5-ASA - colorectal cancer - cell death : an intriguing threesome

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Chapter 1

General introduction and outline of this thesis

General introduction

Cancer of the large bowel, colorectal cancer (CRC), is the second cause of cancer-related death in the Western world, resulting in nearly 500,000 deaths each year worldwide [1]. In spite of better diagnostic possibilities most patients with CRC are beyond reasonable prospect of cure by the time therapy, if any, is instituted; 40-50% of the patients probably will die within 5 year (SEER Cancer Statistics Review USA, NCIN data briefing UK). The most important prognostic factor for patients suffering from CRC is the spread of the tumour at the time of diagnosis, as indicated by the Dukes' staging (*Figure 1*) [2]. Patients with a tumour that has not invaded the submucosa (Dukes' A) have a >90 % 5-year survival rate. Patients with a tumor that has invaded the muscularis propria, but has not spread to adjacent lymph nodes (Dukes' B) have a 70-80 % 5-year survival rate. When tumour cells have spread to adjacent lymph nodes (Dukes' C) or even metastasized to distant organs, (Dukes' D), 5-year survival rates drop to <50% and <10% respectively (SEER Cancer Statistics Review USA, NCIN data briefing UK).

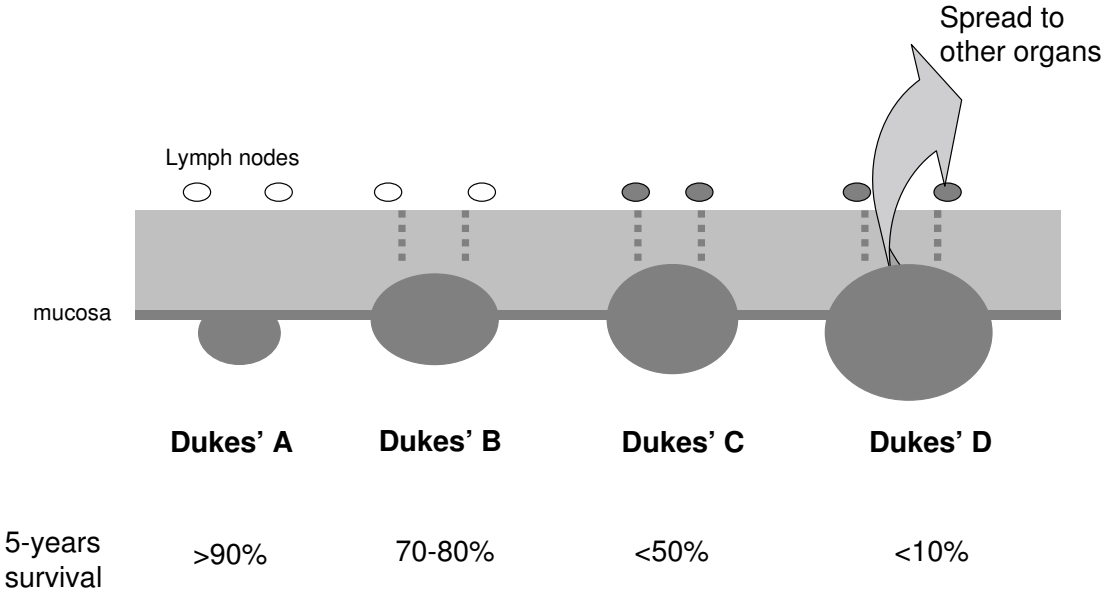

Figure1: Dukes' staging of the progression of CRC with 5-year surival rates indicated below.

The main primary treatment of CRC is removing the bowel segment in which the tumour is located. Despite efforts to improve surgery this is still not ideal, about 50 % of the patients develop recurrence after removal of the primary tumour. Patients are treated with radio- and chemotherapy, before and/or after resection of the primary tumour, but clinical/survival effects are limited [3]. Therefore, the prevention of CRC has become increasingly important, especially in people at higher risk.

Genetic and environmental factors

CRC most commonly occur sporadically, only a small percentage (~5%) occurs in the setting of well-defined inherited syndromes, like familial adenomatous polyposis (FAP) and heriditary-non-polyposis-colorectal cancer (HNPCC) or Lynch syndrome [4-6]. Both syndromes have an autosomal mode of inheritance with the development of CRC at a younger age. FAP individuals develop 100s-1000s adenomatous polyps in their intestine that ultimately progress to carcinoma. CRC is further determined by environmental exposures, i.e., physical inactivity, alcohol consumption, smoking and dietary components [7]. The high fat, low fiber Western type diet is believed to cause the increased incidence of CRC in the Western world [8, 9].

CRC development

Colorectal carcinomas are thought to arise from a precancerous lesion, the benign adenoma (polyp). Progression from a benign adenoma to a malignant carcinoma passes through a series of well-defined histological stages, accompanied by genetic alterations, known as the adenoma-carcinoma sequence (*Figure 2*) [10, 11].

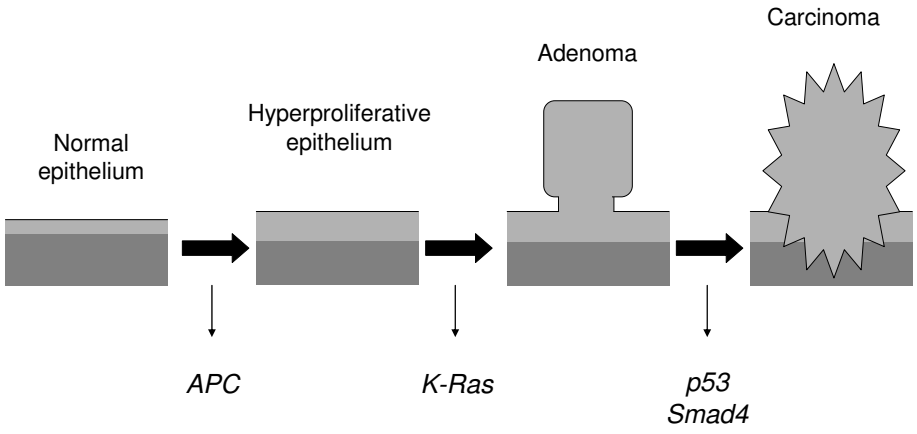

Figure 2: The adenoma-carcinoma sequence introduced by Fearon and Vogelstein [10]

Two different mechanisms of genomic instability give rise to CRC development, microsatellite instability and chromosomal instability. Chromosomal instability is mainly a consequence of genetic alterations that involve the activation of oncogenes (*K-Ras*) or the inactivation of tumor suppressors (*APC, p53*) and is mainly found in sporadic CRC. The inactivation of the adenomatous polyposis coli (*APC*) tumour suppressor gene is found as the initiating mutation in most sporadic colorectal cancers [12]. Patients with FAP have a germ-line mutation in this gene [13, 14].

Mutations in the DNA mismatch repair (MMR)-system result in a failure to repair errors that occur during DNA replication, resulting in an accumulation of frame-shift mutations in small repetitive non-coding sequences, called microsatellites [15, 16]. This microsatellite instability (MIS) is the hallmark of CRC in HNPCC patients, mainly resulting from mutations in one of the MMR genes: *MLH1*, *PMS2*, *MSH2* and *MSH6*, and is more frequently found in tumours of the proximal (right-sided) colon [17].

Wnt signaling

The canonical Wnt pathway is important in regulating multiple aspects of intestinal tissue homeostasis [18]. Upon activation of Frizzled receptors on the cell surface the β-catenin degradation complex, consisting of axins, APC and glycogen synthase kinase-3β (GSK-3β), is disrupted resulting in stabilization and nuclear accumulation of β-catenin. In the nucleus β-catenin binds to the T-cell factor-4 (TCF-4) transcription factor resulting in the transcriptional activation of Wnt/TCF4 target genes, including cyclinD1 and c-MYC (*Figure 3*) [19].

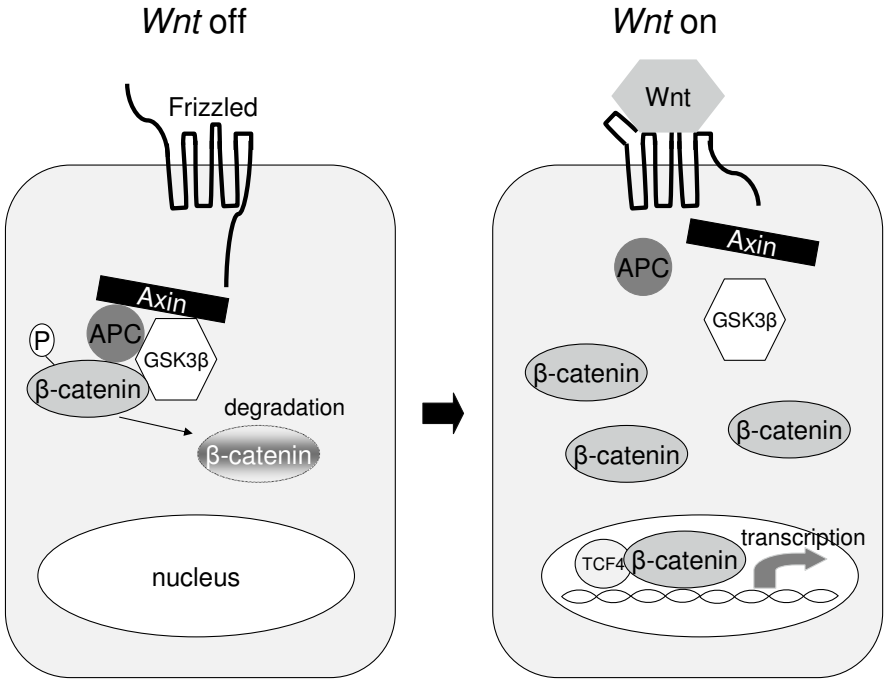

Figure 3: The canonical Wnt signalling pathway.

The majority of CRC is initiated by activating mutations in this pathway and either remove the tumour suppressors APC or axin or activate the proto-oncogene β-catenin, resulting in constant activation of the pathway [20].

Transforming Growth Factor-β signalling

The Transforming Growth Factor (TGF)-β superfamily of proteins consists of TGF-βs, Bone Morphogenetic Proteins (BMPs) and actividins, which play an important role in intercellular communication, cell proliferation, cell motility, functional differentiation and apoptosis [21, 22].

TGF-β binds to a heteromeric complex of transmembrane kinase receptors, TGF-βR-I and TGF-βR-II. Upon TGF-β binding to TGF-βR-II, TGF-βR-I is recruited to the receptor complex and transphosphorylated by TGF-βR-II. TGF-βRI, activin-receptor-like-kinase-5 (ALK-5), in turn phosphorylates the Smad regulatory proteins, Smad2 and Smad3, which associate with the co-Smad, Smad4, and translocate to the nucleus, interacting with other transcription factors, in a cell specific manner, to regulate a panel of TGF-β-responsive genes (*Figure 5*) [23]. Some of the downstream genes are important cell-cycle checkpoint genes, including the cyclin dependent kinase inhibitors p21 and p27 [24,25], which activation leads to a growth arrest. Therefore, TGF-β prevents progression through the cell cycle and induces apoptosis, acting as a tumour suppressor, in normal intestinal epithelium. Other downstream targets, like plasminogen activator inhibitor-1 (PAI-1) and metalloproteinases (MMPs), contribute to the progression of cancer [26,27], reflecting the dual role of TGF-β in cancer. Several mutations in the TGF-β signalling pathway, like in Smad4 and TGF-βR-II, contribute to CRC carcinogenesis, leaving most CRC resistant to TGF-β induced growth inhibition [28-30]. Because CRC usually show a high expression of TGF-β [31-33] it can act as a tumour promoter during the late stages of colorectal carcinogenesis, via promotion of tumour angiogenesis, increased production of extracellular matrix (ECM) and proteolytic enzymes, increased motility and immunosuppression [34]. TGF-β also drives differentiation of fibroblasts into myofibroblasts, which are abundantly present in colorectal carcinomas [35,36], important in the interaction with carcinoma cells in cancer progression and metastasis [37].

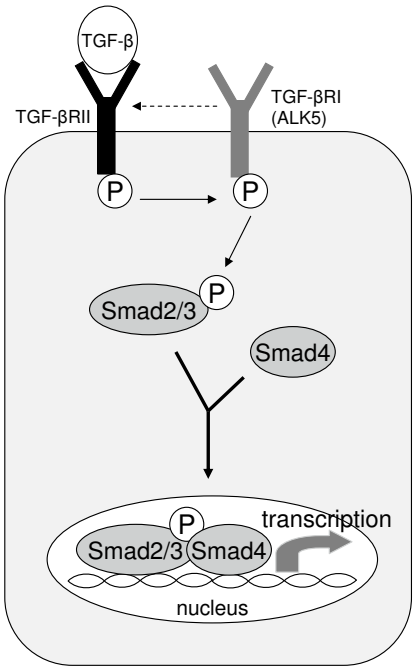

Figure 4: The TGF-β signalling pathway.

Regulated cell death

Programmed cell death, i.e., apoptosis, is an important mechanism to maintain tissue homeastasis as a counter balance of cell proliferation, especially in tissues with a high cell turnover like the intestine, in which the complete epithelial layer is renewed every 4-5 days. In normal intestinal crypts apoptosis is initiated at the top of the crypts, and cell proliferation is stimulated in the bottom of the crypt, by high intracellular Wnt signalling *(Figure 5)* [19,38].

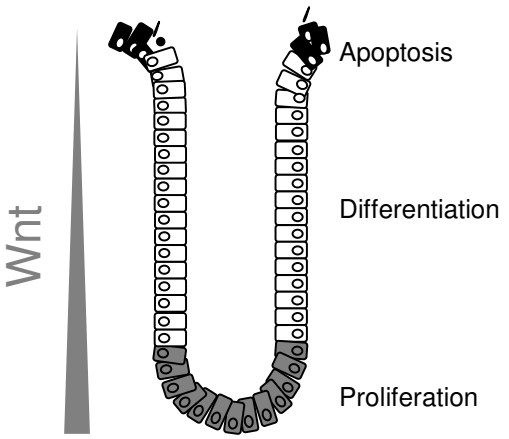

Figure 5: Normal intestinal crypt homeostasis.

The process by which a normal cell transforms in to a tumour cell can be by becoming apoptosis resistant [39]. Apoptosis is accompanied by several distinct morphological features, like chromosomal condensation, cytoplasmic schrinkage, and blebbing of the cell membrane (posphatidylserine externalization) [40], and biochemical features, like the activation of cys-dependent asp proteases, the caspases [41]. Neoplastic cells are usually less sensitive to apoptotic signals, due to deregulation of the two main apoptotic pathways, the extrinsic and intrinsic pathway [42,43]. Activation of surface FAS receptors triggers the extrinsic pathway, activating caspase-3 via activation of caspase-8 (*Figure 6*) [44,45]. In the intrinsic (or mitochondrial) pathway, intracellular stress (genomic stress, defects in DNA repair, replication and cell division) is detected by p53, and causes mitochondrial membrane potentiation, via pro-apoptotic proteins in the mitochondrional membrane (Bcl-2), resulting in the mitochondrional release/outflux of cytochrome-c. Cytochrome-c interacts, together with Apaf-1, in the cytosol activating caspase-9 and eventually caspase-3 [46]. So, both pathways activate the effector caspase-3, which is responsible for cleaving most cellular substrates during the apoptotic process [47].

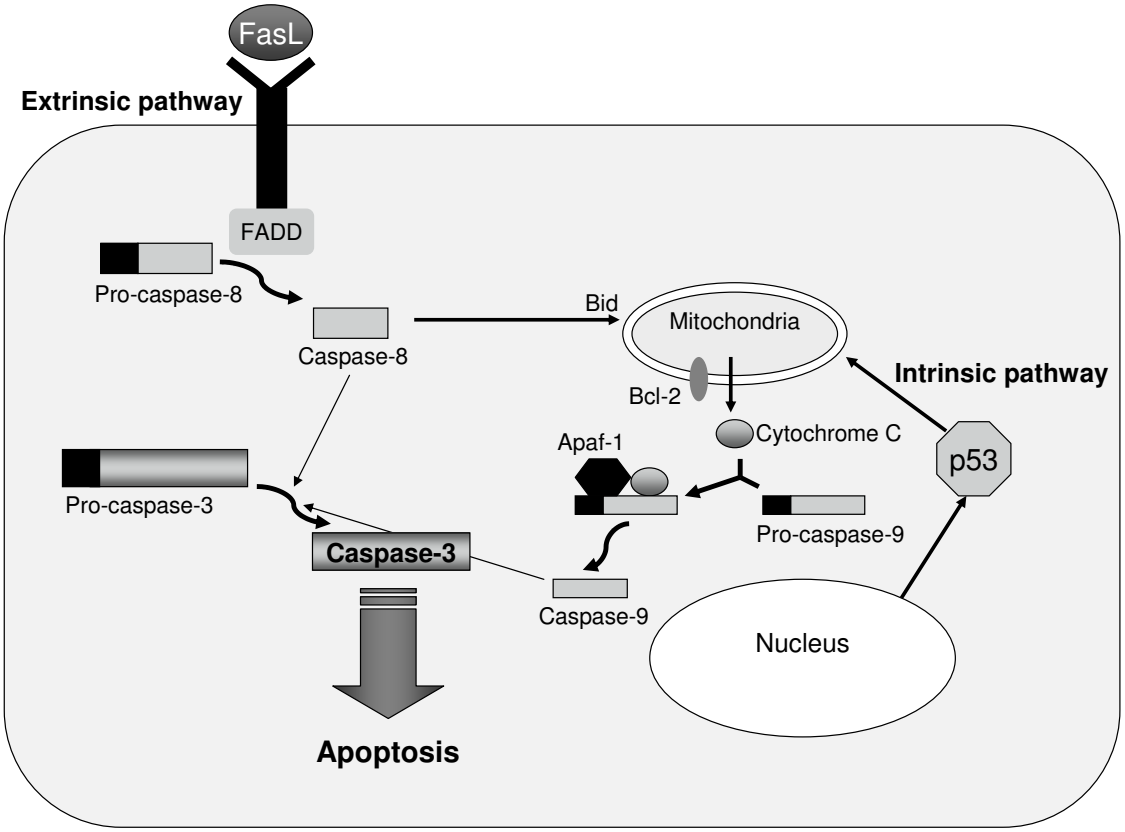

Figure 6: Intrinsic and extrinsic apoptotic pathways.

The prognostic value of apoptosis in CRC

The adenoma-carcinoma sequence is generally accompanied by a resistance to apoptosis [48,49]. Most studies show a relation between apoptosis and proliferation [50-53], which is heavily increased in neoplastic lesions, and therefore the apoptotic level of neoplastic lesions is also higher compared with normal. To evaluate whether tumour cell apoptosis levels have clinical relevance for the patients' outcome several studies have been done [50,54]. Apoptosis can be determined by counting the number of apoptotic tumour cells, either identified by morphological changes on haematoxylin and eosin (H&E) stained slides, but also by immunohistochemical stainings for DNA fragmentation [TdT-mediated dUTP-biotin nick end-labeling (TUNEL)] or caspase-3 degraded cytokeratin 18 (M30). These detection methods have different specificities, TUNEL is not specific for apoptotic epithelial cells, other apoptotic cells and even some necrotic cells with DNA fragmentation are also stained, while M30 immunohistochemistry specifically detects only apoptotic epithelial cells [55,56]. The prognostic relevance of the apoptotic index, as determined with these techniques, is not completely clear; some studies have found that low levels of apoptosis are associated with a

worse clinical outcome (disease recurrence or death), while others have found the opposite or no association [50]. Determination of total cellular apoptosis, by caspase-3 activity assay on CRC protein homogenates has shown significant clinical value for the patient in most studies [57-60], which might indicate the importance of non-epithelial apoptosis in the tumour, as further discussed in **chapter 2**.

Non-apoptotic cell death in tumourigenesis and colorectal cancer

Mitotic catastrophe is a form of cell death that results from an abnormal cell division. Non-proper chromosome segregation and cell division leads to the formation of large non-viable cells with multiple micronuclei which are the feature of mitotic catastrophe [61]. The observation that tumour cells are frequently deficient in cell-cycle checkpoints implies that they are particulary susceptible to the induction of mitotic catastrophe. It is indeed one of the main forms of cell death induced by radiation and chemotherapy in tumour cells [62,63]. The induction of mitotic catastrophe can play a role in tumour regression, as tumours that display a mitotic catastrophe response correspond to enhanced tumour regression after therapy [64]. The clinical value of mitotic catastrophe in general in CRC is unclear as research has been limited due to lack of specific markers.

Colitis-associated CRC

People suffering from the two main forms of chronic inflammatory bowel diseases (IBD), ulcerative colitis (UC) and Crohn's Disease (CD) are at increased risk of developing CRC [65, 66]. As CRC is responsible for up to 15% of all deaths in patients with IBD it is one of the most feared complications of IBD [67]. Several factors are known to increase the risk of CRC, including age of onset, duration and severity of colitis [68,69]. In contrast to sporadic CRC, the precancerous lesions in IBD-associated CRC can be polypoid, flat or diffuse. Detection of the dysplastic lesions by programmed colonscopy screening, followed by surgical resection, is the current approach for prevention of IBD-associated CRC [70-72].

CRC chemoprevention

Non-steroidal-anti-inflammatory-drugs (NSAIDs) are compounds used in the treatment of inflammatory conditions, like rheumatoid arthritis (RA) and IBD. The use of several of these NSAIDs, like sulindac and aspirin, has been found to reduce the CRC risk in large epidemiological, controlled patient, and animal studies [73-83]. Therefore, these NSAIDs have drawn attention as agents that might prevent the development of CRC, i.e.,

chemopreventive agents [79]. Unfortunately, most of these NSAIDs have severe side-effects, like intestinal ulcers, bleeding, perforation and renal toxicity [84-86], and are therefore not useful for a long-term treatment in a chemopreventive strategy to CRC. Moreover, the exact mechanisms by which these NSAIDs reduce CRC risk are unknown [80, 87, 88]. One of the main mechanisms is the inhibition of cyclooxygenase (COX) enzymes [89, 90]. These enzymes are involved in the synthesis of prostanglandin, a molecule involved in pain signalling and maintaining the gastrointestinal lining. COX-1 is present in most tissues as a housekeeper gene and maintains normal gastric mucosa and influences kidney function. COX-2 is inducible by inflammatory mediators, including cytokines, and is upregulated in colorectal adenomas and carcinomas [91-97]. The inhibition of COX-2 reduces inflammatory damage and contributes to the anti-inflammatory effects of NSAIDs, and is also believed to underlie the chemopreventive effect. However, the simultaneous inhibition of COX-1 is related to most of the side-effects of NSAID's [86, 98]. In the 1990s some very promising selective COX-2 inhibitors were developed, like celecoxib [99-101], which were unfortunately found to increase heart failure and strokes [102]. Because some NSAIDs have therapeutic effects besides COX inhibition [80, 87, 103, 104], their therapeutic targets remain to be elucidated and can be of value for the development of new treatment strategies.

Sulphasalazine and 5-aminosalicylic acid

Sulphasalazine has been used for the induction and maintenace of remission in IBD patients for decades [105]. Sulphasalazine is a conjugate of 5-aminosalicylic acid (5-ASA) and sulphapyridine (SP). In the late 1970s 5-ASA was found to be the active moiety in sulphasalazine [106, 107], and this was the starting point of the clinical use of monocomponent 5-ASA or mesalazine therapy. The mode of action of 5-ASA, however, is not complety understood [108]. The effect of 5-ASA is related to the intraluminal concentration of the drug [109]. It is transformed to the inactive acetylated 5-ASA by the intestinal cells and bacteria present [110, 111]. Most epidemiological studies strongly support a chemoporeventive effect of long-term use of 5-ASA in the development of CRC in UC patients, although some studies have failed to show a preventive effect, shown in *Table 1* [69, 112-122].

Table 1: Studies investigating effects of 5-ASA on CRC development in IBD patients.

Author, year	*Study*	*Patients*	*Cases*	*Outcome*	*Medication*	*OR (95 % CI)*
Pinczowksi, 1994	Case control	UC	102	Cancer	Sulphasalazine	**0.38 (0.20-0.69)**
Moody, 1996	Cohort	UC	10	Cancer	Sulphasalazine	**0.08 (0.02-0.29)**
Lashner, 1997	Cohort	UC	29	Both	Sulphasalazine / 5-ASA	0.95 (0.34-2.70)
Eaden, 2001	Case control	UC	102	Cancer	Sulphasalazine / 5-ASA	0.47 (0.22-1.00)
Lindberg, 2001	Cohort	UC	50	Cancer	Sulphasalazine	0.28 (0.06-1.42)
				Dysplasia		0.73 (0.25-2.10)
Bernstein, 2003	Case control	CD/UC	14/11	Cancer	5-ASA	1.22 (0.32-4.62)
Rubin, 2003[&]	Case control	UC	26	Cancer	5-ASA	**0.28 (0.09-0.85)**
Rutter, 2004	Case control	UC	68	Cancer	5-ASA	2.06 (0.61-6.94)
Van Staa, 2005	Case control	IBD	9	Cancer	Sulphasalazine / 5-ASA	**0.54 (0.35-0.86)**
		CD	15			
		UC	76			
Velayos, 2005[#]	Meta-analysis	IBD	-	Cancer	5-ASA	**0.51 (0.37-0.69)**
				Dysplasia		1.18 (0.41-3.43)
				Both		**0.51 (0.38-0.69)**
Velayos, 2006	Case control	UC	188	Cancer	5-ASA	**0.4 (0.2-0.9)**
Terdiman, 2007	Case control	IBD	364	Cancer	5-ASA	0.97 (0.77-1.23)

[&] *Abstract publication.* [#] *Velayos et al. 2005 used all the 9 studies reported before. Statistically different odds ratios (OR) of 5-ASA treated IBD patients are shown in bold.*

5-ASA is an attractive agent in a chemopreventive (and treatment) strategy with respect to CRC, as long standing experience with 5-ASA containing medication has learned that the drug is well tolerated without severe side-effects and gastrointestinal toxicity. The mechanisms behind the chemopreventative effect of 5-ASA are not completely understood, but were first thought to be related to the anti-inflammatory effects of 5-ASA, reducing damage to the colonic mucosa and thereby reducing the CRC risk. More recently, direct anti-cancer effects of 5-ASA have been described, including inhibitory effects on the Wnt pathway [123-137].

Aim and outline of the thesis

The main aim of the studies described in this thesis was to evaluate the effect of 5-ASA on the development of CRC. This thesis, therefore, focuses on the direct anti-cancer effects of 5-ASA on CRC cells *in vitro* and *in vivo*, particularly the induction of apoptosis. In addition, the effect of 5-ASA on the development of both sporadic and IBD-associated CRC was evaluated in a novel mouse model. Also the clinical impact of apoptosis in CRC patients was investigated.

The clinical impact of apoptosis in intestinal tissue on the behaviour of CRC, as described in **Chapter 2,** shows that low levels of CRC apoptosis is an important risk factor in these patients. The products of cell death go into the circulation and the levels measured in plasma of CRC patients are correlated to tumour progression and clinical outcome, as reported in **Chapter 3**. The effects of 5-ASA on CRC cell proliferation and cell death *in vitro* are described in **Chapter 4**. The study on the apoptosis inducing effect of 5-ASA on colorectal tumour cells in human patients *in vivo* is described in **Chapter 5.** The *in vitro* studies described in **Chapter 6** relate to the regulatory effect of 5-ASA on the TGF-β pathway in CRC cells and colonic fibroblasts. A novel mouse model to study CRC was developed and also used in combination with an intestinal inflammation model, in order to evaluate the effect of 5-ASA treatment on the development of both sprodadic and IBD-associated CRC, as described in **Chapter 7**. **Chapter 8** describes another aspect of 5-ASA, that is its modulatory effect on radiotherapy of CRC cells *in vitro*. The observations of the different studies are finally compiled in a summarizing discussion (**Chapter 9**).

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