophils can induce cellular stress at the side of inflammation but can also affect the surrounding epithelial layer which is suggested to be a driving factor for tumourigenesis [76]. It is already known that the release of reactive oxygen species (oxidative stress) can lead to alterations of cellular components like proteins or DNA, but this study should help to answer the question if only oxidative stress (ROS, RNS) or even other molecules like cytokines can cause mutations within colonic epithelial cells. Thus, previous studies that focused exclusively on oxidative-induced mutagenesis did only partially reflect the physiological condition of inflammation-driven colon carcinogenesis.

This *in vitro* study used a specific developed co-culture system using freshly isolated PMNs acting as effectors on different colon epithelial cell lines to simulate the carcinogenic environment in UC. Microsatellite instability is known to be a hallmark of MMR deficiency especially in human colorectal cancers and since MSI is discovered in UC tissue, the inactivation of the important Mismatch Repair system is believed to be an early event in colitis associated carcinogenesis [118]. Frame-shift mutations at microsatellites, such as insertions or deletions due to DNA replication failure, occur throughout the whole eukaryotic genome and several factors, such as length, composition and repeat unit size of DNA repeats influence the stability. Mononucleotide repeats are frequently mutated in MSI [180], but also dinucleotide repeats such as in the epidermal growth factor receptor often show mutations [181], [182]. One example for mononucleotide repeats is the TGF beta receptor 2 (TGFBR2) which harbors an A10 repeat within exon 3 and under normal conditions it is an important tumor suppressor gene. Frame-shift mutations within the polyadenine sequences (A10 repeats), as we have induced by exposure to PMNs, lead to a nonfunctional protein [180].

Different experiments were carried out on various mono-, di- and tetranucleotide repeat harboring cell lines (HCT116+chr3 and HCEC) suggesting an effect of activated PMNs on the occurrence of frame-shift mutations at those target cells. Detection of frame-shifts was done by flow cytometry using the previous described vector-based assay.

The results revealed that colon epithelial cells (cancer cell but also normal epithelial cells) respond to activated PMNs by reduction in proliferation shown by the decrease of cell count of co-cultured clones. 75:1 Effector:Target ratio showed more than 50% reduction in cell count compared to the untreated control. Previous studies suggest that the reduced proliferation is due to an arrest in the G2/M phase of the cell cycle and by induction of apoptosis [163]. The results in this study support the fact that activated PMNs cause a decrease in cell proliferation in colon epithelial cells.

Furthermore, the study revealed that activated PMNs did not only cause a decrease in cell count, but are capable to increase replication errors and mutations which are independent of the nucleotide length and composition of the microsatellite. Thus, the higher the PMN:Target ratio, the stronger the increase of the mutation rate for both cell lines **(Fig22** and **23)**.

This study confirms the assumptions of previous experiments in this field [162], but extends the analysis by using various mono-, di- and tetranucleotide repeats within normal human colonic epithelial reporter cell lines for frame-shift mutations. This new HCEC panel together with the previous established reporter cell lines [162] was utilized for downstream spontaneous and inflammation-induced mutation analyses. The data gained from these new reporter cells differed from previous established ones since in HCT116+chr3 cells the MMR-protein hMSH3 is not expressed. These primary cell lines enables the possibility to check the stability of microsatellite sequences during PMN-induced stress under more physiological conditions even though primary cells are very difficult to transfect and culture.

The most important goal within this study was to identify the factor which is released from activated PMNs that drives MSI. Therefore we utilized a variety of known antagonists in our co-culture experiments and found out that released hydrogen peroxide has to be associated with an increase in replication errors because the exposure of reporter cells to even 1µM H2O2 resulted in a significant increase in mutation rate **(Fig27)** and low dose

catalase (250U/ml) was capable to counteract PMN-induced mutagenesis *in vitro* **(Fig29-32)**.

A future-oriented perspective in this field of research could be the testing of different scavenger combinations and to look if there are synergistic effects between antagonizing compounds. We tested the combination of Superoxide Dismutase together with Catalase but weren't able to see any additive effect (Data not shown). Also a mix of natural compounds which are known to have chemopreventive potential due to their antioxidant abilities could play a key role in IBD medication [183]. This thesis revealed the potential of H_2O_2 as one of the factors driving MSI but we have to consider that catalase itself is very difficult to administer and to use as medication, therefore alternatives must be found. In the year 2008, de Moreno de LeBlanc et al. published the possibility of an oral administration with a catalase-producing gram-positive bacterium as a therapeutic option to prevent induced colon cancer in mice [184]. This could be also interesting for the AOM/DSS model.
Another strategy of this thesis was to block the complete oxidative burst by the use of Apocynin to check whether only ROS is responsible for MSI or even other factors. 75µM Apocynin within the co-culture system decreased the mutation rate by approximately 35% **(Fig34)** which indicates that other factors are also included.

A lot of cytokines and serum proteins are associated with IBD and CRC. Under normal conditions these factors are able to regulate specific cellular functions including homeostasis, survivability, cell migration and anti-inflammatory processes [144]. The Bioplex assay revealed that activated PMNs are able to release a lot of specific pro-inflammatory cytokines like IL-8, TNF-α and IL-6 and many more **(Fig36)** which are known to act not only in a positive way. Several cytokines have a well established role in IBD and this study shows that a specific dose of these small molecules can affect the proliferation rate as well as mutation rate **(Fig38 and 40)** may due to an increased induction of internal ROS production **(Fig41)**.
Many publications indicate the importance of supplementation of natural compounds like Nigella sativa oil (Thymoquinone) or Broccoli extracts and their antioxidant and anti-

inflammatory abilities as alternative options against colon cancer. A new hot topic is also the docosahexaenoic acid (DHA) fraction of fish oil and its ability to reduce the overwhelming expression of TNF-α, IL-6, and IL-8 and thereby lowering their potential role to drive carcinogenesis [185], [186].

Tofacitinib, a potent Jak3 inhibitor was investigated by Pfizer and already tested for the treatment of IBD [178]. We co-treated the HCT116+chr3 CA26.2 reporter clone with 25ng/ml IL-6 and 2μM of the inhibitor and observed a decrease in proliferation with both compounds but a 100% reduction in mutation rate **(Fig39)**. Another hopeful therapeutic approach could be the use of Infliximab, a monoclonal antibody against TNF-α which is already approved by the U.S. Food and Drug Administration (FDA) [43]. Cytokines could be also used as specific serum biomarkers for IBD and colonic carcinogenesis and the development of serum biomarker profiles to specific stages of CRC may can lead to a reduction in mortality by improving diagnosis of early-stage disease [146], [187].

We have to consider that this is an *in-vitro* study; therefore biological effects might be different in *in-vivo* models. Limitations include that freshly isolated PMNs can vary from volunteer to volunteer in their activity but also in the release of different products. It is also very difficult to simulate the exact physiological condition like effector:target ratio and exposure time of our reporter cells within the co-culture system in comparison to natural UC conditions but these results contribute to a better understanding of the origin of colitis driven CRC by giving insight in the role of activated PMNs and their effect on the stability of microsatellite sequences.

5. Materials and Methods

5.1 Cell lines and Growing Conditions

In this study different pre-established reporter clones were utilized to compare mutations of mono- ,di- and tetranucleotide repeats in human colorectal cancer cells (HCT116+chr3 $^{hMLH1+/-}$) as well as normal human colonic epithelial cells (HCEC) [162]. HCT116+chr3 and HCEC were stably transfected with EGFP-based plasmids harboring G16, A10, (CA)13, (CA)26 and (AAAG)17 repeats. MSI-reporter cell lines derived from HCT116+chr3 were grown in Iscove's modified Dulbeco's medium (IMDM, Gibco/Invitrogen) containing 2mM glutamine and 10% fetal bovine serum (Biochrom, Berlin, Germany) in a humidified atmosphere of 5% CO_2, at 37°C. HCEC primary cells were cultured in a 4:1 mixture of Dulbecco's Modified Eagle Medium (DMEM, Gibco) with Medium 199 (M199, Gibco) supplemented with 2% Cosmic Calf Serum (Hyclone), 50 μg/mL gentamicin (Gemini Bioproducts), 1 μg/mL hydrocortisone (Sigma), 20 ng/mL recombinant human epidermal growth factor (EGF, Peprotech), 5 nM sodium selenite (Sigma), 2 μg/mL transferrin (Sigma), and 10 μg/mL insulin (bovine pacreas, Sigma). Additionally the medium for HCEC included 300μg/mL hygromycin B (Gibco/Invitrogen) and the HCT116+chr3 medium contained 150μg/mL hygromycin B as well as 400μg/mL G418 (Gibco/Invitrogen). HCEC clones were cultured in 75cm^2 Primaria PST Sterile Cell Culture Flasks provided by BD Falcon.

5.2 Isolation and activation of polymorphonuclear cells

PMNs were freshly isolated from blood of healthy donors (~1-3x10^6 per mL blood) by mixing with cold ½ Vol. Dextran-Solution (2% Dextran T700, 0.9% NaCl in H2O; Dextran was provided by Pharmacia) followed by sedimentation for 25-30 minutes. Upper yellowish phase was applied on 15ml Ficoll-Paque (Amersham, Uppsala, Sweden) gradient in a 50ml Falcon-Tube and centrifugated for 20 minutes @ 400g (1370rpm), 15°C. Afterwards supernatant was discarded and pellet was resuspended in 15ml icecold 0.2% NaCl for 45 seconds to get rid of remaining erythrocytes (cell lysis). Subsequently 15ml icecold 1.6% NaCl was added to recover physiological osmolarity. Solution was well mixed

and again centrifugated 3 minutes @ 400g, 15°C. Finally remaining pellet (isolated PMNs) was resuspended and washed in 30ml Ca/Mg-free Hanks' balanced salt solution (HBSS - Gibco) and PMN quantity was measured with a Haemocytometer. Needed PMN amount was calculated and activated with 0.5nM Phorbol 12-myristate 13-acetate (500µM stock - Sigma) directly within the well.

5.3 Frameshift mutation assay

In the year 2010 Campregher et al. established a panel of reporter cell lines for frameshift mutations at defined mono-, di-, and tetranucleotide microsatellites by transfection of colon epithelial cells with pIREShyg2-EGFP/MS, an enhanced green fluorescence protein (EGFP) based plasmid [162]. In this plasmid, a microsatellite follows the EGFP start codon and shifts the EGFP reading frame out of position, leading to expression of a non-fluorescent protein. Frameshift mutations that restore the EGFP reading frame [e.g. (CA)13 to (CA)12] allows EGFP expression and quantitation of fluorescent cells by FACS. In these stable transfectants, we were able to differentiate three populations by their fluorescence intensity and mutation profile: non-fluorescent/non-mutant M0-cells, dim fluorescent/intermediate mutant M1-cells and strong fluorescent/definitive mutant M2-fraction [161].

5.4 Cell Sorting and Co-culture

24 hours before co-culture experiments were started, $5x10^3$ HCT116+chr3 and HCEC clones were sorted into 24-well plates ($5x10^3$/well) using FACSAria cell sorter with the CloneCyt Plus software (Becton Dickinson) at the MUW Core Facility Flow Cytometry. All appearing cells were gated and divided into EGFP positive and negative cells. Only EGFP negative (non-fluorescent) clones were sorted into wells and cultured in above mentioned growing conditions.

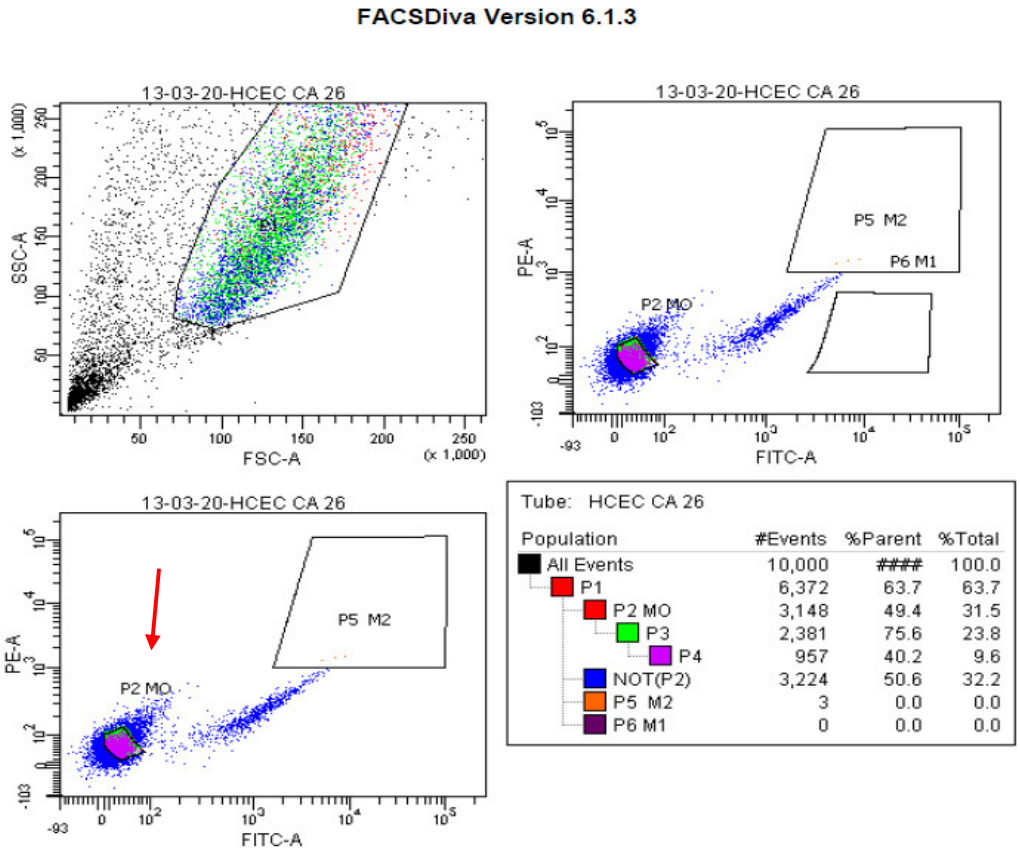


Figure42. HCEC CA26 cell sorting with FACSaria high speed sorter. 5x10^3 EGFP negative cells (P2 MO) were sorted into each well of a 24-well plate.

Twenty-four hours later, isolated PMNs were added to pre-sorted MSI reporter clones at effector:target ratios of 0:1 to 100:1 for again 24h. On the next day, two PBS washing steps were done to get rid of remaining dead PMNs and afterwards all wells were controlled under microscope. Each well was refilled by fresh IMDM or DMEM and plates were placed back into incubator for 6 days.

5.5 Flow cytometric analysis of mutant cells

After a week growing period, cells were detached with 200µL Accutase (PAA Laboratories GmbH, Linz, Austria) and 120µL of this solution was analyzed on a CellLabs Quanta (Beckman Coulter). Cell counts were calculated by 1.6 in order to calculate total number of cells per well. To identify EGFP-positive cells, region 1 (R1) was set to EV/side scatter and region 2 (R2) to FL1 (green) /FL2 (red) scatter [161]. Cells of region 1 and region 2 were

further plotted on a fluorescence intensity histogram, and two different populations were identified (M1 and M2-cells). The population displaying low fluorescence intensity was named M1, with high fluorescence intensity as M2 [161].

5.6 Calculation of mutation rate

Mutation rate is the probability of a cell undergoing a mutation within lifetime, and was calculated per microsatellite per cell per generation [162]. Experiments were done in quadruplicates and repeated twice. Mutation rate was carried out by a statistician (Dipl.-Ing. Theresa Scharl) with two different methods: (1) maximum likelihood method (data shown in result part) [188] and (2) the Luria-Delbruck method (data not shown) [189]. Calculated values were compared by using the Welsh two sample t-test and statistically significant p-values corrected with Benjamini Hochberg method; p-values were considered as ≤0.05.

5.7 Lucigenin-amplified chemiluminescence test

In order to test the activation of PMNs, we took advantage of the Lucigenin-amplified chemiluminescence assay (LcCL) which allows detecting extracellular superoxide anion radicals (O2-) released from PMA activated neutrophils. *N*-methylacridinium nitrate (Lucigenin) is an aromatic compound which is used in many laboratories to detect superoxide. The reaction of lucigenin reduction is not already clear but it is known that a one-electron reduction of the complex takes place to form the lucigenin cation radical $LC^{\cdot+}$ which can further react with superoxide to emit light and the light can then be detected by a photometer. Per sample $75x10^3$ freshly isolated neutrophils were activated with 0.5nM PMA within 500µl HBSS- solution and the chemiluminiescence reaction was started by the addition of 1µl Lucigenin (10mM stock). Different PMA concentrations (0.1-5nM) were tested for their ability to activate PMNs in a sufficient way and after 10 minutes incubation, all samples (+ a non-activated control) were measured at different time points (10'-3h) on a Photometer.

5.8 Hydrogen Peroxide Assay Kit – Measurement of PMN releasing H_2O_2

In order to measure releasing H_2O_2 from PMNs in our system we took advantage of the highly sensitive colorimetric and fluorometric Hydrogen Peroxide Assay Kit provided by Abcam. In this assay the OxiRed Probe reacts with hydrogen peroxide in the presence of Horse Radish Peroxidase (HRP) which ended in a product with color (570nm) or red-fluorescent (Ex/Em=535/587 nm). For this experiment, we followed instructions of the main Abcam Assay Protocol [190]. Briefly, we isolated fresh PMNs and activated a range of $25x10^3$ - $15x10^4$ cells for 30 minutes with 0.5 and 10nM PMA and collected supernatants. Meanwhile we prepared the H_2O_2 standard dilution and the reaction mix according to the protocol. 50µl reaction mix (HRP/OxiRed) was added to each well containing 50µl H_2O_2 standard or samples. After 10 minutes plate was measured on ELISA reader for red-fluorescence and data calculated.

5.9 Bioplex assay

Bioplex cytokine assay kit (BioRad) was utilized to detect released cytokines from PMNs in our co-culture system. This assay is a multiplex bead-based method and was investigated to quantitate multiple cytokines in serum samples but also cell culture supernatants. $2x10^6$ PMNs/ml were activated with 0.5/50nM PMA as well as 1/10µg per ml LPS (all dissolved in DMSO, final 2µl/ml). Cells were activated for 30 minutes, 2 hours and 16 hours, supernatant collected (500µl each - primary remove of PMNs at 400g, secondary remove small particles at fullspeed) and N_2-shock freezed. Bioplex was performed according to the main BioRad Assay protocol [191].

5.10 DCFDH – internal ROS production

To detect intracellular ROS within our cells we used the Abcam DCFDA - Cellular Reactive Oxygen Species Detection Assay. The non-fluorescent 2',7' –dichlorofluorescein diacetate (DCFDA) is a cell permeant reagent and can diffuse into cells where it is deacetylated by celluar esterases to 2', 7'-Dichlorodihydrofluorescin (DCFH). In the presence of ROS, the DCFH can then be oxidized to the highly fluorescent 2',7'dichlorofluorescein (DCF) and

further detected by fluorescence spectroscopy. For the assay we plated $5x10^3$ cell per well on a 96 well plate one day before to reach 70-80% uniform confluence. Only middle 60 wells were consistent so we tried to avoid using the edges of the plate. Next day we treated cells with our compounds (25ng/ml cytokines) for time course (15 min, 30 min, 1h, 2h, and 3h) but also with 100µM H2O2 (10M stock) as positive control. After 3 hours we discarded medium, washed cells twice with PBS. Further on we loaded each well with 100µl of 25µM DCFDA in HBSS- solution and incubated for 30 minutes. Then we washed cells again with cold PBS, added 100µl HBSS- without DCFDA and left plates in incubator for 10 minutes. Subsequently we measured plate on Chameleon V multilabel plate reader (ex. 485, em. 535).

5.11 MTT test

The MTT-test is a colorimetric assay and was utilized to measure cell viability/cytotoxicity of our reporter clones which were treated with different compounds/antagonists to find the maximum tolerable concentration for further experiments. Briefly, the yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent can be reduced to purple formazan in mitochondria. We seeded $1x10^3$ cells per well on 96-well plate and let them grow for 24 hours. Next day we treated cells (triplicates) with selected compounds for 48h. Afterwards we added 20µl of MTT solvent (5mg/ml stock) per 200µl of medium. We incubated cells at 37°C and allowed crystals to develop for 3 hours. After that time medium was discarded and 100µl 1:1 DMSO:Ethanol mix added. Plate was incubated for 10 minutes and measured within 1 hour on a ELISA reader (570nm).

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7. References

[1] D. C. Baumgart and S. R. Carding, "Inflammatory bowel disease: cause and immunobiology.," *Lancet*, vol. 369, no. 9573, pp. 1627–40, May 2007.

[2] D. C. Baumgart and W. J. Sandborn, "Inflammatory bowel disease: clinical aspects and established and evolving therapies.," *Lancet*, vol. 369, no. 9573, pp. 1641–57, May 2007.

[3] R. J. Xavier and D. K. Podolsky, "Unravelling the pathogenesis of inflammatory bowel disease.," *Nature*, vol. 448, no. 7152, pp. 427–34, Jul. 2007.

[4] "Crohn's & Colitis Foundation of America." [Online]. Available: http://www.ccfa.org/.

[5] "Johns Hopkins Children's Center - Gastroenterology and Nutrition." [Online]. Available: https://www.hopkinschildrens.org/.

[6] Iconsinmedicine, "IBD." [Online]. Available: http://iconsinmedicine.wordpress.com/tag/ibd/.

[7] J. Cosnes, C. Gower-Rousseau, P. Seksik, and A. Cortot, "Epidemiology and natural history of inflammatory bowel diseases.," *Gastroenterology*, vol. 140, no. 6, pp. 1785–94, May 2011.

[8] C. Abraham and J. H. Cho, "Inflammatory bowel disease.," *The New England journal of medicine*, vol. 361, no. 21, pp. 2066–78, Nov. 2009.

[9] C. Noble and I. Arnott, *What is the risk that a child will develop inflammatory bowel disease if one or both parents have IBD? Inflamm Bowel Dis.* 2008, pp. 22–23.

[10] R. A. Bennett, P. H. Rubin, and D. H. Present, "Frequency of inflammatory bowel disease in offspring of couples both presenting with inflammatory bowel disease.," *Gastroenterology*, vol. 100, no. 6, pp. 1638–43, Jun. 1991.

[11] J. H. Cho and C. T. Weaver, "The genetics of inflammatory bowel disease.," *Gastroenterology*, vol. 133, no. 4, pp. 1327–39, Oct. 2007.

[12] J. Hampe, A. Franke, P. Rosenstiel, A. Till, M. Teuber, K. Huse, M. Albrecht, G. Mayr, F. M. De La Vega, J. Briggs, S. Günther, N. J. Prescott, C. M. Onnie, R. Häsler, B. Sipos, U. R. Fölsch, T. Lengauer, M. Platzer, C. G. Mathew, M. Krawczak, and S. Schreiber, "A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1.," *Nature genetics*, vol. 39, no. 2, pp. 207–11, Feb. 2007.

[13] M. Parkes, J. C. Barrett, N. J. Prescott, M. Tremelling, C. A. Anderson, S. A. Fisher, R. G. Roberts, E. R. Nimmo, F. R. Cummings, D. Soars, H. Drummond, C. W. Lees, S. A. Khawaja, R. Bagnall, D. A. Burke, C. E. Todhunter, T. Ahmad, C. M. Onnie, W. McArdle, D. Strachan, G. Bethel, C. Bryan, C. M. Lewis, P. Deloukas, A. Forbes, J. Sanderson, D. P. Jewell, J. Satsangi, J. C. Mansfield, L. Cardon, and C. G. Mathew, "Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility.," *Nature genetics*, vol. 39, no. 7, pp. 830–2, Jul. 2007.

[14] J. D. Rioux, R. J. Xavier, K. D. Taylor, M. S. Silverberg, P. Goyette, A. Huett, T. Green, P. Kuballa, M. M. Barmada, L. W. Datta, Y. Y. Shugart, A. M. Griffiths, S. R. Targan, A. F. Ippoliti, E.-J. Bernard, L. Mei, D. L. Nicolae, M. Regueiro, L. P. Schumm, A. H. Steinhart, J. I. Rotter, R. H. Duerr, J. H. Cho, M. J. Daly, and S. R. Brant, "Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis.," *Nature genetics*, vol. 39, no. 5, pp. 596–604, May 2007.

[15] J. C. Barrett, S. Hansoul, D. L. Nicolae, J. H. Cho, R. H. Duerr, J. D. Rioux, S. R. Brant, M. S. Silverberg, K. D. Taylor, M. M. Barmada, A. Bitton, T. Dassopoulos, L. W. Datta, T. Green, A. M. Griffiths, E. O. Kistner, M. T. Murtha, M. D.

Regueiro, J. I. Rotter, L. P. Schumm, A. H. Steinhart, S. R. Targan, R. J. Xavier, C. Libioulle, C. Sandor, M. Lathrop, J. Belaiche, O. Dewit, I. Gut, S. Heath, D. Laukens, M. Mni, P. Rutgeerts, A. Van Gossum, D. Zelenika, D. Franchimont, J.-P. Hugot, M. de Vos, S. Vermeire, E. Louis, L. R. Cardon, C. A. Anderson, H. Drummond, E. Nimmo, T. Ahmad, N. J. Prescott, C. M. Onnie, S. A. Fisher, J. Marchini, J. Ghori, S. Bumpstead, R. Gwilliam, M. Tremelling, P. Deloukas, J. Mansfield, D. Jewell, J. Satsangi, C. G. Mathew, M. Parkes, M. Georges, and M. J. Daly, "Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease.," *Nature genetics*, vol. 40, no. 8, pp. 955–62, Aug. 2008.

[16] C. W. Lees, J. C. Barrett, M. Parkes, and J. Satsangi, "New IBD genetics: common pathways with other diseases.," *Gut*, vol. 60, no. 12, pp. 1739–53, Dec. 2011.

[17] C. A. Anderson, G. Boucher, C. W. Lees, A. Franke, M. D'Amato, K. D. Taylor, J. C. Lee, P. Goyette, M. Imielinski, A. Latiano, C. Lagacé, R. Scott, L. Amininejad, S. Bumpstead, L. Baidoo, R. N. Baldassano, M. Barclay, T. M. Bayless, S. Brand, C. Büning, J.-F. Colombel, L. A. Denson, M. De Vos, M. Dubinsky, C. Edwards, D. Ellinghaus, R. S. N. Fehrmann, J. A. B. Floyd, T. Florin, D. Franchimont, L. Franke, M. Georges, J. Glas, N. L. Glazer, S. L. Guthery, T. Haritunians, N. K. Hayward, J.-P. Hugot, G. Jobin, D. Laukens, I. Lawrance, M. Lémann, A. Levine, C. Libioulle, E. Louis, D. P. McGovern, M. Milla, G. W. Montgomery, K. I. Morley, C. Mowat, A. Ng, W. Newman, R. A. Ophoff, L. Papi, O. Palmieri, L. Peyrin-Biroulet, J. Panés, A. Phillips, N. J. Prescott, D. D. Proctor, R. Roberts, R. Russell, P. Rutgeerts, J. Sanderson, M. Sans, P. Schumm, F. Seibold, Y. Sharma, L. A. Simms, M. Seielstad, A. H. Steinhart, S. R. Targan, L. H. van den Berg, M. Vatn, H. Verspaget, T. Walters, C. Wijmenga, D. C. Wilson, H.-J. Westra, R. J. Xavier, Z. Z. Zhao, C. Y. Ponsioen, V. Andersen, L. Torkvist, M. Gazouli, N. P. Anagnou, T. H. Karlsen, L. Kupcinskas, J. Sventoraityte, J. C. Mansfield, S. Kugathasan, M. S. Silverberg, J. Halfvarson, J. I. Rotter, C. G. Mathew, A. M. Griffiths, R. Gearry, T. Ahmad, S. R. Brant, M. Chamaillard, J. Satsangi, J. H. Cho, S. Schreiber, M. J. Daly, J. C. Barrett, M. Parkes, V. Annese, H. Hakonarson, G. Radford-Smith, R. H. Duerr, S. Vermeire, R. K. Weersma, and J. D. Rioux, "Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47.," *Nature genetics*, vol. 43, no. 3, pp. 246–52, Mar. 2011.

[18] A. I. Thompson and C. W. Lees, "Genetics of ulcerative colitis.," *Inflammatory bowel diseases*, vol. 17, no. 3, pp. 831–48, Mar. 2011.

[19] J. C. Barrett, J. C. Lee, C. W. Lees, N. J. Prescott, C. A. Anderson, A. Phillips, E. Wesley, K. Parnell, H. Zhang, H. Drummond, E. R. Nimmo, D. Massey, K. Blaszczyk, T. Elliott, L. Cotterill, H. Dallal, A. J. Lobo, C. Mowat, J. D. Sanderson, D. P. Jewell, W. G. Newman, C. Edwards, T. Ahmad, J. C. Mansfield, J. Satsangi, M. Parkes, C. G. Mathew, P. Donnelly, L. Peltonen, J. M. Blackwell, E. Bramon, M. A. Brown, J. P. Casas, A. Corvin, N. Craddock, P. Deloukas, A. Duncanson, J. Jankowski, H. S. Markus, M. I. McCarthy, C. N. A. Palmer, R. Plomin, A. Rautanen, S. J. Sawcer, N. Samani, R. C. Trembath, A. C. Viswanathan, N. Wood, C. C. A. Spencer, C. Bellenguez, D. Davison, C. Freeman, A. Strange, C. Langford, S. E. Hunt, S. Edkins, R. Gwilliam, H. Blackburn, S. J. Bumpstead, S. Dronov, M. Gillman, E. Gray, N. Hammond, A. Jayakumar, O. T. McCann, J. Liddle, M. L. Perez, S. C. Potter, R. Ravindrarajah, M. Ricketts, M. Waller, P. Weston, S. Widaa, P. Whittaker, A. P. Attwood, J. Stephens, J. Sambrook, W. H. Ouwehand, W. L. McArdle, S. M. Ring, and D. P. Strachan, "Genome-wide association study of ulcerative colitis identifies three new susceptibility loci, including the HNF4A region.," *Nature genetics*, vol. 41, no. 12, pp. 1330–4, Dec. 2009.

[20] A. Franke, T. Balschun, T. H. Karlsen, J. Sventoraityte, S. Nikolaus, G. Mayr, F. S. Domingues, M. Albrecht, M. Nothnagel, D. Ellinghaus, C. Sina, C. M. Onnie, R. K. Weersma, P. C. F. Stokkers, C. Wijmenga, M. Gazouli, D. Strachan, W. L. McArdle, S. Vermeire, P. Rutgeerts, P. Rosenstiel, M. Krawczak, M. H. Vatn, C. G. Mathew, and S. Schreiber, "Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility.," *Nature genetics*, vol. 40, no. 11, pp. 1319–23, Nov. 2008.

[21] S. Fujino, A. Andoh, S. Bamba, A. Ogawa, K. Hata, Y. Araki, T. Bamba, and Y. Fujiyama, "Increased expression of interleukin 17 in inflammatory bowel disease.," *Gut*, vol. 52, no. 1, pp. 65–70, Jan. 2003.

[22] I. Vind, L. Riis, T. Jess, E. Knudsen, N. Pedersen, M. Elkjaer, I. Bak Andersen, V. Wewer, P. Nørregaard, F. Moesgaard, F. Bendtsen, and P. Munkholm, "Increasing incidences of inflammatory bowel disease and decreasing surgery rates in Copenhagen City and County, 2003-2005: a population-based study from the Danish Crohn colitis database.," *The American journal of gastroenterology*, vol. 101, no. 6, pp. 1274–82, Jun. 2006.

[23] T. R. Yapp, R. Stenson, G. A. Thomas, B. W. Lawrie, G. T. Williams, and A. B. Hawthorne, "Crohn's disease incidence in Cardiff from 1930: an update for 1991-1995.," *European journal of gastroenterology & hepatology*, vol. 12, no. 8, pp. 907–11, Aug. 2000.

[24] C. G. Loftus, E. V Loftus, W. S. Harmsen, A. R. Zinsmeister, W. J. Tremaine, L. J. Melton, and W. J. Sandborn, "Update on the incidence and prevalence of Crohn's disease and ulcerative colitis in Olmsted County, Minnesota, 1940-2000.," *Inflammatory bowel diseases*, vol. 13, no. 3, pp. 254–61, Mar. 2007.

[25] C. N. Bernstein, A. Wajda, L. W. Svenson, A. MacKenzie, M. Koehoorn, M. Jackson, R. Fedorak, D. Israel, and J. F. Blanchard, "The epidemiology of inflammatory bowel disease in Canada: a population-based study.," *The American journal of gastroenterology*, vol. 101, no. 7, pp. 1559–68, Jul. 2006.

[26] S. Thorsteinsdottir, T. Gudjonsson, O. H. Nielsen, B. Vainer, and J. B. Seidelin, "Pathogenesis and biomarkers of carcinogenesis in ulcerative colitis.," *Nature reviews. Gastroenterology & hepatology*, vol. 8, no. 7, pp. 395–404, Jul. 2011.

[27] S. B. Hanauer and D. W. Hommes, "Inflammatory bowel disease.," *Expert review of clinical immunology*, vol. 6, no. 4, pp. 499–500, Jul. 2010.

[28] S. C. TRUELOVE and L. J. WITTS, "Cortisone in ulcerative colitis; final report on a therapeutic trial.," *British medical journal*, vol. 2, no. 4947, pp. 1041–8, Oct. 1955.

[29] S. Danese and C. Fiocchi, "Ulcerative colitis.," *The New England journal of medicine*, vol. 365, no. 18, pp. 1713–25, Nov. 2011.

[30] C. Campregher, "Establishment of an In Vitro Co-culture Model to Study the Molecular Pathways Involved in Ulcerative Colitis Associated Colorectal Cancer," 2008.

[31] T. A. Ullman and S. H. Itzkowitz, "Intestinal inflammation and cancer.," *Gastroenterology*, vol. 140, no. 6, pp. 1807–16, May 2011.

[32] M. Feller, K. Huwiler, A. Schoepfer, A. Shang, H. Furrer, and M. Egger, "Long-term antibiotic treatment for Crohn's disease: systematic review and meta-analysis of placebo-controlled trials.," *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, vol. 50, no. 4, pp. 473–80, Feb. 2010.

[33] C. Prantera and M. L. Scribano, "Antibiotics and probiotics in inflammatory bowel disease: why, when, and how.," *Current opinion in gastroenterology*, vol. 25, no. 4, pp. 329–33, Jul. 2009.

[34] A. J. Tresca, "Medical Therapy For IBD Can Include Medications And Surgery," 2012. [Online]. Available: http://ibdcrohns.about.com/od/treatments/a/treatmentibd.htm.

[35] W. Rower and S. Shehnaz, "Inflammatory Bowel Disease." [Online]. Available: http://www.emedicinehealth.com/inflammatory_bowel_disease/article_em.htm.

[36] W. Kruis, S. Schreiber, D. Theuer, J. W. Brandes, E. Schütz, S. Howaldt, B. Krakamp, J. Hämling, H. Mönnikes, I. Koop, M. Stolte, D. Pallant, and U. Ewald, "Low dose balsalazide (1.5 g twice daily) and mesalazine (0.5 g three times daily) maintained remission of ulcerative colitis but high dose balsalazide (3.0 g twice daily) was superior in preventing relapses.," *Gut*, vol. 49, no. 6, pp. 783–9, Dec. 2001.

[37] PharmGKB, "mesalazine." [Online]. Available: http://www.pharmgkb.org/drug/PA450384#tabview=tab2&subtab=31.

[38] J. A. Katz, "Treatment of inflammatory bowel disease with corticosteroids.," *Gastroenterology clinics of North America*, vol. 33, no. 2, pp. 171–89, vii, Jun. 2004.

[39] A. A. Tanis, "Azathioprine in inflammatory bowel disease, a safe alternative?," *Mediators of inflammation*, vol. 7, no. 3, pp. 141–4, Jan. 1998.

[40] M. C. Dubinsky, "Azathioprine, 6-mercaptopurine in inflammatory bowel disease: pharmacology, efficacy, and safety.," *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association*, vol. 2, no. 9, pp. 731–43, Sep. 2004.

[41] E. Domènech, P. Nos, M. Papo, A. López-San Román, E. Garcia-Planella, and M. A. Gassull, "6-mercaptopurine in patients with inflammatory bowel disease and previous digestive intolerance of azathioprine.," *Scandinavian journal of gastroenterology*, vol. 40, no. 1, pp. 52–5, Jan. 2005.

[42] P. Rutgeerts, G. Van Assche, and S. Vermeire, "Review article: Infliximab therapy for inflammatory bowel disease--seven years on.," *Alimentary pharmacology & therapeutics*, vol. 23, no. 4, pp. 451–63, Feb. 2006.

[43] W. J. Travassos and A. S. Cheifetz, "Infliximab: Use in Inflammatory Bowel Disease.," *Current treatment options in gastroenterology*, vol. 8, no. 3, pp. 187–196, Jun. 2005.

[44] W. J. Sandborn and W. J. Tremaine, "Cyclosporine treatment of inflammatory bowel disease.," *Mayo Clinic proceedings. Mayo Clinic*, vol. 67, no. 10, pp. 981–90, Oct. 1992.

[45] C. G. Loftus, L. J. Egan, and W. J. Sandborn, "Cyclosporine, tacrolimus, and mycophenolate mofetil in the treatment of inflammatory bowel disease.," *Gastroenterology clinics of North America*, vol. 33, no. 2, pp. 141–69, vii, Jun. 2004.

[46] Johnson & Johnson Services, "REMICADE® Becomes First Anti-TNF Biologic Therapy to Treat One Million Patients Worldwide," 2007. [Online]. Available: http://www.jnj.com/connect/NewsArchive/all-news-archive/20071106_141812.

[47] L. Maggiori and Y. Panis, "Surgical management of IBD-from an open to a laparoscopic approach.," *Nature reviews. Gastroenterology & hepatology*, vol. 10, no. 5, pp. 297–306, Apr. 2013.

[48] M. S. Friedlich, M. Guindi, and H. S. Stern, "The management of dysplasia associated with ulcerative colitis: colectomy versus continued surveillance.," *Canadian journal of surgery. Journal canadien de chirurgie*, vol. 47, no. 3, pp. 212–4, Jun. 2004.

[49] D. F. Berg, A. M. Bahadursingh, D. L. Kaminski, and W. E. Longo, "Acute surgical emergencies in inflammatory bowel disease.," *American journal of surgery*, vol. 184, no. 1, pp. 45–51, Jul. 2002.

[50] D. W. Larson and J. H. Pemberton, "Current concepts and controversies in surgery for IBD.," *Gastroenterology*, vol. 126, no. 6, pp. 1611–9, May 2004.

[51] R. Holzheimer and J. Mannick, *Surgical Treatment: Evidence-Based and Problem-Oriented*. Munich, 2001.

[52] Medical-Encyclopedia, "Colon cancer," 17-Nov-2012. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0001308/.

[53] EuropaColon, "CRC Statistics for colorectal cancer." [Online]. Available: http://www.europacolon.com/crcstatistics.php?Action=Crcstatistics.

[54] WHO, "GLOBOCAN PROJECT 2008," 2008. [Online]. Available: http://globocan.iarc.fr/.

[55] Statistik-Austria, "Oesterreichisches Krebsregisteritle," 2009. [Online]. Available: http://www.statistik.at/web_de/statistiken/gesundheit/krebserkrankungen/.

[56] Die Österreichische Krebshilfe, "Darmkrebs in Österreich," 2006. [Online]. Available: http://www.krebshilfe-wien.at/fileadmin/Redakteure/user_upload/Pdf/darmkrebs_08.pdf.

[57] A. K. Rustgi, "The genetics of hereditary colon cancer.," *Genes & development*, vol. 21, no. 20, pp. 2525–38, Oct. 2007.

[58] M. Sant, R. Capocaccia, A. Verdecchia, G. Gatta, A. Micheli, A. Mariotto, T. Hakulinen, and F. Berrino, "Comparisons of colon-cancer survival among European countries: The Eurocare Study.," *International journal of cancer. Journal international du cancer*, vol. 63, no. 1, pp. 43–8, Sep. 1995.

[59] D. M. Parkin, F. Bray, J. Ferlay, and P. Pisani, "Estimating the world cancer burden: Globocan 2000.," *International journal of cancer. Journal international du cancer*, vol. 94, no. 2, pp. 153–6, Oct. 2001.

[60] S. C. Larsson and A. Wolk, "Meat consumption and risk of colorectal cancer: a meta-analysis of prospective studies.," *International journal of cancer. Journal international du cancer*, vol. 119, no. 11, pp. 2657–64, Dec. 2006.

[61] S. A. Bingham, T. Norat, A. Moskal, P. Ferrari, N. Slimani, F. Clavel-Chapelon, E. Kesse, A. Nieters, H. Boeing, A. Tjønneland, K. Overvad, C. Martinez, M. Dorronsoro, C. A. González, E. Ardanaz, C. Navarro, J. R. Quirós, T. J. Key, N. E. Day, A. Trichopoulou, A. Naska, V. Krogh, R. Tumino, D. Palli, S. Panico, P. Vineis, H. B. Bueno-de-Mesquita, M. C. Ocké, P. H. M. Peeters, G. Berglund, G. Hallmans, E. Lund, G. Skeie, R. Kaaks, and E. Riboli, "Is the association with fiber from foods in colorectal cancer confounded by folate intake?," *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, vol. 14, no. 6, pp. 1552–6, Jun. 2005.

[62] A. Moskal, T. Norat, P. Ferrari, and E. Riboli, "Alcohol intake and colorectal cancer risk: a dose-response meta-analysis of published cohort studies.," *International journal of cancer. Journal international du cancer*, vol. 120, no. 3, pp. 664–71, Mar. 2007.

[63] A. A. Moghaddam, M. Woodward, and R. Huxley, "Obesity and risk of colorectal cancer: a meta-analysis of 31 studies with 70,000 events.," *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, vol. 16, no. 12, pp. 2533–47, Dec. 2007.

[64] K. Y. Wolin, Y. Yan, G. A. Colditz, and I.-M. Lee, "Physical activity and colon cancer prevention: a meta-analysis.," *British journal of cancer*, vol. 100, no. 4, pp. 611–6, Mar. 2009.

[65] D. Cunningham, W. Atkin, H.-J. Lenz, H. T. Lynch, B. Minsky, B. Nordlinger, and N. Starling, "Colorectal cancer.," *Lancet*, vol. 375, no. 9719, pp. 1030–47, Mar. 2010.

[66] A. Jemal, R. Siegel, E. Ward, Y. Hao, J. Xu, T. Murray, and M. J. Thun, "Cancer statistics, 2008.," *CA: a cancer journal for clinicians*, vol. 58, no. 2, pp. 71–96.

[67] I. M. Hisamuddin and V. W. Yang, "Genetics of colorectal cancer.," *MedGenMed : Medscape general medicine*, vol. 6, no. 3, p. 13, Jan. 2004.

[68] M. Huncharek, J. Muscat, and B. Kupelnick, "Colorectal cancer risk and dietary intake of calcium, vitamin D, and dairy products: a meta-analysis of 26,335 cases from 60 observational studies.," *Nutrition and cancer*, vol. 61, no. 1, pp. 47–69, Jan. 2009.

[69] L. Yin, N. Grandi, E. Raum, U. Haug, V. Arndt, and H. Brenner, "Meta-analysis: Serum vitamin D and colorectal adenoma risk.," *Preventive medicine*, vol. 53, no. 1–2, pp. 10–6.

[70] Y. Ma, P. Zhang, F. Wang, J. Yang, Z. Liu, and H. Qin, "Association between vitamin D and risk of colorectal cancer: a systematic review of prospective studies.," *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, vol. 29, no. 28, pp. 3775–82, Oct. 2011.

[71] T. Iwama, "NSAIDs and colorectal cancer prevention.," *Journal of gastroenterology*, vol. 44 Suppl 1, pp. 72–6, Jan. 2009.

[72] X. Liao, P. Lochhead, R. Nishihara, T. Morikawa, A. Kuchiba, M. Yamauchi, Y. Imamura, Z. R. Qian, Y. Baba, K. Shima, R. Sun, K. Nosho, J. A. Meyerhardt, E. Giovannucci, C. S. Fuchs, A. T. Chan, and S. Ogino, "Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival.," *The New England journal of medicine*, vol. 367, no. 17, pp. 1596–606, Oct. 2012.

[73] B. Stewart and B. Kleinhues, *World Cancer Report - Colorectal Cancer*. 2003, pp. 198–203.

[74] F. S. Velayos, J. P. Terdiman, and J. M. Walsh, "Effect of 5-aminosalicylate use on colorectal cancer and dysplasia risk: a systematic review and metaanalysis of observational studies.," *The American journal of gastroenterology*, vol. 100, no. 6, pp. 1345–53, Jul. 2005.

[75] S. Segditsas and I. Tomlinson, "Colorectal cancer and genetic alterations in the Wnt pathway.," *Oncogene*, vol. 25, no. 57, pp. 7531–7, Dec. 2006.

[76] J. Terzić, S. Grivennikov, E. Karin, and M. Karin, "Inflammation and colon cancer.," *Gastroenterology*, vol. 138, no. 6, pp. 2101–2114.e5, Jun. 2010.

[77] J. He and J. E. Efron, "Screening for colorectal cancer.," *Advances in surgery*, vol. 45, pp. 31–44, Jan. 2011.

[78] G. Chung-Faye, B. Hayee, S. Maestranzi, N. Donaldson, I. Forgacs, and R. Sherwood, "Fecal M2-pyruvate kinase (M2-PK): a novel marker of intestinal inflammation.," *Inflammatory bowel diseases*, vol. 13, no. 11, pp. 1374–8, Dec. 2007.

[79] J. Xie and S. H. Itzkowitz, "Cancer in inflammatory bowel disease.," *World journal of gastroenterology : WJG*, vol. 14, no. 3, pp. 378–89, Jan. 2008.

[80] T. Armaghany, J. D. Wilson, Q. Chu, and G. Mills, "Genetic alterations in colorectal cancer.," *Gastrointestinal cancer research : GCR*, vol. 5, no. 1, pp. 19–27, Jan. 2012.

[81] E. R. Fearon and B. Vogelstein, "A genetic model for colorectal tumorigenesis.," *Cell*, vol. 61, no. 5, pp. 759–67, Jun. 1990.

[82] M. Ilyas, J. Straub, I. P. Tomlinson, and W. F. Bodmer, "Genetic pathways in colorectal and other cancers.," *European journal of cancer (Oxford, England : 1990)*, vol. 35, no. 3, pp. 335–51, Mar. 1999.

[83] S. D. Markowitz and M. M. Bertagnolli, "Molecular origins of cancer: Molecular basis of colorectal cancer.," *The New England journal of medicine*, vol. 361, no. 25, pp. 2449–60, Dec. 2009.

[84] N. S. Fearnhead, M. P. Britton, and W. F. Bodmer, "The ABC of APC.," *Human molecular genetics*, vol. 10, no. 7, pp. 721–33, Apr. 2001.

[85] P. J. Morin, A. B. Sparks, V. Korinek, N. Barker, H. Clevers, B. Vogelstein, and K. W. Kinzler, "Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC.," *Science (New York, N.Y.)*, vol. 275, no. 5307, pp. 1787–90, Mar. 1997.

[86] A. Goel and C. R. Boland, "Epigenetics of colorectal cancer.," *Gastroenterology*, vol. 143, no. 6, pp. 1442–1460.e1, Dec. 2012.

[87] W. S. Samowitz, "The CpG Island Methylator Phenotype in Colorectal Cancer.," *The Journal of molecular diagnostics : JMD*, Apr. 2007.

[88] N. Jawad, N. Direkze, and S. J. Leedham, "Inflammatory bowel disease and colon cancer.," *Recent results in cancer research. Fortschritte der Krebsforschung. Progrès dans les recherches sur le cancer*, vol. 185, pp. 99–115, Jan. 2011.

[89] J. K. Triantafillidis, G. Nasioulas, and P. A. Kosmidis, "Colorectal cancer and inflammatory bowel disease: epidemiology, risk factors, mechanisms of carcinogenesis and prevention strategies.," *Anticancer research*, vol. 29, no. 7, pp. 2727–37, Jul. 2009.

[90] J. A. Eaden, K. R. Abrams, and J. F. Mayberry, "The risk of colorectal cancer in ulcerative colitis: a meta-analysis.," *Gut*, vol. 48, no. 4, pp. 526–35, Apr. 2001.

[91] A. Ekbom, C. Helmick, M. Zack, and H. O. Adami, "Ulcerative colitis and colorectal cancer. A population-based study.," *The New England journal of medicine*, vol. 323, no. 18, pp. 1228–33, Nov. 1990.

[92] D. Chang, F. Wang, Y.-S. Zhao, and H.-Z. Pan, "Evaluation of oxidative stress in colorectal cancer patients.," *Biomedical and environmental sciences : BES*, vol. 21, no. 4, pp. 286–9, Aug. 2008.

[93] I. L. Aivaliotis, I. S. Pateras, M. Papaioannou, C. Glytsou, K. Kontzoglou, E. O. Johnson, and V. Zoumpourlis, "How do cytokines trigger genomic instability?," *Journal of biomedicine & biotechnology*, vol. 2012, p. 536761, Jan. 2012.

[94] J. P. Issa, N. Ahuja, M. Toyota, M. P. Bronner, and T. A. Brentnall, "Accelerated age-related CpG island methylation in ulcerative colitis.," *Cancer research*, vol. 61, no. 9, pp. 3573–7, May 2001.

[95] Y. Kondo and J.-P. J. Issa, "Epigenetic changes in colorectal cancer.," *Cancer metastasis reviews*, vol. 23, no. 1–2, pp. 29–39.

[96] L. A. E. Hughes, C. A. J. Khalid-de Bakker, K. M. Smits, P. A. van den Brandt, D. Jonkers, N. Ahuja, J. G. Herman, M. P. Weijenberg, and M. van Engeland, "The CpG island methylator phenotype in colorectal cancer: progress and problems.," *Biochimica et biophysica acta*, vol. 1825, no. 1, pp. 77–85, Jan. 2012.

[97] A. S. Fleisher, M. Esteller, N. Harpaz, A. Leytin, A. Rashid, Y. Xu, J. Liang, O. C. Stine, J. Yin, T. T. Zou, J. M. Abraham, D. Kong, K. T. Wilson, S. P. James, J. G. Herman, and S. J. Meltzer, "Microsatellite instability in inflammatory bowel disease-associated neoplastic lesions is associated with hypermethylation and diminished expression of the DNA mismatch repair gene, hMLH1.," *Cancer research*, vol. 60, no. 17, pp. 4864–8, Sep. 2000.

[98] B. M. Fournier and C. A. Parkos, "The role of neutrophils during intestinal inflammation.," *Mucosal immunology*, vol. 5, no. 4, pp. 354–66, Jul. 2012.

[99] D. Zucker-Franklin, *Atlas of blood cells*. 1987, pp. 168–174.

[100] Wikipedia, "Neutrophil granulocyte." [Online]. Available: http://en.wikipedia.org/wiki/Neutrophil_granulocyte.

[101] C. D. Sadik, N. D. Kim, and A. D. Luster, "Neutrophils cascading their way to inflammation.," *Trends in immunology*, vol. 32, no. 10, pp. 452–60, Oct. 2011.

[102] C. Summers, S. M. Rankin, A. M. Condliffe, N. Singh, A. M. Peters, and E. R. Chilvers, "Neutrophil kinetics in health and disease.," *Trends in immunology*, vol. 31, no. 8, pp. 318–24, Aug. 2010.

[103] V. Witko-Sarsat, P. Rieu, B. Descamps-Latscha, P. Lesavre, and L. Halbwachs-Mecarelli, "Neutrophils: molecules, functions and pathophysiological aspects.," *Laboratory investigation; a journal of technical methods and pathology*, vol. 80, no. 5, pp. 617–53, May 2000.

[104] A. S. MRCPath., A. Stevens, J. S. Lowe, B. Young, and P. R. Wheater, *Wheater's Basic Histopathology: A Colour Atlas and Text*. 2002.

[105] Y. Wu, S. Wang, S. M. Farooq, M. P. Castelvetere, Y. Hou, J.-L. Gao, J. V Navarro, D. Oupicky, F. Sun, and C. Li, "A chemokine receptor CXCR2 macromolecular complex regulates neutrophil functions in inflammatory diseases.," *The Journal of biological chemistry*, vol. 287, no. 8, pp. 5744–55, Feb. 2012.

[106] K. Ina, K. Kusugami, T. Yamaguchi, A. Imada, T. Hosokawa, M. Ohsuga, M. Shinoda, T. Ando, K. Ito, and Y. Yokoyama, "Mucosal interleukin-8 is involved in neutrophil migration and binding to extracellular matrix in inflammatory bowel disease.," *The American journal of gastroenterology*, vol. 92, no. 8, pp. 1342–6, Aug. 1997.

[107] K. J. Maloy and F. Powrie, "Intestinal homeostasis and its breakdown in inflammatory bowel disease.," *Nature*, vol. 474, no. 7351, pp. 298–306, Jun. 2011.

[108] B. Amulic, C. Cazalet, G. L. Hayes, K. D. Metzler, and A. Zychlinsky, "Neutrophil function: from mechanisms to disease.," *Annual review of immunology*, vol. 30, pp. 459–89, Jan. 2012.

[109] A. Barzilai and K.-I. Yamamoto, "DNA damage responses to oxidative stress.," *DNA repair*, vol. 3, no. 8–9, pp. 1109–15.

[110] V. Brinkmann, U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D. S. Weiss, Y. Weinrauch, and A. Zychlinsky, "Neutrophil extracellular traps kill bacteria.," *Science (New York, N.Y.)*, vol. 303, no. 5663, pp. 1532–5, Mar. 2004.

[111] B. M. Babior, "NADPH oxidase: an update.," *Blood*, vol. 93, no. 5, pp. 1464–76, Mar. 1999.

[112] K. L. Conway, G. Goel, H. Sokol, M. Manocha, E. Mizoguchi, C. Terhorst, A. K. Bhan, A. Gardet, and R. J. Xavier, "p40phox expression regulates neutrophil recruitment and function during the resolution phase of intestinal inflammation.," *Journal of immunology (Baltimore, Md. : 1950)*, vol. 189, no. 7, pp. 3631–40, Oct. 2012.

[113] T. Assari, "Chronic Granulomatous Disease; fundamental stages in our understanding of CGD.," *Medical immunology (London, England)*, vol. 5, p. 4, Jan. 2006.

[114] A. W. Segal, "How neutrophils kill microbes.," *Annual review of immunology*, vol. 23, pp. 197–223, Jan. 2005.

[115] F. Violi, S. Basili, C. Nigro, and P. Pignatelli, "Role of NADPH oxidase in atherosclerosis.," *Future cardiology*, vol. 5, no. 1, pp. 83–92, Jan. 2009.

[116] A. W. Segal, "The NADPH oxidase and chronic granulomatous disease.," *Molecular medicine today*, vol. 2, no. 3, pp. 129–35, Mar. 1996.

[117] R. D. Kolodner and G. T. Marsischky, "Eukaryotic DNA mismatch repair.," *Current opinion in genetics & development*, vol. 9, no. 1, pp. 89–96, Feb. 1999.

[118] C. R. Boland and A. Goel, "Microsatellite instability in colorectal cancer.," *Gastroenterology*, vol. 138, no. 6, pp. 2073–2087.e3, Jun. 2010.

[119] W. M. Grady and J. M. Carethers, "Genomic and epigenetic instability in colorectal cancer pathogenesis.," *Gastroenterology*, vol. 135, no. 4, pp. 1079–99, Oct. 2008.

[120] Y. Ionov, N. Nowak, M. Perucho, S. Markowitz, and J. K. Cowell, "Manipulation of nonsense mediated decay identifies gene mutations in colon cancer Cells with microsatellite instability.," *Oncogene*, vol. 23, no. 3, pp. 639–45, Jan. 2004.

[121] S. Markowitz, J. Wang, L. Myeroff, R. Parsons, L. Sun, J. Lutterbaugh, R. S. Fan, E. Zborowska, K. W. Kinzler, and B. Vogelstein, "Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability.," *Science (New York, N.Y.)*, vol. 268, no. 5215, pp. 1336–8, Jun. 1995.

[122] I. Sæterdal, S&aelig, I. terdal, M. K. Gjertsen, P. Straten, J. A. Eriksen, and G. Gaudernack, *A TGFβRII frameshift-mutation-derived CTL epitope recognised by HLA-A2-restricted CD8⁺ T cells*, vol. 50, no. 9. 2001, pp. 469 – 476.

[123] D. K. Chang, D. Metzgar, C. Wills, and C. R. Boland, "Microsatellites in the eukaryotic DNA mismatch repair genes as modulators of evolutionary mutation rate.," *Genome research*, vol. 11, no. 7, pp. 1145–6, Jul. 2001.

[124] A. Duval and R. Hamelin, "Mutations at coding repeat sequences in mismatch repair-deficient human cancers: toward a new concept of target genes for instability.," *Cancer research*, vol. 62, no. 9, pp. 2447–54, May 2002.

[125] G. Kurzawski, J. Suchy, T. Debniak, J. Kładny, and J. Lubiński, "Importance of microsatellite instability (MSI) in colorectal cancer: MSI as a diagnostic tool.," *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*, vol. 15 Suppl 4, pp. iv283–4, Jan. 2004.

[126] P. Peltomäki, L. A. Aaltonen, P. Sistonen, L. Pylkkänen, J. P. Mecklin, H. Järvinen, J. S. Green, J. R. Jass, J. L. Weber, and F. S. Leach, "Genetic mapping of a locus predisposing to human colorectal cancer.," *Science (New York, N.Y.)*, vol. 260, no. 5109, pp. 810–2, May 1993.

[127] C. R. Boland, S. N. Thibodeau, S. R. Hamilton, D. Sidransky, J. R. Eshleman, R. W. Burt, S. J. Meltzer, M. A. Rodriguez-Bigas, R. Fodde, G. N. Ranzani, and S. Srivastava, "A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer.," *Cancer research*, vol. 58, no. 22, pp. 5248–57, Nov. 1998.

[128] H. Suzuki, N. Harpaz, L. Tarmin, J. Yin, H. Y. Jiang, J. D. Bell, M. Hontanosas, G. M. Groisman, J. M. Abraham, and S. J. Meltzer, "Microsatellite instability in ulcerative colitis-associated colorectal dysplasias and cancers.," *Cancer research*, vol. 54, no. 18, pp. 4841–4, Sep. 1994.

[129] T. A. Brentnall, D. A. Crispin, M. P. Bronner, S. P. Cherian, M. Hueffed, P. S. Rabinovitch, C. E. Rubin, R. C. Haggitt, and C. R. Boland, "Microsatellite instability in nonneoplastic mucosa from patients with chronic ulcerative colitis.," *Cancer research*, vol. 56, no. 6, pp. 1237–40, Mar. 1996.

[130] C. L. Chang, G. Marra, D. P. Chauhan, H. T. Ha, D. K. Chang, L. Ricciardiello, A. Randolph, J. M. Carethers, and C. R. Boland, "Oxidative stress inactivates the human DNA mismatch repair system.," *American journal of physiology. Cell physiology*, vol. 283, no. 1, pp. C148–54, Jul. 2002.

[131] K. R. Loeb and L. A. Loeb, "Genetic instability and the mutator phenotype. Studies in ulcerative colitis.," *The American journal of pathology*, vol. 154, no. 6, pp. 1621–6, Jun. 1999.

[132] L. J. Hofseth, M. A. Khan, M. Ambrose, O. Nikolayeva, M. Xu-Welliver, M. Kartalou, S. P. Hussain, R. B. Roth, X. Zhou, L. E. Mechanic, I. Zurer, V. Rotter, L. D. Samson, and C. C. Harris, "The adaptive imbalance in base excision-repair enzymes generates microsatellite instability in chronic inflammation.," *The Journal of clinical investigation*, vol. 112, no. 12, pp. 1887–94, Dec. 2003.

[133] J. Jiricny, "The multifaceted mismatch-repair system.," *Nature reviews. Molecular cell biology*, vol. 7, no. 5, pp. 335–46, May 2006.

[134] T. A. Kunkel, "Frameshift mutagenesis by eucaryotic DNA polymerases in vitro," *J Biol Chem*, vol. 261, pp. 13581–13587, 1986.

[135] P. Peltomaki, "Deficient DNA mismatch repair: a common etiologic factor for colon cancer," *Human Molecular Genetics*, vol. 10, no. 7, pp. 735–740, Apr. 2001.

[136] R. Fishel and T. Wilson, "MutS homologs in mammalian cells.," *Current opinion in genetics & development*, vol. 7, no. 1, pp. 105–13, Feb. 1997.

[137] S. Gradia, S. Acharya, and R. Fishel, "The human mismatch recognition complex hMSH2-hMSH6 functions as a novel molecular switch.," *Cell*, vol. 91, no. 7, pp. 995–1005, Dec. 1997.

[138] S. Gradia, D. Subramanian, T. Wilson, S. Acharya, A. Makhov, J. Griffith, and R. Fishel, "hMSH2-hMSH6 forms a hydrolysis-independent sliding clamp on mismatched DNA.," *Molecular cell*, vol. 3, no. 2, pp. 255–61, Feb. 1999.

[139] G. Poulogiannis, I. M. Frayling, and M. J. Arends, "DNA mismatch repair deficiency in sporadic colorectal cancer and Lynch syndrome.," *Histopathology*, vol. 56, no. 2, pp. 167–79, Jan. 2010.

[140] A. Umar and T. A. Kunkel, "DNA-replication fidelity, mismatch repair and genome instability in cancer cells.," *European journal of biochemistry / FEBS*, vol. 238, no. 2, pp. 297–307, Jun. 1996.

[141] R. M. Pokorny, A. Hofmeister, S. Galandiuk, A. B. Dietz, N. D. Cohen, and H. L. Neibergs, "Crohn's disease and ulcerative colitis are associated with the DNA repair gene MLH1.," *Annals of surgery*, vol. 225, no. 6, pp. 718–23; discussion 723–5, Jun. 1997.

[142] G. Plotz, J. Raedle, A. Spina, C. Welsch, A. Stallmach, S. Zeuzem, and C. Schmidt, "Evaluation of the MLH1 I219V alteration in DNA mismatch repair activity and ulcerative colitis.," *Inflammatory bowel diseases*, vol. 14, no. 5, pp. 605–11, May 2008.

[143] S. I. Grivennikov and M. Karin, "Inflammatory cytokines in cancer: tumour necrosis factor and interleukin 6 take the stage.," *Annals of the rheumatic diseases*, vol. 70 Suppl 1, pp. i104–8, Mar. 2011.

[144] M. C. Fantini and F. Pallone, "Cytokines: from gut inflammation to colorectal cancer.," *Current drug targets*, vol. 9, no. 5, pp. 375–80, May 2008.

[145] L. Klampfer, "Cytokines, inflammation and colon cancer.," *Current cancer drug targets*, vol. 11, no. 4, pp. 451–64, May 2011.

[146] T. M. Long and J.-P. Raufman, "The diagnostic and prognostic role of cytokines in colon cancer," *Gastrointestinal Cancer:Targets and Therapy*, 2011.

[147] T. Tanaka, H. Kohno, R. Suzuki, Y. Yamada, S. Sugie, and H. Mori, "A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate.," *Cancer science*, vol. 94, no. 11, pp. 965–73, Nov. 2003.

[148] M. De Robertis and E. Massi, "The AOM/DSS murine model for the study of colon carcinogenesis: From pathways to diagnosis and therapy studies," *Journal of Carcinogenesis*, vol. 10, 2011.

[149] Y. Yan, "Temporal and Spatial Analysis of Clinical and Molecular Parameters in Dextran Sodium Sulfate Induced Colitis," *PloS one*, 2009.

[150] Y. Fan, R. Mao, and J. Yang, "NF-κB and STAT3 signaling pathways collaboratively link inflammation to cancer.," *Protein & cell*, vol. 4, no. 3, pp. 176–85, Mar. 2013.

[151] H. Knüpfer and R. Preiss, "Serum interleukin-6 levels in colorectal cancer patients--a summary of published results.," *International journal of colorectal disease*, vol. 25, no. 2, pp. 135–40, Feb. 2010.

[152] J.-P. Herbeuval, E. Lelievre, C. Lambert, M. Dy, and C. Genin, "Recruitment of STAT3 for production of IL-10 by colon carcinoma cells induced by macrophage-derived IL-6.," *Journal of immunology (Baltimore, Md. : 1950)*, vol. 172, no. 7, pp. 4630–6, Apr. 2004.

[153] S. Grivennikov, E. Karin, J. Terzic, D. Mucida, G.-Y. Yu, S. Vallabhapurapu, J. Scheller, S. Rose-John, H. Cheroutre, L. Eckmann, and M. Karin, "IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer.," *Cancer cell*, vol. 15, no. 2, pp. 103–13, Feb. 2009.

[154] Y. Ning and H.-J. Lenz, "Targeting IL-8 in colorectal cancer.," *Expert opinion on therapeutic targets*, Apr. 2012.

[155] Wikipedia, "TNFalpha." [Online]. Available: http://en.wikipedia.org/wiki/Tumor_necrosis_factor_alpha.

[156] M. M. Garrity-Park, E. V Loftus, S. C. Bryant, W. J. Sandborn, and T. C. Smyrk, "Tumor necrosis factor-alpha polymorphisms in ulcerative colitis-associated colorectal cancer.," *The American journal of gastroenterology*, vol. 103, no. 2, pp. 407–15, Feb. 2008.

[157] B. Yan, H. Wang, Z. N. Rabbani, Y. Zhao, W. Li, Y. Yuan, F. Li, M. W. Dewhirst, and C.-Y. Li, "Tumor necrosis factor-alpha is a potent endogenous mutagen that promotes cellular transformation.," *Cancer research*, vol. 66, no. 24, pp. 11565–70, Dec. 2006.

[158] B. K. Popivanova, K. Kitamura, Y. Wu, T. Kondo, T. Kagaya, S. Kaneko, M. Oshima, C. Fujii, and N. Mukaida, "Blocking TNF-alpha in mice reduces colorectal carcinogenesis associated with chronic colitis.," *The Journal of clinical investigation*, vol. 118, no. 2, pp. 560–70, Feb. 2008.

[159] R. Parsons, G. M. Li, M. J. Longley, W. H. Fang, N. Papadopoulos, J. Jen, A. de la Chapelle, K. W. Kinzler, B. Vogelstein, and P. Modrich, "Hypermutability and mismatch repair deficiency in RER+ tumor cells.," *Cell*, vol. 75, no. 6, pp. 1227–36, Dec. 1993.

[160] M. Koi, A. Umar, D. P. Chauhan, S. P. Cherian, J. M. Carethers, T. A. Kunkel, and C. R. Boland, "Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces N-methyl-N'-nitro-N-nitrosoguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation.," *Cancer research*, vol. 54, no. 16, pp. 4308–12, Aug. 1994.

[161] C. Gasche, C. L. Chang, L. Natarajan, A. Goel, J. Rhees, D. J. Young, C. N. Arnold, and C. R. Boland, "Identification of frame-shift intermediate mutant cells.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 4, pp. 1914–9, Mar. 2003.

[162] C. Campregher, T. Scharl, M. Nemeth, C. Honeder, T. Jascur, C. R. Boland, and C. Gasche, "The nucleotide composition of microsatellites impacts both replication fidelity and mismatch repair in human colorectal cells.," *Human molecular genetics*, vol. 19, no. 13, pp. 2648–57, Jul. 2010.

[163] C. Campregher, M. G. Luciani, and C. Gasche, "Activated neutrophils induce an hMSH2-dependent G2/M checkpoint arrest and replication errors at a (CA)13-repeat in colon epithelial cells.," *Gut*, vol. 57, no. 6, pp. 780–7, Jun. 2008.

[164] M.-S. Chang, B.-C. Chen, M.-T. Yu, J.-R. Sheu, T.-F. Chen, and C.-H. Lin, "Phorbol 12-myristate 13-acetate upregulates cyclooxygenase-2 expression in human pulmonary epithelial cells via Ras, Raf-1, ERK, and NF-kappaB, but not p38 MAPK, pathways.," *Cellular signalling*, vol. 17, no. 3, pp. 299–310, Mar. 2005.

[165] C. Gasche, C. L. Chang, J. Rhees, A. Goel, and C. R. Boland, "Oxidative stress increases frameshift mutations in human colorectal cancer cells.," *Cancer research*, vol. 61, no. 20, pp. 7444–8, Oct. 2001.

[166] B. C. Dickinson and C. J. Chang, "Chemistry and biology of reactive oxygen species in signaling or stress responses.," *Nature chemical biology*, vol. 7, no. 8, pp. 504–11, Aug. 2011.

[167] O. I. Aruoma, B. Halliwell, B. M. Hoey, and J. Butler, "The antioxidant action of taurine, hypotaurine and their metabolic precursors.," *The Biochemical journal*, vol. 256, no. 1, pp. 251–5, Nov. 1988.

[168] M. López-Lázaro, "Dual role of hydrogen peroxide in cancer: possible relevance to cancer chemoprevention and therapy.," *Cancer letters*, vol. 252, no. 1, pp. 1–8, Jul. 2007.

[169] B. Vogelstein and K. W. Kinzler, "Cancer genes and the pathways they control.," *Nature medicine*, vol. 10, no. 8, pp. 789–99, Aug. 2004.

[170] J. L. Hirpara, M. V Clément, and S. Pervaiz, "Intracellular acidification triggered by mitochondrial-derived hydrogen peroxide is an effector mechanism for drug-induced apoptosis in tumor cells.," *The Journal of biological chemistry*, vol. 276, no. 1, pp. 514–21, Jan. 2001.

[171] M. C. Symons, S. Rusakiewicz, R. C. Rees, and S. I. Ahmad, "Hydrogen peroxide: a potent cytotoxic agent effective in causing cellular damage and used in the possible treatment for certain tumours.," *Medical hypotheses*, vol. 57, no. 1, pp. 56–8, Jul. 2001.

[172] P. Chelikani, I. Fita, and P. C. Loewen, "Diversity of structures and properties among catalases.," *Cellular and molecular life sciences : CMLS*, vol. 61, no. 2, pp. 192–208, Jan. 2004.

[173] Wikipedia, "Cytokine." [Online]. Available: http://en.wikipedia.org/wiki/Cytokine.

[174] W. C. Burhans and M. Weinberger, "DNA replication stress, genome instability and aging.," *Nucleic acids research*, vol. 35, no. 22, pp. 7545–56, Jan. 2007.

[175] S. Reuter, S. C. Gupta, M. M. Chaturvedi, and B. B. Aggarwal, "Oxidative stress, inflammation, and cancer: how are they linked?," *Free radical biology & medicine*, vol. 49, no. 11, pp. 1603–16, Dec. 2010.

[176] A. J. Schetter, N. H. H. Heegaard, and C. C. Harris, "Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways.," *Carcinogenesis*, vol. 31, no. 1, pp. 37–49, Jan. 2010.

[177] D. J. J. Waugh and C. Wilson, "The interleukin-8 pathway in cancer.," *Clinical cancer research : an official journal of the American Association for Cancer Research*, vol. 14, no. 21, pp. 6735–41, Nov. 2008.

[178] L. Vuitton, S. Koch, and L. Peyrin-Biroulet, "Janus Kinase Inhibition With Tofacitinib: Changing the Face of Inflammatory Bowel Disease Treatment.," *Current drug targets*, Apr. 2013.

[179] M. B. Grisham and D. N. Granger, "Neutrophil-mediated mucosal injury. Role of reactive oxygen metabolites.," *Digestive diseases and sciences*, vol. 33, no. 3 Suppl, p. 6S–15S, Mar. 1988.

[180] M. Planck, E. Wenngren, A. Borg, H. Olsson, and M. Nilbert, "Somatic frameshift alterations in mononucleotide repeat-containing genes in different tumor types from an HNPCC family with germline MSH2 mutation.," *Genes, chromosomes & cancer*, vol. 29, no. 1, pp. 33–9, Sep. 2000.

[181] H.-S. Han, H. J. Chang, Y. S. Hong, S. Y. Kim, K. S. Lee, and K. H. Jung, "Epidermal growth factor receptor expression discrepancies in metastatic colorectal cancer patients treated with cetuximab plus irinotecan-based chemotherapy refractory to irinotecan and oxaliplatin.," *Diseases of the colon and rectum*, vol. 52, no. 6, pp. 1144–51; discussion 1152–3, Jun. 2009.

[182] V. J. Bubb, L. J. Curtis, C. Cunningham, M. G. Dunlop, A. D. Carothers, R. G. Morris, S. White, C. C. Bird, and A. H. Wyllie, "Microsatellite instability and the role of hMSH2 in sporadic colorectalcancer.," *Oncogene*, vol. 12, no. 12, pp. 2641–9, Jun. 1996.

[183] K. N. Chidambara Murthy, G. K. Jayaprakasha, and B. S. Patil, "Citrus limonoids and curcumin additively inhibit human colon cancer cells.," *Food & function*, vol. 4, no. 5, pp. 803–10, Apr. 2013.

[184] A. de Moreno de LeBlanc, J. G. LeBlanc, G. Perdigón, A. Miyoshi, P. Langella, V. Azevedo, and F. Sesma, "Oral administration of a catalase-producing Lactococcus lactis can prevent a chemically induced colon cancer in mice.," *Journal of medical microbiology*, vol. 57, no. Pt 1, pp. 100–5, Jan. 2008.

[185] J. X. Kang and K. H. Weylandt, "Modulation of inflammatory cytokines by omega-3 fatty acids.," *Sub-cellular biochemistry*, vol. 49, pp. 133–43, Jan. 2008.

[186] I. Vedin, T. Cederholm, Y. Freund Levi, H. Basun, A. Garlind, G. Faxén Irving, M. E. Jönhagen, B. Vessby, L.-O. Wahlund, and J. Palmblad, "Effects of docosahexaenoic acid-rich n-3 fatty acid supplementation on cytokine

release from blood mononuclear leukocytes: the OmegAD study.," *The American journal of clinical nutrition*, vol. 87, no. 6, pp. 1616–22, Jun. 2008.

[187] X. Li, L. Conklin, and P. Alex, "New serological biomarkers of inflammatory bowel disease.," *World journal of gastroenterology : WJG*, vol. 14, no. 33, pp. 5115–24, Sep. 2008.

[188] P. L. Foster, "Methods for determining spontaneous mutation rates.," *Methods in enzymology*, vol. 409, pp. 195–213, Jan. 2006.

[189] S. E. Luria and M. Delbrück, "Mutations of Bacteria from Virus Sensitivity to Virus Resistance.," *Genetics*, vol. 28, no. 6, pp. 491–511, Nov. 1943.

[190] Abcam, "Abcam Hydrogen Peroxide Assay Kit." [Online]. Available: http://www.abcam.com/ps/products/102/ab102500/documents/ab102500 Hydrogen Peroxide Assay Kit Protocol v2 (website).pdf.

[191] BioRad, "BioRad Bioplex Cytokine assay." [Online]. Available: http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4110004F.pdf.

[192] A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward, and D. Forman, "Global cancer statistics.," *CA: a cancer journal for clinicians*, vol. 61, no. 2, pp. 69–90.

[193] T. Jess, C. Rungoe, and L. Peyrin-Biroulet, "Risk of colorectal cancer in patients with ulcerative colitis: a meta-analysis of population-based cohort studies.," *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association*, vol. 10, no. 6, pp. 639–45, Jun. 2012.

[194] R. C. Langan, P. B. Gotsch, M. A. Krafczyk, and D. D. Skillinge, "Ulcerative colitis: diagnosis and treatment.," *American family physician*, vol. 76, no. 9, pp. 1323–30, Nov. 2007.

[195] M. D. Evans and M. S. Cooke, "Factors contributing to the outcome of oxidative damage to nucleic acids.," *BioEssays : news and reviews in molecular, cellular and developmental biology*, vol. 26, no. 5, pp. 533–42, May 2004.

[196] N. A. Yamada and R. A. Farber, "Induction of a low level of microsatellite instability by overexpression of DNA polymerase Beta.," *Cancer research*, vol. 62, no. 21, pp. 6061–4, Nov. 2002.

8. Anhang

8.1 Summary

Colorectal cancer (CRC) is the third most frequent cancer in men and women and ranks 4th in cancer mortality worldwide [65], [192]. The risk of developing CRC is increased in those patients suffering from chronic inflammatory conditions such as ulcerative colitis (UC) which is a disease of the large intestine that is noticeable by inflammations and ulcerations of the colon mucosa [193], [194]. It is characterized by the accumulations of polymorphonuclear neutrophiles (PMNs) and an increased transepithelial migration leading to the formation of crypt abscesses [103], [193]. Oxidative stress induced by degranulating PMNs is suggested to be one of the major contributing factors driving colon carcinogenesis by altering cellular components including proteins and DNA [195]. Activated PMNs do not only release reactive oxygen species (ROS), but also reactive nitrogen species (RNS), lactoferrin and other proteins including several cytokines [103]. Microsatellite instability (MSI) is a hypermutable phenotype caused by an impaired DNA mismatch repair (MMR) system [196] leading to frame-shift mutations in coding regions of cancer-related genes (e.g transforming growth factor β receptor type II) which may contribute to the development of colitis associated CRC (CAC) [122].

For this study we adapted a pre-established co-culture system [163] in order to simulate the mutagenic environment in UC and to check the stability of microsatellite sequences during PMN-induced stress. Therefore we utilized PMNs as effectors and frameshift-reporter cells as targets which were transfected with the pIREShyg2-EGFP/MS plasmid harboring mono-, di- and tetranucleotide repeats. Our working hypothesis was that exposure of reporter cells to secreted products from degranulating PMNs leads to replication errors and frameshift mutations within such microsatellites. Therefore our aim was to identify specific PMN-released molecules driving MSI in order to get a better insight of this disease and to develop specific inhibitors of colitis-associated CRC development.

8.2 Zusammenfassung

Darmkrebs ist die dritthäufigste Krebserkrankung bei Männern und Frauen und die 4th häufigste Todesursache weltweit. Colitis Ulcerosa ist eine chronisch entzündliche Darmerkrankung mit erhöhtem Darmkrebsrisiko. Die Krankheit ist gekennzeichnet durch eine Entzündung des Dickdarms und Ulzerationen der Darmschleimhaut. Häufig kommt es zu einer Anhäufung von polymorphkernige neutrophile Granuolzyten (PMN) und folglich zur Formation von sogenannten Kryptenabszessen. Es wird angenommen, dass Oxidativer Stress einer der treibenden Faktoren für die Entwicklung von Darmkrebs ist. Dieser Stress wird durch die Degranulation von aktivierten Neutrophilen induziert und kann zu Veränderungen zellulärer Komponenten wie zB Proteine und DNA führen. Es konnte bisher nachgewiesen werden, dass aktivierte PMNs nicht nur Reaktive Sauerstoff-Spezies (ROS), sondern auch viele andere potenziell DNA schädigende Faktoren wie zB Reaktive Stickstoff-Spezies (RNS), Lactoferrin und Cytokine freisetzen können. Mikrosatelliteninstabilität (MSI) ist ein hypermutabler Phänotyp und entsteht durch ein defektes DNA Reparatursystem was zu sogenannten „frame-shift“ Mutationen (Leserahmen-Mutationen) in kodierenden Regionen von Krebs-zusammenhängenden Genen (zB. transforming growth factor β receptor type II) führen kann und folglich zur Entwicklung von colitis-assoziierten Darmkrebs beiträgt.

Für diese Studie haben wir ein bereits etabliertes KoKultur System übernommen und adaptiert um das mutagene Milieu in Colitis Ulcerosa zu simulieren. Desweiteren wollten wir die Stabilität von Mikrosatellit-Sequenzen unter PMN-induzierten Stress testen. pIRESHyg2-EGFP/MS Plasmid transfektierte Darmepithelzellen wurden hierfür als Zielzellen und frisch isolierte PMNs als Effektorzellen verwendet. Unsere Hypothese war, dass EGFP-Reporter Darmepithelzellen, die ausgeschütteten Produkten von degranulierenden PMNs ausgesetzt werden, Replikationsfehler und frameshift Mutationen innerhalb dieser Mikrosatelliten entwickeln und diese biologischen Veränderungen gemessen werden können. Das Ziel dieser Studie war, mit Hilfe dieses Systems spezifische PMN assoziierte Moleküle, die mit MSI Entwicklung in Zusammenhang gebracht werden können, zu entdecken und einen besseren Einblick in die molekularen Hintergründe der Krankheit Colitis Ulcerosa zu bekommen.

8.3 Curriculum Vitae

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Publications / Abstracts:

Kyle W. Dammann, Vineeta Khare, Michaela Lang, **Nicolas Granofszky**, Christoph Gasche *"PAK1 Mediates NF-KB Signaling in Colitis and Colitis-Associated Cancer"*, Abstract DDW May 2013; Gastroenterology Vol. 144, Issue 5, Supplement 1, Page S-819