The influence of inflammatory mediators on the expression of OATPs in colorectal cancer cells as a possible target for the therapy

Marian Bolis

Magistra der Pharmazie (Mag.pharm.)

Wien, 2016 / Vienna, 2016

A 449

Diplomstudium Pharmazie

Ao. Univ.-Prof. Mag. Dr. Walter Jäger
Acknowledgment

First, I would like to express my thanks to Ao. Univ.-Prof. Mag.pharm. Dr. rer.nat. Walter Jäger (Department of Pharmaceutical Chemistry, Division of Clinical Pharmacy and Diagnostics, University of Vienna) for giving me an opportunity to gain experience in this research field and for his encouragement.

Furthermore, I would like to express my sincerest gratitude to my supervisor Ao. Univ-Prof. Mag.pharm. Dr. Theresia Thalhammer (Institute of Pathophysiology and Allergy Research, Medical University of Vienna) for her permanent support throughout my work time and for reviewing my thesis. She has been answering my questions patiently, helping me with her advice and always provided a kind working atmosphere.

Moreover, my great appreciation goes to Lukas Klameth, MSc, for teaching me all methodological skills required for my research and for his constant supportive role. In addition to that, I am grateful for Ms. Erika Bajna, technical assistant, teaching me the necessary techniques for immunohistochemical stainings. I also thank for her help with the TissueFAXS® and HistoQuest® software.

My colleagues at the Institute of Pathophysiology and Allergy Research also deserve special thanks for providing a kind and motivating atmosphere.

Dedication

I would like to express my profound gratitude to my siblings and my parents for their ceaseless support throughout my study years. Their endless help, motivation, strength, encouragement and confidence in me allowed me to get further in order to reach a new peak in my educational career. Therefore, I dedicate this work to my parents.
# List of Contents

Abstract.................................................................................................................I

Abstract (German).................................................................................................III

1. Introduction...........................................................................................................1

   1.1. Colorectal cancer..............................................................................................1
       1.1.1. Definition..................................................................................................1
       1.1.2. Anatomy, Histology and Function .........................................................1
       1.1.3. Epidemiology .........................................................................................2
       1.1.4. Pathogenesis .........................................................................................3
       1.1.5. Risk factors .........................................................................................10
       1.1.6. Prevention ............................................................................................13
       1.1.7. Clinical symptoms ...............................................................................18
       1.1.8. Staging/ Tumor classification ...............................................................18
       1.1.9. Prognosis .............................................................................................21
       1.1.10. Therapy ..............................................................................................22

   1.2. OATP-Transporters..........................................................................................25
       1.2.1. Structural features..................................................................................25
       1.2.2. Classification and Nomenclature ..........................................................25
       1.2.3. Distribution and Function ...................................................................27
       1.2.4. Expression in cancer tissue ...................................................................29

2. Aim of the Study .................................................................................................33

3. Materials and methods.....................................................................................35

   3.1. Cell Culture....................................................................................................35

   3.2. Cell treatment.................................................................................................38

   3.3. RNA-Isolation...............................................................................................39

   3.4. Reverse Transcription....................................................................................41

   3.5. Quantitative Real Time PCR (qPCR)............................................................42
       3.5.1. TaqMan Method......................................................................................44

   3.6. Indirect Immunofluorescence Staining.......................................................46
       3.6.1. TissueFAXS®/ TissueQuest®.................................................................48

   3.7. Indirect Immunohistochemistry Staining.....................................................49
       3.7.1. HistoQuest®...........................................................................................51
4. Results

4.1. Measurement of OATP4A1 mRNA expression in the colorectal cell line HT-29

4.1.1. mRNA- Expression of OATP4A1 in HT-29 cells after IL-6 treatment

4.1.2. mRNA- Expression of OATP4A1 in HT-29 cells after TNFα treatment

4.1.3. mRNA- Expression of OATP4A1 in HT-29 cells after treatment with IL-6 and TNFα

4.2. Measurement of OATP1B3 mRNA expression in the colorectal cell line HT-29

4.2.1. mRNA- Expression of OATP1B3 in HT-29 cells after IL-6 treatment

4.2.2. mRNA- Expression of OATP1B3 in HT-29 cells after TNFα treatment

4.2.3. mRNA- Expression of OATP1B3 in HT-29 cells after a combination treatment of both IL-6 and TNFα

4.3. Indirect Immunofluorescence staining of Ht-29 cells after treatment

4.3.1. OATP4A1 in cells after IL-6 treatment

4.3.2. OATP4A1 in cells after TNFα treatment

4.3.3. OATP4A1 in cells after treatment with IL-6 and TNFα

4.4. Indirect Immunohistochemistry staining of colorectal cancer tissue

5. Discussion

5.1. Role of inflammatory mediators in CRC

5.1.1. Interleukin-6 (IL-6)

5.1.2. Tumor necrosis factor - α (TNF- α)

5.1.3. Effect of IL-6 and TNF-α on OATP expression

5.2. Role of anti-inflammatory agents in CRC therapy

List of Figures

List of Tables

References
Abstract

**Background:** Colorectal Cancer is among the most diagnosed malignant diseases worldwide. Among other transport proteins, which could be important for colorectal cancer progression, members of the organic anion transporter polypeptide family (OATPs) were found to be expressed in normal and cancerous colon tissue including colorectal cancer. They are responsible for cellular uptake of endo- and xenobiotics. The question whether their expression pattern of OATP4A1 can be used as diagnostic factor or whether they may influence the therapeutic effects of anticancer drugs should be examined. Furthermore, the impact of inflammatory mediators on the expression of OATPs should be examined.

**Methods:** The expression of OATP4A1 in colorectal cancer cell line HT-29 after exogenous addition of TNF-α and IL-6 is determined both on the mRNA- and protein level. OATP4A1 levels are measured after 6, 12, 24 and 48 hours of treatment with either TNF-α or IL-6 or both inflammation mediators. The effect of TNF-α and IL-6 on the expression of OATP1B1 on mRNA-level is examined as well. The presence of immune cells adjacent to the tumor cells is analysed as well.

**Results:** After addition of IL-6, an increase of the expression rate of OATP4A1 was seen after 6, 24 and 48 hours of treatment on the mRNA- as well as protein level. TNF-α addition caused the highest expression rate of OATP4A1 protein after 24 hours of treatment. Both mediators caused a remarkable elevation of the OATP4A1 expression after 24 and 48 hours of treatment on mRNA-as well as protein-level. IL-6 resulted in a moderate raise of the OATP1B1 expression on mRNA-level while TNF-α caused a higher increase. A significant elevation of the expression of OATP1B1 on mRNA-level was noticed after the addition of both mediators. The staining of human biopsies samples for OATP4A1 demonstrated that it was more pronounced in immune cells surrounding cancer cells than in the stroma.

**Conclusion:** The presence of the inflammatory mediators IL-6 and TNF-α have an impact on the expression mode of OATP4A1 and OATP1B1 in the colorectal cancer cell line HT-29. The influence pro-inflammatory tissue factors on the OATP expression should be examined in future studies, because these factors might be therapeutic targets.
Abstract (German)

Einleitung: Das kolorektale Karzinom zählt zu den am häufigsten diagnostizierten malignen Erkrankungen weltweit. Transportproteine, aus der Familie der Organischen Anionen Transporter (OATPs) werden sowohl im normalen als auch im kanzerösen Gewebe exprimiert. Als Memranproteine sind sie für die Aufnahme von Endo- und Xenobiotika in die Zelle verantwortlich. Es sollte untersucht werden, ob ihre Expressionsrate als Faktor in der Diagnostik verwendet werden kann bzw. ob ihre Expression therapeutische Wirkungen von Arzneistoffen beeinflusst. Weiters soll die Wirkung von Entzündungsmediatoren auf die Expressionsrate der OATPs untersucht werden.

Methoden: Die Expression von OATP4A1 in der kolorektalen Zelllinie HT-29 nach der Zugabe von TNF-α und IL-6 wird auf mRNA- und Proteinebene bestimmt. Die Messungen werden nach 6, 12, 24 und 48 Stunden der Behandlung mit TNF-α oder IL-6 alleine, oder mit der Kombination der beiden Entzündungsmediatoren, gemessen. Der Effekt von TNF-α und IL-6 auf die Expression von OATP1B1 auf mRNA-Ebene wird ebenfalls untersucht. Im Divertikulitisproben von Patienten erfolgt die Analyse von OATP4A1 in Immunzellen, welche die Tumorzellen umgeben.


Conclusio: Zugabe der Entzündungsmediatoren IL-6 und TNF-α erhöht die Expression von OATP4A1 und OATP1B1 in der kolorektalen Zelllinie HT-29. Die Auswirkungen der erhöhten Expressionsrate, welche durch die Entzündungsfaktoren
bedingt ist, könnten therapeutische Targets darstellen und sollten weiter untersucht werden.
1. Introduction

1.1. Colorectal cancer

1.1.1. Definition

Any non-benign tumor which starts in the colon or the rectum is classified as colorectal cancer. Both, colon and rectum belong to the gastrointestinal system which consists of esophagus, stomach, small and large intestine.

![Anatomy of the colon](image)

Figure 1: Anatomy of the colon

It demonstrates the four sections of the large intestine: the ascending colon, the transverse colon, the descending colon and the sigmoid colon. [1]

1.1.2. Anatomy, Histology and Function

The colon counts as the biggest part of the large bowel and is about five to six feet long and ends with the rectum. This muscular tube is divided in four sections: the ascending colon, the transverse colon, the descending colon and the sigmoid colon. Its key role includes water and salt extraction from solid wastes before elimination from the human body. The colonic wall is made up of four layers: the mucosa, submucosa, muscularis propria, and serosa. The mucosa forms the innermost layer where abnormal cell growth mostly starts. As a connective layer the submucosa is located between the muscularis mucosae and the muscularis propria. The muscularis propria takes responsibility for contractility and the serosa is the outermost layer covered by mesothelial cells. [2], [3]
**Figure 2: The Histology of the colonic wall**

Mainly, it consists of four layers: the mucosa, submucosa, muscularis propria and serosa with thin connective tissue in-between. [4]

As a terminal part of the gastrointestinal tract the rectum is about 10 to 12 cm long on average. It begins within the sigmoid colon and ends with the anus. The temporary storage of faeces before elimination causing expansion of the rectal walls leading the stretch receptors to stimulate the urge for defecation counts as the mean function of the rectum. The histological structure of the rectum is similar to the colon concerning layers which are made up from non-keratinising, non-ciliated columnar epithelium. The mucosa forms rectal columns which terminate at the anorectal junction. [5],[6],[7]

Abnormal cell proliferation whether in colon or rectum occurs mostly in one of the layers and reaches some or all layers.

1.1.3. Epidemiology

Colorectal cancer is globally as well as in Austria the second most diagnosed cancer in females and the third in males although the incidence proportion in men is higher than in women. In 2008, there were over 1.2 million new colon cancer cases discovered worldwide and 608,700 deaths occurred. Among these patients 436,000 cases were diagnosed and 212,000 experienced CRC-related death in Europe. The burden of this disease is mainly higher in industrialised countries according to lifestyle-associated risk factors such as obesity and the lack of physical activity as well as of balanced diet. In Europe, the rate is slightly higher in west and north than in the south and east. [8],[9],[10],[11]
CRC constitutes the third place of men and the second of women newly diagnosed with cancer. In the ranking as a cause of death CRC takes the fourth place for men and the third for women. Source: GLOBOCAN 2008 [11]

The risk of acquiring colorectal cancer increases with age. According to Austrian statistics more than 70% of patients are older than 65 years. In general, the chance of survival depends on the time of detection and the stage of the tumor. Although the relative survival rate in Austria reaches about 87% for patients with local malignancy without any involvement of lymph nodes, it is only 13% for those with metastasis. In some western countries the incidence of colorectal carcinoma has been decreasing because of the early diagnosis resulting from the increasing awareness of the society. In Austria, as an example, the chances of a survival increased from 14% to 26% between 1987 and 2007. Patients with metastasis are an exception. As stated in the Austrian statistical report 2014 regarding the cancer incidence and mortality, colorectal tumors might be early detected through screenings such as colonoscopy preventing the expansion of the carcinoma. Another benefit would be the early recognition of polyps which can get removed before they develop a cancerous growth. [12]

1.1.4. Pathogenesis

The slow development of the cancerous expansion over decades without any observable symptoms is regarded as the danger of this disease. In the majority of cases, benign polyps in the colonic or rectal mucosa transform from non-cancerous tissue to malignant carcinoma based on several pathways. Polyps appear on the
surface of the lining of colon or rectum caused by abnormal growth of epithelial cells. The two histological kinds of polyps are adenomatous and hyperplastic polyps. Hyperplastic lesions are usually harmless because of their small size and their classification as non-neoplastic polyps. Adenomatous polyps, also called adenomas, are categorized as tubular, villous or tubulovillous due to specific histological aspects. Serrated polyps which were counted before as hyperplastic polyps due to the similar morphology, however, are described nowadays as a mix of the hyperplastic and adenomatous type with malignant potential. Two precursor lesions, sessile serrated and traditional serrated adenomas, have recently been established as subgroups of serrated polyps with different locations and characteristics. [13],[14],[15],[16],[17]

An increase in the number of adenomatous lesions is associated with a higher risk of colorectal cancer. The development of carcinoma depends on the size, shape and the histology of the adenoma. Villous adenomas, as an example, are linked to higher morbidity and mortality among all types of lesions. [15],[18],[19]

CRC is classified into two different types, the sporadic and more frequent type and the familial one, which relies on the genetic background of patients. Both forms have various molecular features and genetic failures which define each category.

1.1.4.1. Sporadic Colorectal Cancer
The non-hereditary malignancy is initiated by accumulation of genetic mutations causing chromosomal instability, several modifications of the DNA and tumor initiation and tumor growth in the end. In the majority of cases, multiple allelic imbalances were observed inducing loss of heterozygosity. This indicates high rates of occurrence of allelic losses at specific chromosomal regions. This is described as the classical pathway whereas another mechanism of tumor development is known based on the inactivation of specific genes which maintain the normal DNA replication. [20],[14]

1.1.4.1.1. The classical genetic pathway for sporadic CRC
Approximately 85% of the sporadic colorectal cancer is described by the adenoma-carcinoma sequence with a primary bi-allelic mutation of the adenomatous polyposis of coli (APC) tumor suppressor gene as an initiating event. The APC gene is located
on chromosome 5q and is responsible for cell growth regulation and apoptosis. It triggers the progression of adenomas as a result of homozygous somatic mutations leading to the activation of the Wnt signalling pathway. Consequentially, β-catenin dependent genes are activated and the coding protein promote cell differentiation and proliferation. Even so, further genetic alterations are necessary for the malignant transition. [21],[22]

Another mutation affects the proto-oncogene, k-ras, on chromosome 12 which has a vital function as an encoder for a protein that transduces extracellular signals to the nucleus. Specific changes of the oncogene are discovered in more than 50% of large polyps and colorectal cancers. This suggests its role as promoter for the intermediate stage of carcinogenesis. [22],[23],[24]

An additional genetic alteration influences the DCC (deleted in colon cancer) gene which is located on chromosome 18q. Its physiologic function in addition to tumor suppression comprises regulation of neural cell adhesion and acceleration of apoptosis. Additionally, deletions of two main genes on chromosome 18q, namely SMAD-2 and SMAD-4, which show tumor suppression activity by affecting the TGF-beta signalling pathway. The loss of these genes is connected with the transformation of intermediate to late-stage adenomas. [20],[22],[23]

An inactivation of the suppressor gene p53 via mutations is considered as critical for the transition of the late-stage adenoma to the early malignant tumor. The loss of normal p53 function as regulator of the G1 cell cycle and apoptosis is induced by a missense point mutation of one allele and the deletion of the other. [23],[25]

This complicated and multifactorial process results in an accumulation of various genetic abnormalities leading to transformation of normal mucosa to malignant tissues. Due to the numerous chromosomal aberrations incorporated into carcinogenesis, this pathway is known as the chromosomal instability pathway. [17],[22],[26]
Figure 4: Key molecular events associated with the chromosomal instability pathway

The alteration of normal colonic mucosa to early adenoma is triggered by the inactivation of the APC (adenomatous polyposis coli) tumor suppressor gene, afterwards mutations of the proto-oncogene k-ras cause the transition to an intermediate adenoma. The deficiency of the genes DCC (deleted in colon cancer) and SMAD-2&-4 (portmanteau word from MAD- mothers against decapentaplegic and SMA- small body size) leads to the development of late-stage adenoma. The main promoter to the growth of carcinoma is the mutation and loss of suppressor gene p53.

1.1.4.1.2. DNA mismatch repair deficient sporadic CRC

The remaining cases (about 10-15% of sporadic tumors) arise from a distinctive genetic pathway, which is also involved in the pathogenesis of hereditary CRC. The alternative way is characterized as the microsatellite instability (MSI) phenotype. [27],[28]

Microsatellites are short repetitive sequences all over the genome whereat replication errors often appear during DNA replication. Normally, these mistakes are corrected by the mismatch repair system. This corrective system is regulated by several genes, such as MLH1, MSH2 or MSH6, which recognize amplification errors to repair them specifically. Mutational inactivation of these genes results in the loss of DNA repair mismatch repair function. The hypermethylation of CpG islands of the promoter region MLH1 leads to epigenetic modifications to transcriptional silencing of the repair-mismatch genes. CpG islands are clusters of cytosine-guanosine residues, in which
approximately 50% of promoters of all genes are included. The aberrant methylation of cytosines at these loci induces epigenetic silencing of gene expression and relates to a phenomenon called the CIMP (CpG island methylator phenotype). Not only the \textit{MLH1}-gene but also other tumor suppressor gene promoters, e.g. insulin like growth factor 2 receptors (\textit{IGF2R}), transforming growth factor beta receptor II (\textit{TGFBR2}) or BCL2-associated X protein (BCL) are affected by methylation. [16],[27],[28],[29],[30]

Approximately half of the cases of sporadic colorectal carcinoma with MSI show BRAF point mutations as well. These mutations activate the serine-threonine kinase resulting in initiating the mitogen-activated protein kinase (MAPK) signalling pathway. Consequently, the cascade is not turned off and leads to a continuous proliferation. [16],[31]

In summary, sporadic CRC with MSI relies on the CIMP background and has additionally BRAF mutations. Due to the observed increasing methylation with advanced age, these tumors occur mostly in older patients. Gender is also a factor among MSI-positive patients, so females are more likely to be diagnosed with this type. The poor differentiation, lymphocytic infiltration and location at the proximal colon are counted among the distinctive features. This category is associated with better prognosis and drug-resistance towards 5-fluorouracil. [27],[30],[32],[33],[34]

\textbf{Figure 5: Main events involved in the initiation of CRC with MSI}

The CpG islands belonging to the promotor region of the \textit{MLH1}-gene are hypermethylated conducting a microsatellite instability (MSI). This disorder causes the loss of DNA repair mismatch function. Mutations of the proto-oncogene BRAF and the hypermethylation of insulin like growth factor 2 receptors (\textit{IGF2R}), transforming growth factor beta receptor II (\textit{TGFBR2}) or BCL2-associated X protein (BCL) contribute CRC’s development.
1.1.4.2. Hereditary colorectal cancer syndromes

Colorectal cancer occurrence is linked in >20% of the cases to inherited genetic mutations. Individuals are more likely to develop CRC if family members have been diagnosed with this disease. The possibility of cancer expansion increases with the degree of relatedness, which means that persons with affected first-degree relatives are at the doubled risk compared to the general population. Therefore the family history should be considered as a risk factor in the anamneses. [28],[35]

The two relevant syndromes are familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC).

1.1.4.2.1. Hereditary non-polyposis colon cancer (HNCPP)

HNCPP is an autosomal dominant disease, which is recognized in 2-4% of hereditary colorectal cancer patients. The misleading name gives the impression of non-existing adenomas, although usually a small number for up to 100 are found in these patients. Also widely known as Lynch syndrome, appears at younger age and tumors arises in the right colon. Although transformation of small adenomas into malignant tissue takes about 8-10 years for most cases, it may be reduced to 2-3 years in HNCPP. [28],[35],[36]

Significant features such as lymphocytic infiltration and poor differentiation are similar to those manifested in the DNA mismatch repair deficient sporadic CRC. For that reason the identification is more complex due to the overlapping characteristics. Lynch syndrome is caused by germline mutations of genes involved in DNA mismatch-repair system, so more or less all patients with HCNPP have microsatellite instability. The absence of BRAF mutations in this specific cancer type is considered as the main difference between sporadic CRC with microsatellite instability and the lynch syndrome. The genes MSH2 and MLH1 are more frequently altered than MSH6, PMS1 and PMS2 and make up about 90% of mutations in Lynch syndrome families. Due to accumulation of mutations within the genome based on MSH2 inactivation, the risk of developing cancer increases for CRC and also for extracolonic tumors. Accompanied malignancies include endometrium, stomach, small bowel, hepatobiliary tract, pancreas and brain tumors. [37],[38],[39]
For risk evaluation clinical classification guidelines were generated to help diagnosing HNPCC allowing a better surveillance and an appropriate management. The “Amsterdam criteria” were established for research intentions in order to improve discovering homogenous genetic conditions of Lynch syndrome families. The criteria comprise the following points: a) at least three relatives are affected with HNPCC-related CRC or an extracolonic malignancy. One has to be a first-degree family member, b) diagnosis of CRC in a person before the age of 50 years, c) CRC manifestation at least in two generations in a row, d) exclusion of familial adenomatous polyposis, e) tumors should be verified by pathological examination. The aim of “Bethesda guidelines” is to determine which individuals should be examined for MSI. According to these guiding principles people are eligible for initial screening for MSI if a) the patient is younger than 50 years at the time of cancer diagnosis, b) CRC was diagnosed in at least one first-degree relative with HNPCC-associated tumor with one of the cases was diagnosed before the age of 50, c) high-level microsatellite instability was histologically recognized in a CRC patient younger than 60 years, d) CRC was detected in two or more first-degree or second-degree family members going along with Lynch-syndrome-connected carcinoma regardless of age. [40],[41],[42] A new category is specified as Type X familial colorectal cancer which fulfils the terms of the Amsterdam criteria but without any molecular genetic features. These patients lack both germline mutations and MSI in their tumors. The carcinogenesis pathway of this tumor type is not clear yet. The major differences between Lynch syndrome and this cancer type is the late onset of the disease and the lower risk for relatives. [43],[44]

1.1.4.2.2. Familial Adenomatous Polyposis (FAP)
The autosomal dominant inheritance of mutations in the APC gene is the origin of FAP, which was previously named Polyposis coli. Approximately 1% of all CRC cases are connected with this disorder, which induces the proliferation of cells to hundreds to thousands benign adenomas. The number of polyps depends on the location of the mutation within the APC gene, which determines the severity and manifestation of the cancer. Accumulation of additional mutations leads to the inactivation of the tumor suppressor gene p53 and the progression of the polyposis into the malignancy. The development of lesions starts at an early age, mostly in the puberty, and occurs in
various areas of the large bowel. Without clinical awareness, regular medical surveillance and treatment, the non-malignant tissue will gradually transform into cancer presenting at the age of 35-45. [45]

Deletions of few bases or premature truncations alter the APC gene, which is located on chromosome 5q. This causes the characteristic phenotypic features of FAP. The significant substrate for the interaction with the APC gene is β-catenin. High β-catenin levels are responsible for stimulation of cell proliferation and they influence cell adhesion. The APC protein antagonizes the effect of β-catenin by blocking cell growth and promoting apoptosis. Through inactivating mutations of the APC gene, the degradation of β-catenin is blocked and this leads to high intracellular concentrations of β-catenin resulting in the formation of adenomas. An important aspect are the extra-colonic manifestations of which FAP. Patients often suffer from retinal lesions, osteomas, hepatoblastoma, adrenal adenoma, duodenal or ampulary polyps, desmoid disease, brain tumors, thyroid cancer and other disorders, although the incidence of developing other cancers among FAP patients is low. [20],[46],[47]

1.1.5. Risk factors

In many cases exogenous factors interact with the genetic features to promote carcinogenesis. People with a familial background have higher tendency to develop cancer according to the same lifestyle conditions. Nearly all non-genetic risk factors mentioned in various studies are accompanied by controversial statements because of the difficulty of the evaluation. Therefore, lifestyle-related factors as described below- are defined by experts and might increase the risk of CRC. But they are actually linked to an increased risk for several other cancer types as well.

1.1.5.1. Lifestyle factors

With regard to CRC, lifestyle factors play a role because a higher occurrence of CRC is seen in western nations when compared with those in developing countries. [48]
➢ Dietary patterns

The analysis of dietary practices of patients is quite complex. It is nearly impossible for researchers to trace the dietary regimes of affected persons after cancer diagnosis. To assess the association between the diet and the risk of colorectal cancer, the amount, frequency and sort of food consumed in 20 to 30 years before the discovery of the disease and not only at the time of diagnosis should be examined. However, the consumption of red and processed meat as well as saturated fatted acids might correlate with a higher incidence of colorectal tumors. It is claimed that high fat might induce oxidative stress in colon leading to inflammation. [49],[50]

➢ Obesity

Overweight and obese patients (BMI>25 and 30) are more likely to develop colorectal tumors but a correlation between body mass index and the burden of this disease has not been established yet. The increased risk appears to be gender-specific because obese men are more affected. [51]

➢ Physical activity

Colonic but not rectal tumors are suggested to occur more often in physical inactive individuals. The antioxidant defence system is enhanced through exercise because of the modulation of the MAPK and NFκB pathways causing an improved cell defence. [49],[52]

➢ Alcohol and Smoking

Excessive alcohol consumption is estimated to increase the risk of CRC compared to non- or occasional alcohol intake. The higher danger is linked to the daily number of drinks, but not to the type of alcohol beverage. [53]

Smoking is also expected to relate to the widespread presence of adenomatous polyps after an induction period of three to four decades. [54]
1.1.5.2. Other Risks
There are additional factors which have an effect on the prevalence of CRC, e.g.:

➢ Age
Age has an influence on the development of non-hereditary colorectal carcinoma. As stated before, the incidence of the disease is high among older human beings. A possible reason might be the increased production of free radicals during aging, inducing alterations and changes of sensitive cellular mechanisms. As a consequence the predisposition for several diseases including cancer expands with ageing. [49]

➢ Family history and inherited syndromes
Inherited genes and the common circumstances of family members have a significant role regarding the susceptibility to CRC. The presence of affected relatives with adenomatous polyps or cancer is connected to a higher risk.

➢ Inflammatory bowel diseases
Ulcerative colitis and Crohn’s disease are considered as the main chronic inflammatory bowel diseases (IBD). The risk of cancer initiation for patients with the burden of IBD is higher than for the general population. The increased risk is connected to the duration of the chronic diseases. The relation between inflammatory diseases and CRC was studied in mouse models. The results show that both, the toll-like receptors and tumor necrosis factor-α activate the NFκB pathway generating the transcription of tumorigenesis-associated genes. Mutations of the suppressor gene p53 trigger the progress of mild dysplasia to carcinoma. IBD- related cancer is characterized by numerous of features such as the general spread at the time of diagnosis, the lower age of patients and the poor prognosis. The major question concerns the management of IBD and whether the medication can provide a sufficient protection against the malignant transition. [55]
- **Previous malignancies**

  Individuals with preceding tumors such as lymphoma, testicular cancer or endometrial cancer have an increased risk as the consequence of genetic alterations or of the treatment of the first carcinoma. [56]

- **Metabolic syndrome**

  The link between the risk for CRC and the metabolic syndrome is not fully elucidated yet. The fact that this disease includes a variety of metabolic aberrations makes it difficult. Abdominal obesity, elevated blood pressure, diabetes mellitus and dyslipidaemia are a clusters of metabolic abnormalities which define the metabolic syndrome. Independently, some of these irregularities may raise the incidence of CRC. [57]

- **Ethnical background**

  Ashkenazi Jews as an ethnic group suffer from CRC more than any other population. Gene mutations in I1307K are supposed to be the reason of the high incidence. [58],[59]

1.1.6. **Prevention**

There are various strategies recommended for prevention of CRC. Lifestyle risk factors should be avoided using plans for a healthy encouragement for the general population and especially for the high risk groups. This should be supported by other disease-preventing programs in order to identify individuals with a genetic predisposition. Regular screenings would enable early detection of adenomas before their transformation to non-benign lesions.

1.1.6.1. **Primary prevention**

The earliest prevention methods consider basically lifestyle factors just as diet and drinking habits, physical activity and smoking. A potential relation was established
between some drugs, e.g. Aspirin, and an inverse risk of CRC. In any case, a healthy way of life is beneficial for all general conditions, not only for cancer prevention.

- **Diet**

A contribution to a decreased risk might be provided through a diet rich in vegetables and fruits. It is suggested that the Mediterranean diet habits may offer profitable effects towards CRC risk because of their regular consumption of vegetables, fruits, nuts, fish, cereals and their frequent usage for olive oil as a source of lipids. Furthermore, the protective role of fibre supplements against CRC seems to be highly debatable. [60],[61]

Micronutrient antioxidants such as vitamins A, C and E, flavonoids, polyphenols or β-carotene together with protease inhibitors, calcium, vitamin D and folate might also reduce the incidence of colorectal cancer. A number of studies demonstrate benefits of the above mentioned compounds. Antioxidants stimulate signalling pathways leading to maintenance of the regular cell cycle. Vitamin E shows antiproliferative effects in cancer cell lines, higher intake of vitamin A and C and calcium prevent the recurrence of adenoma polyps as a consequence of their assumed efficacy of reducing cell replication in the intestinal mucosa. Few trials show that levels of pre-diagnostic 25-(OH)-Vitamin D concentrations are inversely associated with the risk of colorectal cancer. Nevertheless, further investigations are needed to bring a stronger evidence that an additive intake of these supplements leads to a significant risk minimization. [62],[63],[64],[65]

- **Drugs**

Nonsteroidal anti-inflammatory drugs (NSAIDs) as a widely-used medication are believed to lower the CRC risk. NSAIDs affect the cell-cycle by reducing cell proliferation and promoting apoptosis. The proposed mechanism of action relating to the suppression of adenoma formation involves the inhibition of cyclooxygenase (COX) enzymes, which are necessary for the generation of prostaglandins, thromboxane and prostacyclin. The COX-1 isoform is classified as a “house-keeping gene”, which is mainly responsible for the synthesis of THXA₂ causing platelet activation and aggregation. The isozyme COX-2 is upregulated in CRC resulting in an enhancement
of prostanoids production. The COX-2-derived prostaglandin E$_2$ takes part in pathogenic processes contributing to the initiation of cancer. The pro-inflammatory PGE2 plays a role in the stimulation of tumor growth via an increased cell adhesion and resistance to apoptosis. [66],[67],[68]

Aspirin as a non-selective COX-inhibitor remains the most analysed NSAID concerning colorectal cancer prevention. An evidence for potential profit could be provided for Aspirin, though investigators do not agree on the dosage to achieve valuable results. Some researchers claim that recognizable protective effects are shown in doses ≥300mg/ daily for at least five years. Other trials demonstrate a reduction of the 20-year risk of CRC by 24% with a low-doses of aspirin (75-500 mg/d). The inhibition of the transformation of normal mucosa to adenoma is suggested by a low-dose acetylsalicylic acid. According to further studies the treatment duration is of particular importance and a greater advantage is associated with a regular use. Clinically, cardiovascular disease patients seem to benefit more from the aspirin intake than the high-risk CRC patients regarding the colorectal cancer prevention. [67],[69]

Sulindac- a newer non-and selective NSAID- was found to cause the regression of adenomas in FAP patients without preventing their occurrence or affecting the elimination. [70]

The selective inhibitors of COX-2, Celexocib and Rofecoxib, may make a contribution to the decreased recurrence of colorectal adenomas.[70]

However, NSAIDs-related adverse effects should be taken into account before a recommendation for CRC prevention. The compounds might cause dose-dependent life-threatening events, primarily gastrointestinal and intracranial bleeding. After all, several clinical points still remain to be determined if the benefits outweigh the side effects or not. The most appropriate age to start a treatment, the effective duration of the application and the optimal dosage for a preventive schedule need to be better defined.

1.1.6.2. Secondary prevention

Various screening tests allow the early detection of polyps and early stage cancers making these diagnostic strategies the relevant options for colorectal cancer
prevention in asymptomatic individuals. Humans older than 50 years without other risk factors for developing CRC belong to the medium-risk population. [70],[71]

Screening strategies need to fulfil some criteria before extensive utilization in population programs. The measure of usefulness comprises sufficient specificity and sensitivity of the test, adequate reduction in CRC incidence and mortality, acceptance by the population and low cost. The three most frequently used tests are faecal occult blood test (FOBT), colonoscopy and sigmoidoscopy. [71]

In general, there are differences in screening programs concerning the test intervals and the age of the target group. In Europe, similar strategies are implemented. Most countries recommend FOBT as a screening method every two years. In case of positive results, colonoscopy should be conducted for confirmation. [72]

> **Faecal Occult Blood Test (FOBT)**

Based on the damage of the blood vessels existing on the surface of colorectal polyps, sporadic blood in stool can be detected by FOBT. For this home test, two samples from three consecutive stools are required and then sent to a laboratory for processing. There are two types of this stool examination: the guaiac test (gFOBT) and the immunologically based one (FIT). Both methods are widely used and extensively studied. The gFOBT is not specific to human haemoglobin, therefore some dietary limitations are necessary for higher specificity. In order to evade false positive results, drugs such as NSAIDs and Vitamin C supplements should be avoided. However, some investigators do not support any food restriction. Dependent on the different gFOBT offered on the market, clinically reliable results of sensitivity and specificity are not proven. [28],[71],[73],[74]

The immunologically based test FIT uses monoclonal or polyclonal antibodies which are specific for human haemoglobin. Additional to non-existing dietary restrictions, fewer samples are required for the examination. For that reason, FIT is better accepted by the population than gFOBT. The problem of this test concerns the storage in refrigerator because of possible changes of the results through exposure to high temperatures. Similar to gFOBT, the sensitivity and specificity in various investigations
is variable. Nevertheless, referring to the detection results and the participation rate, FIT is superior to gFOBT. [71],[75],[76],[77]

The false positive or negative outcome either from gFOBT or FIT remains as the leading problem of this screening method. [71]

➢ Colonoscopy

Colonoscopy is the method of choice for the diagnosis of colorectal polyps both in high and average risk target population. Significant reduction of the CRC mortality and incidence is demonstrated in various studies. However, this screening test is regarded as uncomfortable. Bowel cleansing preparation including specific dietary restriction, 48 hours before examination, anxiety to sedation or to the invasive character of the exploration and possible serious complications count as the main reasons for the low acceptance from the general population. [78],[79],[80]

Colonoscopy presides over all other screening methods presenting the best results in both sensitivity and specificity. Additionally, the examination provides consequential information on the size, number and location of polyps. Important clues about the malignant potential of existing polyps are offered at colonoscopy as well. Therefore, colonoscopy is categorized as the gold standard test for the observation of any pathologic events in the bowel and rectum. [14],[71],[81]

Current recommendation involve a colonoscopy every ten years for the average risk population. Surveillance intervals for patients with genetic predisposition should be minimized. [71]

➢ Sigmoidoscopy

With reference to the variety of scanning methods, advantages and disadvantages are always set side by side to offer the best surveillance for the medium and high risk population. The main benefits of sigmoidoscopy are the reduced requirement of a complicated preparation and the performance without an intake of a sedative medication. The great limitation of this test is its ability of detecting neoplasms exclusively in the distal colon. Furthermore, the sensitivity is inferior to that of
colonoscopy. For these reasons, its role in screening and diagnosis of CRC is more and more limited. A one-time screening for average-risk population aged between 55 and 64 years may cause a reduction in the CRC incidence and mortality. [14],[71],[82]

1.1.7. Clinical symptoms
The difficulty of early diagnosis of CRC is the lack of specific clinical symptoms. In the majority of cases, CRC is discovered at advanced stages. Potential predictive values for early detection are abdominal pain, change in bowel habits, rectal bleeding (hematochezia) and melena. Weight loss, bloating and anaemia are among the frequent symptoms reported to be associated with this cancer type. The essence of the complication is the heterogeneity of clinical trials which leads to the absence of statistical and evidential conclusions. [83],[84],[85]

1.1.8. Staging/ Tumor classification
The American Joint Committee on Cancer (AJCC) has developed a classification system for cancer staging. Based on the cancer location and spread in the body, staging systems are designed to help physicians selecting an effective treatment and predicting survival. TNM stands for Tumor, Node and Metastasis, and the TNM system is widely used for colorectal cancer classification. T describes the extent of the tumor through the layers of the colon and rectum. N stands for the appearance or non-appearance of metastasis in nearby lymph nodes. M indicates the extension of metastasis at distant organs. [4],[86]

This categorization is divided in two types:
- cTNM describes the clinical classification which is made after the physical examination, imaging tests, and biopsies of affected areas.
- pTNM represents the pathologic classification which is determined after removal surgery of the cancer. It summarizes the outcome of both the clinical staging as well as the surgery results.

The AJCC committee seeks the update of the staging classification schemes for better evaluation of cancer control measures resulting in more advantageous survival rates.
### Table 1: The TNM staging system for CRC classification-7th edition

<table>
<thead>
<tr>
<th><strong>Primary tumor (T)</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Primary tumor cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ: intraepithelial or invasion of lamina propria</td>
</tr>
<tr>
<td>T1</td>
<td>Tumor invades submucosa</td>
</tr>
<tr>
<td>T2</td>
<td>Tumor invades muscularis propria</td>
</tr>
<tr>
<td>T3</td>
<td>Tumor invades through the muscularis propria into the pericolorectal tissues</td>
</tr>
<tr>
<td>T4a</td>
<td>Tumor penetrates to the surface of the visceral peritoneum</td>
</tr>
<tr>
<td>T4b</td>
<td>Tumor directly invades or is adherent to other organs or structures</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Regional lymph nodes (N)</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NX</td>
<td>Regional lymph nodes cannot be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1</td>
<td>Metastasis in 1-3 regional lymph nodes</td>
</tr>
<tr>
<td>N1a</td>
<td>Metastasis in 1 regional lymph node</td>
</tr>
<tr>
<td>N1b</td>
<td>Metastasis in 2-3 regional lymph nodes</td>
</tr>
<tr>
<td>N1c</td>
<td>Tumor deposit(s) in the subserosa, mesentery, or non-peritonealised pericolic or perirectal tissues without regional nodal metastasis</td>
</tr>
<tr>
<td>N2</td>
<td>Metastasis in 4 or more lymph nodes</td>
</tr>
<tr>
<td>N2a</td>
<td>Metastasis in 4-6 regional lymph nodes</td>
</tr>
<tr>
<td>N2b</td>
<td>Metastasis in 7 or more regional lymph nodes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Distant metastasis (M)</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
<tr>
<td>M1a</td>
<td>Metastasis confined to 1 organ or site (e.g. liver, lung, ovary, non-regional node)</td>
</tr>
<tr>
<td>M1b</td>
<td>Metastasis in more than 1 organ/site or the peritoneum</td>
</tr>
</tbody>
</table>

The level of tumor extent in the colonic and rectal layers is characterised through T. N describes the existence of metastasis in nearby lymph nodes. M stands for the expansion of metastasis at distant organs.
Another well-known classification system according to Duke is also established as a staging system. The classification is not as precise as the TNM-system: it consists of only four stages which describe the extension of carcinoma spread in the body. [87]

Table 2: The staging system due to Duke

<table>
<thead>
<tr>
<th>Dukes A</th>
<th>The tumor is limited to mucosa and submucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dukes B</td>
<td>The tumor already infiltrates all layers</td>
</tr>
<tr>
<td>Dukes C</td>
<td>Local Lymph nodes</td>
</tr>
<tr>
<td>Dukes D</td>
<td>Distant metastasis</td>
</tr>
</tbody>
</table>

The combination of both staging systems allows a better evaluation of the extension of the carcinoma.

Table 3: A combined classification scheme based on the TNM- & Duke staging system

<table>
<thead>
<tr>
<th>TNM stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
<th>Dukes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
<td></td>
</tr>
<tr>
<td>Stage IIA</td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
<td>B</td>
</tr>
<tr>
<td>Stage IIB</td>
<td>T4a</td>
<td>N0</td>
<td>M0</td>
<td></td>
</tr>
<tr>
<td>Stage IIC</td>
<td>T4b</td>
<td>N0</td>
<td>M0</td>
<td></td>
</tr>
<tr>
<td>Stage IIIA</td>
<td>T1-T2</td>
<td>N1/N1c</td>
<td>M0</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>N2a</td>
<td>M0</td>
<td></td>
</tr>
<tr>
<td>Stage IIIB</td>
<td>T3-T4a</td>
<td>N1/N1c</td>
<td>M0</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>T2-T3</td>
<td>N2a</td>
<td>M0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T1-T2</td>
<td>N2b</td>
<td>M0</td>
<td></td>
</tr>
<tr>
<td>Stage IIIC</td>
<td>T4a</td>
<td>N2b</td>
<td>M0</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>T3-T4a</td>
<td>N2b</td>
<td>M0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T4b</td>
<td>N1-N2</td>
<td>M0</td>
<td></td>
</tr>
</tbody>
</table>

Stage I in the TNM-system matches up Duke’s A, whereas Duke’s B keeps up with Stage II. All subgroups of Stage III are equal to Duke’s C.
Table 4: A combined classification scheme based on the TNM- & Duke staging system - Continuation

<table>
<thead>
<tr>
<th>Stage</th>
<th>T</th>
<th>N</th>
<th>M1a</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage IVA</td>
<td>Any T</td>
<td>Any N</td>
<td>M1a</td>
<td>D</td>
</tr>
<tr>
<td>Stage IVB</td>
<td>Any T</td>
<td>Any N</td>
<td>M1b</td>
<td></td>
</tr>
</tbody>
</table>

Surprisingly Duke’s D is not linked to Stage IV of the TNM-classification system. [86]

1.1.9. Prognosis

The survival of cancer patients depends at first line on the timing of diagnosis. For colorectal cancer the tumor location also plays a key role regarding prognosis. The presence or absence of clinical symptoms is claimed to be involved in the survival rate.

Statistical results as stated below are on the basis of the sixth edition (Stage IV is not divided into subgroups) system defined by AJCC. Representative data about Surveillance, Epidemiology, and End Results (SEER) is used for assessment of survival rates linked to the defined stages. The 5-year survival rate of patients diagnosed with colon cancer between 1991 and 2000 is presented in the following table. [88]

Table 5: Statistical 5-years survival rate for patients due to their clinical and pathological stage

<table>
<thead>
<tr>
<th>Stage</th>
<th>5-year survival rate in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>93.2%</td>
</tr>
<tr>
<td>Stage IIA</td>
<td>84.7%</td>
</tr>
<tr>
<td>Stage IIB</td>
<td>72.2%</td>
</tr>
<tr>
<td>Stage IIIA</td>
<td>83.4%</td>
</tr>
<tr>
<td>Stage IIIB</td>
<td>64.1%</td>
</tr>
<tr>
<td>Stage IIIC</td>
<td>44.3%</td>
</tr>
<tr>
<td>Stage IV</td>
<td>8.1%</td>
</tr>
</tbody>
</table>

The chances of survival is influenced by the time of diagnosis and the pathological stage of the carcinoma. Therefore advanced stages are associated with reduced survival rate but stages IIB and IIIA.
The table shows a surprising statistic about patients staged IIB, which have a significantly worse prognosis than those in stage IIIA. This effect might relate to the therapy concept of these patients.

Patients with tumors located in the sigmoid colon have a slightly improved 5-year survival (69.8%) than those with tumors in the left colon (65.1%), transverse colon (65.0%) and right colon (63.7%). [88]

Asymptomatic patients have a lower 5-year survival rate than those who developed symptoms. This might associate with the time of diagnosis and the stage. [85]

1.1.10. Therapy

The treatment strategies for colorectal cancer basically depend on the stage of disease at diagnosis. The common proceeding after detection is the surgical intervention. The choice of whether a total resection, laparoscopic colectomy, polypectomy, local excision, transanal endoscopic microsurgery or any other type of surgery is determined by the size and extent of spread of the tumor and involvement of regional lymph nodes. After surgery, a systematic treatment with chemotherapeutic agents is often necessary to improve the outcomes. [89]

There are different treatment regimens which are approved by the FDA. As first-line therapy, the IFL scheme consisting of irinotecan, bolus fluorouracil, and leucovorin is used. Another scheme, abbreviated FOLFIRI, includes irinotecan, infusional fluorouracil, and leucovorin is also applied as first-line therapy. The FOLFOX regime containing oxaliplatin, infusional fluorouracil, and leucovorin is put to use as first- and second-line therapy. For the management of advanced colorectal tumors biologicals are used as adjuvant therapy. [90]

1.1.10.1. Chemotherapy

Fluorouracil

The thymidylate synthetase inhibitor, a fluorinated pyrimidine, remains as the cornerstone of the treatment of colorectal cancer. In order to stabilize the binding between fluorouracil and its enzyme, it is usually administered with a reduced folate,
leucovorin. The combination of both agents leads to an enhancement of the inhibition of the DNA synthesis, thereby to a size reduction of the tumor and prolonging the survival time. The method of administration has a major impact on the occurrence of side effects. The most common adverse effects after administration of bolus fluorouracil and leucovorin for five days in a row every four to five weeks are neutropenia and stomatitis. Diarrhea is more frequent in regimens of weekly bolus doses of fluorouracil and leucovorin. A continuous infusion of fluorouracil is less involved with hematologic and gastrointestinal toxic effects. This way of administration is significantly associated with higher incidence of palmar-plantar erythrodysesthesia, also called “hand-foot syndrome”. [90],[91]

- **Oral Fluoropyrimidines**

After intact absorption of capecitabine, the oral prodrug, through the gastrointestinal mucosa, it undergoes the enzymatic conversion to fluorouracil in three steps. The side effect profile is similar to that seen after protracted infusion. In order to avoid the catabolic enzyme of fluorouracil, the dihydropyrimidine dehydrogenase, located in the mucosa, another regime is developed. Tegafur uracil combines an oral fluoropyrimidine (tegafur) with a DPD inhibitor (uracil) allowing thereby an improvement of absorption and bioavailability. [90],[91]

- **Irinotecan**

Irinotecan is a prodrug which is converted to its metabolite SN-38 by hepatic carboxylesterases. The semi-synthetic derivative of the natural alkaloid camptothecin inhibits the enzyme topoisomerase I. SN-38 stabilizes the DNA breaks caused by the topoisomerase I enzyme leading thereby to DNA fragmentation and cell apoptosis. Diarrhea, myelosuppression, nausea, vomiting and alopecia are among the frequent adverse events associated with irinotecan. [90],[91]
Oxaliplatin

The third-generation platinum compound is structurally described as diaminocyclohexane platinum derivative. It induces programmed cell death by forming DNA adducts which results in an impaired DNA replication. A co-administration of oxaliplatin with fluorouracil and leucovorin is beneficial. The downregulation of thymidylate synthase by oxaliplatin causes a synergistic efficacy. The primary toxic effect is neuropathy, especially the sensory type. The paresthesias of the hands and feet diminishes after the cessation of treatment. [90],[91]

1.1.10.2. Biologicals

Another strategy for the treatment of CRC includes the inhibition of neoangiogenesis and the new formation of new blood vessels by inhibiting the vascular endothelial growth factor (VEGF). The proliferation of blood vessels is stimulated by VEGF. Its receptor is a transmembrane glycoprotein which is overexpressed in CRC and is associated with poorer prognosis. [90],[91]

Bevacizumab

The humanized monoclonal antibody is directed against VEGF. In advanced colorectal cancer Bevacizumab is combined with the different treatment regimens. A significant increase in the rate of response and prolongation of median survival rate is apparent after addition of the antibody to the IFL and FOLOFOX schemes. The most common adverse effects observed are reversible hypertension and proteinuria. [90],[91]

Cetuximab

The monoclonal antibody is directed against the extracellular binding domain of the epidermal growth factor receptor. The addition of Cetuximab to chemotherapeutic agents demonstrate a therapeutic synergism. In advanced CRC, cetuximab overcomes the resistance of tumor cells towards irinotecan. The main toxicities associated with cetuximab are acne-like rash, hypomagnesemia and in some cases hypersensitivity infusion reactions. [90],[91]
1.2. OATP-Transporters

The Organic Anion Transporting Polypeptides (OATP) are classified as members of the “Solute Carrier for organic Anions” (SLCO) gene superfamily. As corresponding proteins of the SLCO transporter family, OATPs are expressed either specifically in a single organ or ubiquitously.

1.2.1. Structural features

Based on the structure prediction by computational analysis, OATPs have 12-transmembrane domains. Six extracellular and five intracellular loops with intracellular C- and the N- termini separate the helices. The transport activity is assumed to be determined by the fifth loop. The formation of disulfide bonds as a result of the alignment with cysteines -contained in the sequence- affect the surface expression. [92], [93], [94]

OATPs contain a Kazal-2-type serine protease inhibitor (Kazal_SLC21) domain, located as well in the extracellular fifth loop region. The secreted proteins influence physiological processes such as blood coagulation, inflammation and immune response. [92]

Six N-terminal and six C-terminal helices around the central pore of OATP1B3 and OATP2B1 are responsible for the formation of a positive electrostatic pore which enables the transport of anions. [92]

For the plasma membrane localization of OATP1A2, OATP3A1 and OATP1C1 an interaction with the C-terminal PDZ consensus domain seems to be essential. [95]

The “OATP superfamily signature” is characterised by a length of 13 amino acids. The specific sequence “D-X-RW-(I, V)-GAWW-XG-(F, L)-L” is at the borderline between the extracellular third domain and the transmembrane domain 6. [96]

1.2.2. Classification and Nomenclature

Due to the increasing number of identified OATP, there is urge for a systematic nomenclature to keep track of the discovered members. The old classification system-
which shown in the figure below- is based on the organ existence and causes confusion.

**Figure 6: Old nomenclature system of the human and rodent OATP/Oatp Family**

Human OATPs (blue) are lettered in capital letters. Rodent Oatps (yellow) star with an initial capital followed by small letters. Mouse proteins (orange) are written with “m” for differentiation between mouse and rat Oatps. [96]

Hagenbuch et al. established a new nomenclature system analogue to the classification model of the Cytochrome P450 enzymes. The HUGO Gene Nomenclature Committee approved the usage of the new scheme where the OATP superfamily is divided into families and subfamilies in correlation with the homology of the amino acid sequences. OATPs/Oatps are categorized within the same family if they share ≥40% amino acid sequence identities. As a result, human OATPs are classified in six families which are grouped in subfamilies sharing ≥60% amino acid sequence identities. [96]

**Figure 7: New nomenclature of the OATP/Oatp superfamily**

Proteins with amino acid sequence identities of more than 40% belong to the same family (OATP1-OATP6). The categorization into a subfamily (OATP1A-C; OATP2A-B etc.) is based on the identities of proteins of more than 60%. The eleven human OATP are highlighted in blue while rodent proteins in yellow and Mouse Oatps in orange. [96]
1.2.3. Distribution and Function

The transporter proteins mediate the sodium-independent uptake of a variety of structurally unrelated endo-& xenobiotics. OATP substrates include bile salts, hormones and their conjugates, bilirubin and its glucuronides, various drugs and toxins. The following table gives an overview of the organ distribution of OATPs and their identified substrates.

Table 6: List of OATPs with their organ distribution and substrates

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organ distribution</th>
<th>Identified substrates</th>
</tr>
</thead>
</table>
| OATP1A2   | Liver, Blood-BRAIN border, Kidney, Cholangiocytes | **Bile acids**- Cholate  
**Bilirubin**  
**Steroid hormones conjugates** (Estradiol-17β-glucuronide, DHEA-S)  
**Thyroid hormones** (T3, T4, rT3)  
**Prostaglandins** (E2)  
**Drugs:**  
• β-Blocker- Atenolol  
• Statins- Pravastatin  
• Antibiotics- Ciprofloxacin  
• Antiretroviral drugs- Saquinavir  
• Chemotherapy- Imatinib, Methotrexate |
| OATP1B1   | Liver              | **Bile acids**- Cholate  
**Bilirubin**  
**Steroid hormones conjugates** (Estradiol-17β-glucuronide, DHEA-S)  
**Thyroid hormones** (T3, T4, rT3)  
**Prostaglandins** (E2)  
**Drugs:**  
• Statins- Atorvastatin  
• ARBs- Valsartan  
• ACE-Inhibitors- Enalapril  
• Antiretroviral drugs- Lopinavir  
• Antibiotics- Cefazolin, Rapamycin, Rifampicin  
• Chemotherapy- Methotrexate, Flavopirido  
• Others- Bosentan, Troglitazone, Ezetemibe, Caspofungin |
Table 7: List of OATPs with their organ distribution and substrates – Continuation

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organ distribution</th>
<th>Identified substrates</th>
</tr>
</thead>
</table>
| OATP1B3 | Liver              | **Bile acids** - Cholate  
                       |                     | **Bilirubin**      
                       |                     | **Steroid hormones conjugates** (Estradiol-17β-glucuronide, DHEA-S)  
                       |                     | **Thyroid hormones** (T3, T4, rT3)  
                       |                     | **Drugs:**  
                       |                     |   - Statins - *Rosuvastatin*  
                       |                     |   - ARBs - *Valsartan*  
                       |                     |   - ACE-Inhibitors - *Enalapril*  
                       |                     |   - Cardiac glycoside: *Digoxin*  
                       |                     |   - Antiretroviral drugs - *Lopinavir*  
                       |                     |   - Antibiotics- *Benzylpenicillin, Cefazolin, Rapamycin, Rifampicin*  
                       |                     |   - Chemotherapy- *Methotrexate, Paclitaxel, Imatinib*  
                       |                     |   - Others- *Bosentan*  
| OATP1C1 | Brain, Testis, Heart, Retina | **Steroid hormones conjugates** (Estradiol-17β-glucuronide, DHEA-S)  
                       |                     |   - **Thyroid hormones** (T3, T4, rT3)  
| OATP2A1 | Ubiquitous         | **Prostaglandins** (E1,2, F2α, H2)  
                       |                     | **Thromboxane** (B2)  
| OATP3A1 | Ubiquitous         | **Steroid hormones conjugates** (Esterone-3-sulfate)  
                       |                     | **Thyroid hormones** (T4)  
                       |                     | **Prostaglandins** (E1,2, F2α)  
                       |                     | **Vasopressin**  
                       |                     | **Drugs:**  
                       |                     |   - Antibiotics- *Benzylpenicillin*  
| OATP4A1 | Ubiquitous         | **Bile acids** - Cholate  
                       |                     | **Steroid hormones conjugates** (Estradiol-17β-glucuronide, Esterone-3-sulfate)  
                       |                     | **Thyroid hormones** (T3, T4, rT3)  
                       |                     | **Prostaglandins** (E2)  

Table 8: List of OATPs with their organ distribution and substrates – Continuation

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organ distribution</th>
<th>Identified substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP4A1</td>
<td></td>
<td>Drugs:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Antibiotics: <em>Benzylpenicillin</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Cardiac glycoside: <em>Digoxin</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Antidiabetic drugs: <em>Sitagliptine</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Chemotherapy: <em>Methotrexate</em></td>
</tr>
<tr>
<td>OATP4C1</td>
<td>Kidney, Lung, Skin</td>
<td>Thyroid hormones (<em>T3, T4</em>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Drugs:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Cardiac glycoside: <em>Digoxin, Ouabain</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Chemotherapy: <em>Methotrexate</em></td>
</tr>
<tr>
<td>OATP5A1</td>
<td>Heart, Brain, skeletal muscle, Breast</td>
<td>Not examined yet</td>
</tr>
<tr>
<td>OATP6A1</td>
<td>Testis</td>
<td>Not examined yet</td>
</tr>
</tbody>
</table>

**Abbreviations:**

*DHEA-S*: dehydroepiandronsterone sulfate; *T3*: Triiodothyronine; *T4*: Thyroxine; *rT3*: Reverse Triiodothyronine

*ARB*: Angiotensin Receptor blocker; *ACE-Inhibitors*: Angiotensin Converting Enzyme-Inhibitors [92],[94],[97]

### 1.2.4. Expression in cancer tissue

Tumor cells show altered expression of OATPs indicating the important role of the proteins in the diagnosis and progression of malignancies. Their relevance includes also the response to anticancer chemotherapy thereby affecting the clinical outcome of tumors. The altered expression under pathological conditions may be of benefit for their usage as targets for therapy but also may confer resistance to anticancer drugs.

- **OATP1A2**

While high expression levels are confirmed in breast, prostate and bone carcinoma cells, a decreased expression is found in colon polyps and cancer. OATP1A2 is postulated as a potential target for the treatment of prostate cancer. This is based on the fact that androgen-sensitive prostate cancer is influenced by the transport of DHEA-S into cells, which is mediated by OATP1A2, and the following conversion of
DHEA-S into the growth stimulating DHEA by steroid sulfatase. The efficacy of the therapy can also be influenced by interactions of OATP1A2 substrates leading to severe side effects. Mutations cause altered expression of the protein in the kidney which contribute to altered renal clearance and drug-induced toxicity of drugs being OATP1A2 substrates. [97],[98]

**OATP1B1/1B3**

Both proteins are classified as “liver-specific”. Surprisingly their expression is decreased in liver tumors. However, tumors of the gastrointestinal tract, breast, lung, pancreas, ovary and prostate show high expression levels of OATP1B1 and OATP1B3. A better prognosis and a decreased recurrence is detectable for breast cancer patients expressing OATP1B3 in the tumor. An inverse correlation exists between its expression levels and the tumor size. A high intratumoral androgen level in hormone resistant metastases is associated with an increased expression of several OATPs, among them OATP1B1/1B3. OATP1B3 mediates the uptake of testosterone into prostate cancer cells, which is converted afterwards in dihydrotestosterone (DHT) leading to the proliferation of cancer cells. Overexpression of OATP1B3 lead to a poor response to testosterone deprivation therapy. OATP1B3 protein expression is found within all tumor stages of colon cancer. However, it is upregulated in earlier-stage, lower-grade and better differentiated tumors causing an improved 5-year survival. In p53 (wild type) expressing tumors, overexpression of OATP1B3 in colon cancer demonstrates a survival disadvantage. The apoptotic effect after camptothecin and oxaliplatin treatment is reduced due to the alteration of p53-dependent survival pathways caused by OATP1B3 upregulation. This indicates a possible correlation between chemotherapeutic resistance and an overexpression of OATP1B3 in colon cancer cells. Functional defects of OATP1B1 and OATP1B3 may exert influence on docetaxel disposition but not its clearance. During the first-line treatment of ovarian cancer, the expression levels of both proteins may be responsible for the sensitivity to paclitaxel. [96],[97],[98]
➢ **OATP1C1**

The protein is considered as crucial for the transport of thyroid hormones because of its high affinity for the hormones. It is not clearly explained in which cancers this protein plays an important role. OATP1C1 expression was found in samples from osteosarcomas, kidney cancer metastasis and aneurysmal bone cysts. [97],[98]

➢ **OATP2A1**

The prostaglandin carrier is upregulated at the mRNA level in bone metastases from kidney cancer and in many other tumors including breast, ovary, lung and liver. In colorectal cancer the expression is decreased. [97],[98]

➢ **OATP2B1**

The expression of this protein is regulated by steroid hormones, therefore high levels are demonstrated in breast and prostate carcinoma. A higher grade of breast cancer is associated with increased protein expression. Nevertheless, the expression level does not have an impact on the clinical progression. Osteocarcinoma show higher mRNA expression of OATP2B1 than bone cysts. Furthermore, protein expression is detected at the blood-brain barrier and blood-tumor barrier in human gliomas. [97],[98]

➢ **OATP3A1**

Its expression is upregulated in liver and prostate cancers. Still, significant high levels of OATP3A1 are demonstrated in breast cancer. [98]

➢ **OATP4A1**

The expression pattern of OATP4A1 in carcinomas is comparable to that of OATP3A1. An upregulation is discovered in breast, lung, ovarian, prostate and colon cancers and in metastasis of colorectal cancer in liver. Additionally, OATP4A1 is highly expressed in colon adenocarcinoma, bone cysts and osteosarcoma. [97],[98]
➢ **OATP4C1**

Its expression levels in cancer tissue is poorly characterized. It is found in colon cancer, in normal and cancerous breast cancer tissue, but further studies are required. [97],[98]

➢ **OATP5A1**

The protein is upregulated in primary and secondary liver cancer, and in urothelial and renal tumors as well. Insignificant concentrations are found in colorectal, and pancreas cancers. At the mRNA level its expression is also detected in bone and breast cancer. OATP5A1 localisation in normal breast tissue differs from that in breast cancer. Whereas the protein's location in normal tissue is at the membrane of epithelial cells around the milk ducts, in malignant breast tissue the membrane localisation is lost and immunoreactivity is found in the cytoplasm of milk duct cells. However, drug-resistant lung cancer cell lines (SCLC) show expression of this protein indicating its role in the disposition of satraplatin. [97],[98],[99]

➢ **OATP6A1**

A number of cancers, such as brain, urinary bladder, oesophagus and lung cancer show protein expression. It is identified as "*a cancer-testis antigen in lung tumors and lung tumor cell lines*". High expression levels of this protein are noticed in SCLC cells suggesting its usage as a marker in lung cancer. Primary SCLC cells ((NCI-H417) show increased OATP6A1 gene expression at high cisplatin doses and at low doses of etoposide. The gene expression of OATP6A1 in metastatic SCLC cells is decreased at low dosage of etoposide. Topotecan downregulates the protein expression both on mRNA- and protein level. [97],[98],[100]
2. Aim of the Study

Colorectal cancer counts as the second most discovered malignancy in females and the third in males. The major risk concerns the time of diagnosis since the development and spread of the tumor occurs unnoticeable and a better prognosis is associated with an early detection.

Citizens of industrialised countries are much more affected compared to those of other countries. This prevalence is attributable to lifestyle-related aspects, for instance obesity, lack of exercise, alcoholism and smoking. However, the raised awareness of the society in western countries results lately in a decreased incidence. Age is also considered as a crucial risk factor of CRC. Due to statistical analysis, patients older than 65 years represent 70% of the total number of CRC patients. Tumors of the colon and rectum are grouped into two types: the non- and hereditary carcinoma. Both forms are specified through several characteristic molecular features and genetic failures. The accumulation of genetic mutations leading to chromosomal instability and various modifications of the DNA provide the initiation of the non-hereditary sporadic CRC, which occurs more often. Two critical syndromes, the familial adenomatous polyposis (FAP) and the hereditary non-polyposis colorectal cancer (HNPCC) rely on inherited genetic mutations, cause the development of hereditary tumors.

Chronic inflammatory bowel diseases (IBD) are regarded as dominant risk factors which contribute to an early transition to malignant tissue and poor prognosis. Inflammatory mediators induce the activation of the NFκB pathway leading to cell proliferation, decrease of cell death, cell invasion and metastasis. Moreover, activated immune cells trigger the production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) inducing damages and mutations in the DNA. It is well observed that tumor cells are mostly infiltrated by immune cells. Therefore, carcinogenesis is tremendously related to the inflammation microenvironment and patients with chronic IBD are at higher risk.

The correspondent proteins of the “Solute Carrier for organic Anions” (SLCO) gene superfamily are the Organic Anion Transporting Polypeptides (OATPs). Their expression is found either specifically or ubiquitously, in normal and cancerous tissue.
The transport proteins have 12 domains separated by 5 intra- and 6 extracellular loops. The sodium-independent transport of diverse structurally unlinked endo- & xenobiotics into cells is mediated by OATPs. Bile salts, hormones and their conjugates, bilirubin and its glucuronides, various drugs and toxins are counted among their substrates. The expression pattern is altered in tumors; the precipitative causes are still unknown. Previous studies demonstrate the influence of inflammatory mediators on the expression mode of OATPs.

The central aim of this study is the examination of the expression pattern of OATP4A1 in the HT-29 colorectal cancer cell-line after the exogenous addition of the inflammation cytokines, Interleukin 6 (IL-6) and Tumor Necrosis Factor alpha (TNF-α). The research are conducted on both mRNA- and protein level. In addition, the impact of the above-mentioned inflammatory factors on the expression of OATP1B1 on mRNA- level is examined. Apart from that, malignant diverticulitis tissue is stained with vimentin to study the infiltration of tumor cells with activated immune cells.

Therefore, the first essential step will be the cultivation of the HT-29 cells for the treatment procedure. Cells will be stimulated either with IL-6 or TNF-α or a mixture of both for a certain period of time. The duration of stimulation will remain for 6, 12, 24 and 48 hours. Afterwards, the RNA should be isolated for the reverse transcription to provide the cDNA. The alteration of the OATP4A1 and OATP4A1 expression on mRNA-level will be determined through quantitative Real Time (qRT) - TaqMan polymerase chain reaction (PCR). For the immunofluorescence staining the same protocol of cell stimulation should be used. Afterwards, the OATP4A1- positive cells will be scanned with the TissueFAXS® microscopic workstation and analysed with the TissueQuest® software. The number of OATP4A1 expressing cells should be thereby quantified and the intensity of the staining will also be measured. The influence of the added inflammation mediators on the expression rate of OATP4A1 will be investigated. Besides, the effect of IL-6 and TNF-α on the expression of OATPP1B1 will be studied as well. The distribution of immune cells around tumor cell will be researched through immunohistochemistry staining of malignant diverticulitis tissue. The images will be scanned with TissueFAXS®, subsequently the analysis will be performed by the HistoQuest® program.
3. Materials and methods

3.1. Cell Culture

- **Materials**
  - 25cm² cell culture flasks (TPP, Trasadingen, CH)
  - Pipettes 10ml, 25ml (Biozym Scientific GmbH, Oldendorf, GER)
  - Pasteur pipettes (SIGMA-ALDRICH, Buchs, CH)
  - Safe-seal tips (Biozym Scientific GmbH, Oldendorf, GER)
  - 15ml centrifugation tubes (TPP, Trasadingen, CH)
  - DMEM- medium with [+4,5g/L D-Glucosse, [+ L-Glutamine, [- Pyruvate (Gibco®, Life Technologies, Carlsbad, California, USA)
  - Fetal Bovine Serum FBS superior (Biochrom AG Biotechnologie, Berlin, GER)
  - HEPES Buffer Solution (1M) (Gibco®, Life Technologies, Carlsbad, CA, USA)
  - Calcium and magnesium free 1x PBS- pH 7.4
  - 10x Trypsin - EDTA solution Nr. 59418 (SIGMA-ALDRICH, Buchs, CH)

- **Instruments**
  - Lamina flow (Thermo Fisher Scientific, Asheville, US)
  - Heraeus cytoperm 2 (Thermo Fisher Scientific, Asheville, US)
  - Olympus CK2 inverted microscope with phase contrast (Spach Optics, Rochester, NY)
  - Hettich Rotante centrifuge (Hettich Bäch, CH)
  - Luna™-Automated Cell Counter (Biozym Scientific GmbH, Oldendorf, GER)

- **HT-29**

HT-29 is a cell line which was isolated from a 44 years old Caucasian female patient with primary colorectal adenocarcinoma. The adherent cells of HT-29 were used for all experiments done in this thesis. [101]
The entire work with HT-29 cells was completed in the cell culture room, a separated room where all materials and reagents used are sterilized to avoid any contamination. The access to this room is only permitted with white coats, house shoes and wearing gloves is also essential. Before starting work hands and forearms should be disinfected with Mikrozid AF liquid. The working bench needs to be washed with Microzid before as well as after usage.

The cells were brought into cultivation in 25cm² flasks in 5 ml DMEM-medium and incubated at a temperature of 37°C, 95% humidity and 5% CO₂. Every second day the cell density was checked under the microscope and the old medium was replaced with a fresh one to guarantee good conditions for cell growth. After reaching a confluence of about 80% cells were considered to get splitted.

Cell Splitting

Splitting Cells is a process where cells are separated into different culture flasks to get multiplied. In this way premature aging of the cells - which occurs through high cell density- is prevented. By stopping cell splitting important nutrients are barely available and the cells die. HT-29 cells were splitted every five days. [102]

The different steps of the procedure are explained point by point in the upcoming part. First of all the reagents were pre-warmed to room temperature to evade stressing the cells. The old medium was aspirated and the cells were rinsed gently with 5ml of PBS to remove dead cells. After elimination of PBS 1ml Trypsin was added and the cell flask was incubated for four minutes at 37°C. Through Trypsin adherent cells were unglued from the flask ground. Then 3ml of DMEM-medium were added to the trypsin-cell-suspension and it was transferred into a 15ml tube for centrifugation at 450g for three minutes. The supernatant was aspirated and the pellet was re-suspended in 3ml fresh medium. Afterwards cells were counted by the cell counter and an appropriate amount of cells was plated in a new 25 cm² flask with 5 ml fresh DMEM-medium. The cell density requested was 100000 Cells/flask.
**Figure 8: Cell count Report**

Its results show the concentration and number of live as well as dead cells. In addition cell specific information, e.g. cell size and roundness are obtained. The protocol also generates graphics of histograms of the gained data.
3.2. Cell treatment

- **Materials**
  - Chamber Slides (Thermo Fisher Scientific Inc., Waltham, MA, USA)
  - Pipettes 10ml, 25ml (Biozym Scientific GmbH, Oldendorf, GER)
  - Pasteur pipettes (SIGMA-ALDRICH, Buchs, CH)
  - Safe-seal tips (Biozym Scientific GmbH, Oldendorf, GER)
  - 1x PBS- pH 7.4
  - PFA (Sigma-Aldrich Chemie GmbH, Steinheim, GER)
  - DMEM- medium with [+4,5g/L D-Glucose, [+1 L-Glutamine, [-] Pyruvate (Gibco®, Life Technologies, Carlsbad, California, USA)
  - HEPES Buffer Solution (1M) (Gibco®, Life Technologies, Carlsbad, CA, USA)
  - IL6 (Sigma-Aldrich Chemie GmbH, Steinheim, GER)
  - TNFα (Sigma-Aldrich Chemie GmbH, Steinheim, GER)

- **Instruments**
  - Lamina flow (Thermo Fisher Scientific, Asheville, US)
  - Heraeus cytoperm 2 (Thermo Fisher Scientific, Asheville, US)
  - Olympus CK2 inverted microscope with phase contrast (Spach Optics, Rochester, NY)
  - Hettich Rotante centrifuge (Hettich Bäch, CH)
  - Luna™-Automated Cell Counter (Biozym Scientific GmbH, Oldendorf, GER)

Cells were splitted and a certain amount of cells (usually 100,000 cells) was transferred into chamber slides. HT-29 cells were cultivated for several days before testing. After the cultivation period, the old medium was exchanged with a fresh serum-free medium. Then the test compound was added. Either 1 µl TNF-α (10ng/ml) or 2.5 µl IL-6 (100ng/ml) or a mixture of both was applied to the cells cultivated on each chamber slide. Subsequently, the cell response towards the additives (regarding the expression of OATP4A1) was monitored after 6, 12, 24 and 48 hours of stimulation. Finally, 4% (w/v) PFA in PBS was utilized to stop the reactions after the specific time periods to
the chamber slides. This reagent was added for eight minutes and was then replaced by 1x PBS. In the end, the cells were stored at 4°C until staining was performed.

Table 9: Scheme of Chamber slides

<table>
<thead>
<tr>
<th>Slide 1</th>
<th>Slide 2</th>
<th>Slide 3</th>
<th>Slide 4</th>
<th>Slide 5</th>
<th>Slide 6</th>
<th>Slide 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con 1</td>
<td>TNFα 6h_1</td>
<td>IL6 12h_1</td>
<td>Combo 12h_1</td>
<td>IL6 24h_1</td>
<td>IL6 48h_1</td>
<td>Combo 48h_1</td>
</tr>
<tr>
<td>Con 2</td>
<td>TNFα 6h_2</td>
<td>IL6 12h_2</td>
<td>Combo 12h_2</td>
<td>IL6 24h_2</td>
<td>IL6 48h_1</td>
<td>Combo 48h_2</td>
</tr>
<tr>
<td>IL6 6h_1</td>
<td>Combo 6h_1</td>
<td>TNFα 12h_1</td>
<td>Combo 24h_1</td>
<td>TNFα 24h_1</td>
<td>TNFα 48h_1</td>
<td></td>
</tr>
<tr>
<td>IL6 6h_2</td>
<td>Combo 6h_2</td>
<td>TNFα 12h_2</td>
<td>Combo 24h_2</td>
<td>TNFα 24h_2</td>
<td>TNFα 48h_2</td>
<td></td>
</tr>
</tbody>
</table>

Each chamber is stimulated with a single cytokine (IL-6 or TNF-α) or a combination of both for a certain interval (6h, 12h, 24h or 48h). IL-6 concentration is 100ng/ml; TNF-α concentration is 10ng/ml

3.3. RNA-Isolation

- **Materials**
  - 25cm² cell culture flask (Nunc, Roskilde, DK)
  - Cell scrapers (TPP, Trasadingen, CH)
  - Reaction tubes 1.5ml (Biozym Scientific GmbH, Oldendorf, GER)
  - Safe-seal tips (Biozym Scientific GmbH, Oldendorf, GER)
  - peqGold Trifast (PEQLAB Biotechnologie GmbH, Erlangen, GER)
  - GIBCO distilled water, RNase/DNase free (Life Technologies, Carlsbad, CA, USA)
  - Ethanol (EtOH) (AnalaR NORMAPUR, West Sussex, UK)
  - Isopropanol (Merck, Darmstadt, GER)
• Chloroform (Merck, Darmstadt, GER)

**Instruments**
- Centrifuge 5415R (Eppendorf AG, Hamburg, GER)
- Thermomixer comfort (Eppendorf AG, Hamburg, GER)
- Nanodrop Spectrophotometer ND-100 (Thermo Fisher Scientific, Asheville, USA)

RNA is very instable and can easily degrade. Therefore, the isolation should be done in sterile work place and with RNase free reagents.

For RNA isolation, cell cultivation and stimulation was done on six-well plates. After harvesting, cells were lysed by 1ml TriFast. The incubation lasted five minutes at RT to ensure complete lysis. After addition of 200µl chloroform per 1ml TriFast, tubes were shook 15-20 times manually. Thereafter, the cells in the mixture were homogenized. Samples were left standing at RT for ten minutes, before they were centrifuged for 15 minutes at 12000g and 4°C for the separation of RNA from proteins and genomic DNA. As a result, the RNA was distributed to the aqueous phase on the top of the fluid, while chloroform and TriFast (red phase) were remaining on the bottom. In-the interphase genomic DNA and proteins are present. The colourless fluid with the RNA was transferred to a fresh tube and mixed with 500µl isopropanol. Samples were incubated on ice for ten minutes and after that centrifugation was done for 15 minutes at 12000g and 4°C. RNA was precipitated and formed a pellet. After removal of isopropanol, the RNA was washed with 70% ethanol. The tubes were then centrifuged again for ten minutes at 12000g and 4°C. Subsequently, the supernatant was aspirated and for a complete vaporization of ethanol, the samples were heated to 55°C for three minutes. Dependent on its size, the pellet was dissolved in 20-80µl RNase-free water by passing a few times through a pipette. At the end, the RNA containing mixture was incubated at 55°C for ten minutes. The RNA concentration and the purity of samples (260/280 & 260/230) were evaluated by NanoDrop. Finally the RNA samples were stored at -80°C.
3.4. Reverse Transcription

- **Materials**
  - PCR-Softstrips 0,2ml (Biozym Scientific GmbH, Oldendorf, GER)
  - Safe-seal tips (Biozym Scientific GmbH, Oldendorf, GER)
  - GIBCO distilled, RNase/DNAse free H2O (Life Technologies, Carlsbad, CA, USA)
  - High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA)
    - RT Buffer
    - dNTP Mix (100mM)
    - RT Random Primers
    - MultiScribe™ Reverse Transcriptase

- **Instruments**
  - Biometra personal cycler (Gatt-Koller, Innsbruck, AT)

The reverse transcription of RNA generates the complementary cDNA. It is generated through the reverse transcriptase enzyme. Here, the “High Capacity cDNA Reverse Transcription Kit” was used to achieve the transcription. Random primers and the enzyme Reverse Transcriptase are essential for the synthesis of the cDNA.

A certain amount of soluble RNA—which was calculated based on the RNA concentration and its purity- was dissolved in RNase free water to give an end volume of 10µl. The Master Mix was prepared according to the table below:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT Buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>RT Random Primers</td>
<td>2 µl</td>
</tr>
<tr>
<td>dNTP Mix</td>
<td>0,8 µl</td>
</tr>
<tr>
<td>MultiScribe™ Reverse Transcriptase</td>
<td>1 µl</td>
</tr>
<tr>
<td>RNase/DNase free H2O</td>
<td>4,2 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Table 10: The composition of the Master Mix
10µl Master Mix was added to each RNA sample and incubated in the cycler using the following program:

- Ten minutes at 25°C: Reverse Transcriptase is activated
- Two hours at 37°C: Reverse transcription
- Five minutes at 85°C: Reverse transcriptase is deactivated
- Unlimited time at 4°C: cDNA is stored

Figure 9: The conversion of mRNA to cDNA by Reverse Transcription

By binding of the Oligo dT primer to the mRNA the reverse transcription is initiated. After the copy of the first cDNA strand, the reverse transcriptase enzyme copies the second strand by digestion and displacement of the mRNA. [103]

To reach a cDNA concentration of 10ng/µl, 80µl RNase free water were put in and the cDNA was stored at -20°C.

3.5. Quantitative Real Time PCR (qPCR)

- **Materials**
  - 2x TaqMan Gene Expression Master Mix (Life Technologies, Carlsbad, CA)
  - GIBCO distilled, RNase/DNAse free H2O (Life Technologies, Carlsbad, CA)
• TaqMan Primer/Probes (Life Technologies, Carlsbad, CA, USA)
• OATP4A1 (OATP1B3)
• geNorm Reference (housekeeping) gene selection kit (PrimerDesign, Southampton, UK)
• Safe-seal tips (Biozym Scientific GmbH, Oldendorf, GER)
• MicroAmp Clear Adhesive Film (Life Technologies, Carlsbad, CA, USA)
• MicroAmp Fast Optical 96-well Reaction Plate (Life Technologies, Carlsbad, CA, USA)

➢ Instruments
  • Heraeus Megafuge40 (Thermo Scientific, Fremont, USA)
  • A7900HT-Fast Real Time PCR System with SDS 2.4 Software Film (Life Technologies, Carlsbad, CA, USA)
  • Microsoft Excel 2013 (Microsoft Corporation, Redmond, USA)

Real Time PCR (Polymerase Chain Reaction) is a method which allows the quantitative analysis of gene expression in cells by measuring DNA amplification. “It is easy to perform, provides the necessary accuracy and produces reliable as well as rapid quantification results”. [103]

The high-speed and specific process collects fluorescent signals while exponentially amplifying DNA sequences over a range of cycles. The signal is directly proportional to an increase of the DNA amount. Every PCR amplification has four phases shown as baseline, followed by the linear and exponential phase, and at the end the plateau phase. The linear phase where amplification is dependent on the concentration of the template is taken for the calculations.
Figure 10: The PCR growth curve

The baseline region demonstrates the initial cycles of PCR without any remarkable changes in fluorescence signals. The doubling of the product during the exponential phase creates a significant signal analogue to the exponential growth at every cycle. Due to the limitation of the reaction components the signal does not grow exponentially anymore at the linear phase. The plateau phase is reached when no fluorescence signals are generated because of the exploitation of the reaction components. [105]

3.5.1. TaqMan Method

In the absence of double-stranded DNA, SYBR Green emits low fluorescence signals, however when DNA is synthetized, the dye intercalates into the double-strand and the fluorescence intensity increases.

The TaqMan’s assay is based on the fluorescence resonance energy transfer, abbreviated as FRET. This mechanism relies on two components, where one is able to emit fluorescent signals and is called donor. The other component is an acceptor chromophore which can quench the donor signal by absorbing it. This concept is used for the TaqMan’s probes where one end the molecule contains a reporter (donor) dye on the 5’site and a quencher (acceptor) on the 3’site. In solution, reporter and quencher are close enough, therefore the quencher absorbs the reporter’s fluorescent signals. After the binding of the TaqMan’s probe to the template during the annealing phase, the 5’-3’ exonuclease of the Taq-polymerase gets activated and separates the reporter from the quencher. As a consequence of the hydrolysis the fluorescent signals emitted from the reporter which correlate with the amount of DNA-can be detected by the Real Time PCR system. [107],[108]
**Figure 11: Fluorescence detection through the TaqMan Method**

The fluorescent TaqMan probe binds to the target cDNA during annealing. During the extension the probe is displaced by the DNA polymerase. After the cleavage of the fluorophore from the quencher the distance increases between them leading to fluorescence. [109]

➢ **Preparation**

First, a MasterMix was mixed for every housekeeping gene and the genes of interest as well. 96-well plates were always used. Table 7 shows the components of MasterMix for 1 well.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan Primer/ Probe</td>
<td>1 µl</td>
</tr>
<tr>
<td>2x TaqMan Gene expression MasterMix</td>
<td>10 µl</td>
</tr>
<tr>
<td>RNase free H2O</td>
<td>7 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>18 µl</strong></td>
</tr>
</tbody>
</table>

Next, 2µl of cDNA were pipetted in each well. Thereafter, 18µl of the MasterMix were put in every well and the 96-well plate was isolated with an adhesive film.

The plate was centrifuged for 1 min at 200 rpm and then the fluorescence signals were examined with the Real Time PCR System using the following settings:
1. 2 minutes at 50°C
2. 10 minutes at 95°C
3. 15 seconds at 95°C (40 times)
4. 1 minute at 60°C (40 times)

➢ Data Analysis

There are two main quantifications for results acquired from the real time PCR analysis: the absolute and relative quantification. With the absolute quantification, data refer to a standard curve. The principle of the relative method is based on the comparison of the relative expression values of target genes to reference values. Most of the reference genes used are housekeeping genes, such as GAPDH, or YWAZ. They are expressed in all nucleated cell types due to their essential role for cell survival. Their mRNA synthesis is regarded as stable thus they are basics for the normalization of the results. For quantification the Ct-value is used. The cycle threshold is defined by the number of necessary cycles to gain values over the background fluorescent signals. A low Ct value means an early increase in the fluorescence signals because of a high amount of templates and vice versa. [104],[110]

The calculation of data is carried out through ∆∆Ct-method. An optimum efficiency in all samples is assumed. The three steps below are computed for every sample:

1. ∆Ct = Ct target gene – geometric value of reference gene
2. ∆∆Ct= ∆Ct treatment – ∆CP control
3. Ratio=2^{−∆∆Ct}

3.6. Indirect Immunofluorescence Staining

➢ Materials

- 1x PBS- pH 7.4
- NH₄Cl 50mM (Merck, Darmstadt, GER)
- Tween 20 (SIGMA-ALDRICH, Buchs, CH)
- BSA (PAN™ BIOTECH GmbH, Aidenbach, GER)
- 4´,6-Diamidin-2´-phenylindoldihydrochlorid, DAPI (Roche Diagnostics GmbH, Basel, CH)
• Fluoromount-G (Southern Biotech, Alabama, US)
• Cover Glass 50mm, Thickness no.1 (VWR International, Radnor, PA)
• Antibodies

Table 12: List of Antibodies used for immunofluorescence staining of cells

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLCO 4A1</td>
<td>1:200 in PBS</td>
<td>Atlas Antibodies, Stockholm, SE</td>
</tr>
<tr>
<td>Anti-rabbit IgG Alexa 488</td>
<td>1:1000 in PBS</td>
<td>Invitrogen Life Technologies, Carlsbad, California, US</td>
</tr>
</tbody>
</table>

SLCO 4A1: Solute carrier organic anion transporter family member 4A1

➢ Instruments

• Centrifuge 5415R (Eppendorf AG, Hamburg, GER)
• Microscope Zeiss AXIO Imager Z1, Axio Cam HRc2 Color CCD Digitalcamera, Autovision 4.6 Software (Zeiss, Jena, GER)

Cells were washed three times for five minutes with PBS. Then they were permeabilized by adding 0.2% Tween in PBS for 20 minutes. Then they were washed twice for five minutes with the buffer. Afterwards, NH₄Cl was added for 15 minutes to quench free aldehyde groups which could prevent the following blocking. Cells were washed again twice for five minutes. Next, the blocking solution (3% BSA in 1x PBS) was given to the cells for 45 minutes. All these steps were done at RT. Following that procedure, cells were incubated with the primary antibody overnight in a 4°C cold room.

On the following day, the staining procedure was continued at RT. After washing cells three times for five minutes with buffer, cells were incubated with the secondary antibody for one hour. As from this step on, the work had to be done in the dark to prevent bleaching. Cells were washed twice for five minutes before adding DAPI solution (1:1000 in PBS) for 15 minutes for nuclear staining. In the last step, cells were washed twice for five minutes with buffer, then the slides were rinsed briefly with bidest water, mounted with one drop of Fluoromount and covered with a cover slide.
3.6.1. TissueFAXS®/ TissueQuest®

The TissueFAXS® (TissueGnostics) is an automated fluorescence microscopic workstation which allows the scanning and analysis of samples in the fluorescence mode. It contains a high quality microscope (Zeiss, Leica, and Nikon), the computer hardware with the software and two high resolution screens. Eight slides in a row can be scanned via the automated system. The TissueFAXS® scanning software detects single cells due to some algorithms. 4 objectives (1x, 2.5x, 20x and 40x) are used and 5 fluorescence channels with different wave-length for the immunofluorescence evaluation of DAPI, FITCS, Texas Red, Cy5, Cy7 are provided. For the statistical analysis of generated data by TissueFAXS®, the TissueQuest® software is used after scanning. Regions of interest (ROI) are defined for the evaluation. The statistical report about the intensity of staining and the number of cells expressing OATP4A1 is transferred to an Excel sheet for further assessment. [111]

![Figure 12: Workstation consisting of the TissueFAXS® System and TissueQuest® Software (TissueGnostics GmbH, Vienna, Austria) [111]](image)

- **Adjustments of TissueFAXS® Cell Analysis System (Version 4.1.5.2 PCO) and TissueQuest® (Version 4.0.1.0127).**

Table 10 gives a description of the used microscopic system.

**Table 13: Settings used for the scanning**

<table>
<thead>
<tr>
<th>Camera</th>
<th>Pixel Fly, Rear Port</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV- Lamp</td>
<td>100%</td>
</tr>
<tr>
<td>Channels used</td>
<td>DAPI, GFP</td>
</tr>
<tr>
<td>Objective used</td>
<td>20x, 40x, 63x</td>
</tr>
</tbody>
</table>
To receive the scanning quality some settings need to be adjusted, for instance the exposure time and thresholds. The upper and lower threshold describe the values of the layer thickness in cells which measurements are taken of.

Table 14: Specific settings for DAPI and GFP used while scanning

<table>
<thead>
<tr>
<th></th>
<th>DAPI</th>
<th>GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure time</td>
<td>40</td>
<td>330</td>
</tr>
<tr>
<td>Upper Threshold</td>
<td>10273</td>
<td>8505</td>
</tr>
<tr>
<td>Lower Threshold</td>
<td>231</td>
<td>2500</td>
</tr>
</tbody>
</table>

*DAPI: 4',6- diamino-2-phenylindole; GFP: Green Fluorescent Protein*

3.7. **Indirect Immunohistochemistry Staining**

- **Materials**
  - Xylol (Merck, Darmstadt, DE)
  - 100% Ethanol (Merck, Darmstadt, GER)
  - 20x Epitope Retrieval Solution DEPP-9 pH 9 (Eubio, Wien, AT)
  - 1x PBS pH 7.4
  - Tween® 20 (Sigma-Aldrich Chemie GmbH, Steinheim, GER)
  - Dako Pen (DakoCytomation, Glostrup, DK)
    - UltraVision Protein Block
    - HRP Polymer
    - DAB Plus Chromogen
    - DAB Plus Substrate
  - ChemMate™ Hematoxylin (DakoCytomation, Glostrup, DK)
  - Vimentin (Thermo Fisher Scientific, Fremont, US)
  - BSA (PAN™ BIOTECH GmbH, Aidenbach, DE)
  - H₂O bidest
  - Cover Glass 24x 50mm, Thickness no.1 (VWR International, Radnor, PA)
  - Fluoromount-G (Southern Biotech, Alabama, US)
- **Instruments**
  - Centrifuge 5415R (Eppendorf AG, Hamburg, DE)
  - Microscope Zeiss AXIO Imager Z1, Axio Cam HRc2 Color CCD Digitalcamera, Autovision 4.6 Software (Zeiss, Jena, DE)
  - TissueFAXS® and HistoQuest® Software (TissueGnostics GmbH, Vienna, AT)

Immunohistochemistry is a widely used method of staining of tissue sections either with a colour or a fluorescent dyes bound to an antibody. The staining procedure of tissues enables the localization and detection of proteins in cell compartments via antigen-antibody reaction. It is based on the affinity of antibodies to specific epitopes of the tissue. There are two methods available-direct and indirect microscopy. The direct and rapid way is adequate for the demonstration of various proteins within one sample. The primary antibody is marked with a fluorophore which facilitates the detection directly after binding to the protein. The indirect and more sensitive method consists of two steps where at first a specific primary antibody binds to the target protein. Then the second antibody-which is conjugated to an enzyme-triggers an enzyme-substrate reaction after binding to the primary antibody and causes the synthesis of a fluorescent dye. [112]

*Figure 13: Schematic principle of Indirect Immunofluorescence*

The fluorophore-marked secondary antibody binds to the primary antibody after its specific binding to the target protein/antigen
➢ **Staining method**

First of all slides were put into Xylol and EtOH 100% for three minutes each. In the next step, DEPP-9 Buffer was added for deparaffinization and slides were incubated in the microwave for ten minutes. After cooling down for 25 minutes at room temperature, slides were washed three times for three minutes with 1xPBS + 0.1% Tween. Next, every tissue on the slides was encircled by Dako Pen. Then the “UltraVision Protein Block” was put on the tissues for seven minutes. Meanwhile the primary antibody against vimentin was prepared with a dilution of 1:50 in 1xPBS + 10% BSA and slides were incubated for one hour at RT. Thereafter, slides were washed again for three times for three minutes with 1xPBS + 0.1% Tween. Subsequently, the antibody enhancer was added for ten minutes and the washing step (three times for three minutes with 1xPBS + 0.1% Tween) was repeated. As for the next step, the coverage of tissues with HRP Polymer lasted 15 minutes followed by washing the slides for three times for three minutes with 1xPBS + 0.1% Tween. In the meantime, a mixture of “DAB Plus Chromogen” and “DAB Plus Substrate” was prepared and added to the slides for three minutes at RT. After washing the slides (three times for one minute with H₂O-bidest) hematoxylin was added for one minute to stain the nuclei. In the next step, slides were washed first with water for five minutes, then briefly with H₂O-bidest. In the final step, tissue sections were imbedded in Fluoromount and were covered with a cover glass.

3.7.1. HistoQuest®

Analogue to the TissueQuest® software, HistoQuest® allows the quantitative analysis of stained tissue samples. After scanning the TissueFAXS® the gained images were assessed with the HistoQuest® program. First, regions of interest (ROIs) were specified and regions were classified into tumor, stroma and immune cells. After that, information about the percentage and staining intensity of vimentin in the categories mentioned before were obtained.
4. Results

4.1. Measurement of OATP4A1 mRNA expression in the colorectal cell line HT-29

First of all, the aim of these investigations was to monitor the expression of the membrane protein OATP4A1 on mRNA-level in the colorectal cancer cell line HT-29. In this cell line, the role of inflammatory mediators interleukin 6 (IL-6) and tumor necrosis factor α (TNFα) separately or combined on the mRNA expression of OATP4A1 was examined.

The reference genes with a stable expression even under treatment with cytokines were assessed before the experiment with the cytokines. Therefore the expression was first determined. In the first experimental approach, GAPDH (glyceraldehyde-3 phosphate dehydrogenase gene), 18S (ribosomal subunit) and YWHAZ (14-3-3 protein zeta/delta gene) were used. Because of a very unstable expression in the presence of cytokines, YWHAZ, which was used in other studies for controls, was not used anymore in the following investigations. The data analysis was done as stated before (Chapter 2.7.1).

4.1.1. mRNA- Expression of OATP4A1 in HT-29 cells after IL-6 treatment

The experiments were performed in duplicates. The concentration of IL-6 was 100ng/ml in all experiments and different periods of treatment were done. The investigation was carried out three times.

Figure 14 shows that mRNA expression of OATP4A1 is slightly higher after 6 hours of treatment. It is followed by a decrease in the expression after 12 hours. However, after 24 hours of treatment the expression rate increases again.
In Figure 14, it is shown that a marginally higher mRNA expression of OATP4A1 occurs after 6 hours of treatment, but a reduction in the expression rate after 12, 24, 48 hours of treatment was observed.

In Figure 15, it is shown that a marginally higher mRNA expression of OATP4A1 occurs after 6 hours of treatment, but a reduction in the expression rate after 12, 24, 48 hours of treatment was observed.
Figure 16 shows that after 24 hours of stimulation with IL-6, the mRNA expression rate of OATP4A1 is nearly the double compared with the expression rate of OATP4A1 in untreated control cells. After 48 hours a reduced expression is observed, but the expression is still about 1.5 fold higher than in control cells.

![Figure 16: mRNA-Expression of OATP4A1 in the HT29- cancer cell-line](image)

In summary, mRNA expression of OATP4A1 was quite variable between the experiments. However in all three experiments, an increase of the expression rate was seen after 6 (Experiment 1, 2) and 24 hours (Experiment 1, 3). After 12 hours (Experiment 1, 2) of treatment the mRNA expression is lower than in the untreated control.

4.1.2. mRNA- Expression of OATP4A1 in HT-29 cells after TNFα treatment

The experiments were performed in duplicates. The concentration of tumor necrosis factor α was 10ng/ml. The investegiation was carried out three times.

Figure 17 shows differences between the mRNA expression in the control cells and the cells stimulated with TNFα for 6 hours. After 12 hours of treatment, a decreased mRNA expression is visible, while, a higher expression of OATP4A1 is observed after 24 hours.
A slight increase of the expression is seen after 6 hours of treatment with TNFα. After 12 and 24 hours a reduced expression rate is seen, while, after 48 hours the expression of OATP4A1 is nearly the same as the control (Figure 18).

Figure 17: mRNA-Expression of OATP4A1 in the HT29- cancer cell-line

Control (without treatment) vs. single treatment with tumor necrosis factor α (10ng/ml) for 6, 12 and 24 hours, Standards used: 18S, GAPDH, YWHAZ

Figure 18: mRNA-Expression of OATP4A1 in the HT29- cancer cell-line

Control (without treatment) vs. single treatment with tumor necrosis factor α (10ng/ml) for 6, 12, 24 and 48 hour, Standards used: 18S, GAPDH
Similar to the date with the interleukin 6 treatment, the mRNA-expression of OATP4A1 after stimulation with TNFα is highly variable. As shown in Figure 19 the expression rate is about 2.5 fold higher than the control after 24 hours. After 48 hours, a reduced expression is detected, although the expression is almost as double as in untreated cells.

![Graph showing mRNA expression of OATP4A1](image)

**Figure 19: mRNA-Expression of OATP4A1 in the HT29- cancer cell-line**

Control (without treatment) vs. single treatment with tumor necrosis factor α (10ng/ml) for 24 and 48 hours, Standards used: 18S, GAPDH

To summarize the data, the mRNA expression of the membrane protein OATP4A1 is highly variable after the treatment schemes. Nevertheless, a decreased expression is seen after 12 hours (Experiment 1, 2), while the treatment after 24 and 48 hours leads to a tendency of a higher mRNA-expression.

### 4.1.3. mRNA- Expression of OATP4A1 in HT-29 cells after treatment with IL-6 and TNFα

The experiments were performed as duplicates. The concentration of interleukin 6 was 100ng/ml and of tumor necrosis factor α 10 ng/ml. The investigation was carried out three times.

The Figure 20 shows that there is almost no difference between the expression of OATP4A1 in the untreated control cells and in those stimulated with IL-6 and TNFα for 6 hours. A notable increase (80% & 89%) is detected after 12 and 24 hours of treatment.
In Figure 21, it is shown that an increase of OATP4A1 expression is seen in all stimulated cells. The highest expression is observed in cells treated for 12 and 48 hours. The expression rate of OATP4A1 mRNA after 6 hours of treatment is similar to that in the control. After 24 hours treatment with IL-6 and TNFα a higher expression than the control is seen.

Control (without treatment) vs. combo treatment with both interleukin 6 (100ng/ml) and tumor necrosis factor α (10ng/ml) for 6, 12 and 24 hours, Standards used: 18S, GAPDH, YWHAZ
In Figure 22, the data obtained in the previous experiments are confirmed. After a combination treatment the mRNA-expression indeed increases by 80%. The OATP4A1 mRNA expression is after 24 hours 2.5 fold and after 48 hours, it is 4.3 fold higher than in control cells.

![OATP4A1- Experiment 3](image)

**Figure 22: mRNA-Expression of OATP4A1 in the HT29- cancer cell-line**

Control (without treatment) vs. combo treatment with both interleukin 6 (100ng/ml) and tumor necrosis factor α (10ng/ml) for 24 and 48 hours, Standards used: 18S, GAPDH

To summarize the data, the treatment with IL-6 and TNFα together induces the mRNA expression of OATP4A1. This was observable in all three experiments.

### 4.2. Measurement of OATP1B3 mRNA expression in the colorectal cell line HT-29

In control experiments, we also examined the effect of IL-6 and TNFα on the mRNA expression of other OATPs in the colorectal cancer cell line HT-29. Therefore, the expression of OATP1B3 mRNA was investigated.

#### 4.2.1. mRNA- Expression of OATP1B3 in HT-29 cells after IL-6 treatment

The experiments were performed as duplicates. The concentration of IL-6 was 100ng/ml.
As the Figure 23 shows, a treatment with IL-6 for 24 and 48 hours leads to a modest increase of the expression of OATP1B3 on mRNA-level.

![Figure 23: mRNA-Expression of OATP1B3 in the HT29- cancer cell-line](image)

Control (without treatment) vs. single treatment with interleukin 6 (100ng/ml) for 24 and 48 hours, Standards used: 18S, GAPDH

**4.2.2. mRNA- Expression of OATP1B3 in HT-29 cells after TNFα treatment**

The experiments were performed as duplicates. The concentration of tumor necrosis factor α added was 10ng/ml.

As demonstrated in the diagram (Figure 24), TNFα has an obvious influence on the expression of OATP1B3. The expression rate of the membrane transporter increases both after 24 and 48 hours of stimulation. The increment is about the double after a treatment time of 1 day and 3.2 fold higher than the control after 2 days.
4.2.3. mRNA- Expression of OATP1B3 in HT-29 cells after a combination treatment of both IL-6 and TNFα

The experiments were performed as duplicates. The concentration of the used interleukin 6 was 100ng/ml and of tumor necrosis factor α was 10 ng/ml.

The influence of a combination treatment on the mRNA- expression of OTP1B3 is astonishing. The increase of the expression after 24 and 48 hours is self-evident (Figure 25).

Figure 24: mRNA-Expression of OATP1B3 in the HT29- cancer cell-line

Control (without treatment) vs. single treatment with tumor necrosis factor α (10ng/ml) for 24 and 48 hours, Standards used: 18S, GAPDH

Figure 25: mRNA-Expression of OATP1B3 in the HT29- cancer cell-line

Control (without treatment) vs. combo treatment with both interleukin 6 (100ng/ml) and tumor necrosis factor α (10ng/ml) for 24 and 48 hours, Standards used: 18S, GAPDH
To give the main points of the analysis, single treatment of IL-6 or TNFα affects the expression rate of OATP1B3 on mRNA-level leading to an increase. A combination of IL-6 and TNFα cause an enormous increase of expression of OATP1B3 especially after 48 hours of stimulation. Still, more studies are needed for confirmation.

### 4.3. Indirect Immunofluorescence staining of Ht-29 cells after treatment

Subsequent to the measurement of the OATP4A1 expression via real time PCR the Ht-29 cells were treated and stained as introduced before (Chapter 2.6) for further researches. The scanning of the samples was conducted by the TissueFAXS® as microscope, which allows the detection of stained cells. The scanned images of cells were evaluated by the statistical analysis program TissueQuest® enabling the analysis of staining intensity of OATP4A1 and quantifying OATP4A1-positive cells.

#### 4.3.1. OATP4A1 in cells after IL-6 treatment

The treatment was performed in the Ht29-cancer cell line. The concentration of IL-6 added to cells was the same as for qPCR experiments (100ng/ml). The experiments were performed as duplicates.

As Figure 26 shows, the intensity of stained cells is only higher after stimulation with IL-6 for 6 hours. Afterwards, the intensity decreases.
Figure 26: The intensity of OATP4A1 stained cells after interleukin 6 treatment

Cells were stimulated with interleukin 6 (100ng/ml) for 6, 12 and 24 hours. The statistical report was provided via TissueQuest. “Mean Value” refers to the intensity of OATP4A1-positive cells expressed as a percentage compared to the non-stimulated control cells.

Figure 27 highlights the percentage of cells which have an OATP4A1 expression after stimulation with IL-6. The number of OATP4A1-positive cells increases after 6 and 24 hours of treatment. However, the percentage of OATP4A1-stained cells is decreased after 12 hours of stimulation.

Figure 27: Percentage rate of HT29-cells with the protein OATP4A1 after interleukin 6 treatment

Cells were stimulated with interleukin 6 (100ng/ml) for 6, 12 and 24 hours. The statistical report was provided via TissueQuest. “Mean Value” refers to the number of OATP4A1-positive cells expressed as a percentage compared to the non-treated control cells.
The expression of OATP4A1 under the influence of IL-6 is given in the Figure 28. Compared to the control cells, after 24 hours of stimulation the number of OATP4A1-positive stained cells is much higher than in the controls.

4.3.2. OATP4A1 in cells after TNFα treatment

The treatment was performed in the Ht29-cancer cell line. The concentration of TNFα added to cells was the same as for qPCR experiments (10ng/ml). The experiments were performed in duplicates.

Figure 29 depicts the influence of TNFα on the OATP4A1-staining intensity of positively stained cells. There was no visible difference to the controls.
As shown in Figure 30, after 6 and 24 hours of treatment with TNFα the number of positively stained cells showing OATP4A1 expression is much higher than in control cells. The increase after 6 and 24 hours is about 20% and 90% respectively.

Figure 30: Percentage rate of HT29-cells with the protein OATP4A1 after tumor necrosis factor α treatment

Cells were stimulated with tumor necrosis factor α (10ng/ml) for 6, 12 and 24 hours. The statistical report is provided through TissueQuest. “Mean Value” refers to the number of OATP4A1- positive cells expressed as a percentage compared to the non-treated control cells.
Figure 31 highlights the development of OATP4A1 expression in presence of TNFα after diverse treatment intervals. The most effect is seen after 24 hours of treatment. However, it is important to mention that the evaluation of the outcome is problematic due to the high background colour.

4.3.3. OATP4A1 in cells after treatment with IL-6 and TNFα
The treatment was performed in the Ht29-cancer cell line. The concentration of, interleukin 6 and tumor necrosis factor α was 100 ng/ml and 10 ng/ml respectively. The experiments were performed in duplicates.

As demonstrated in Figure 32, the intensity of OATP4A1-stained cells seems to decrease with a longer stimulation period. At the first treatment interval, the intensity is around 17% higher than that of the control, with longer duration it is reduced to 90% of the control values after 24 hours of stimulation.
The number of positively stained cells expressing the membrane protein OATP4A1 is about 50% higher than the control after 6 hours of treatment (Figure 33). After 12 hours, it reaches the maximum rate and decreases again after 24 hours.

Cells were stimulated with both interleukin 6 (100ng/ml) and tumor necrosis factor α (10ng/ml) for 6, 12 and 24 hours. The statistical report is provided through TissueQuest. "Mean Value" refers to the intensity of OATP4A1- positive cells expressed as a percentage compared to the non-stimulated control cells.

Cells were stimulated with both interleukin 6 and tumor necrosis factor α (10ng/ml) for 6, 12 and 24 hours. The statistical report is provided through TissueQuest. "Mean percent" refers to the number of OATP4A1- positive cells expressed as a percentage compared to the non-treated control cells.
Under the influence of both IL-6 and TNFα the best intensity is observed. The assessment of the staining for cells treated for 12 hours is difficult according to the high background colour.

**Figure 34: selected image sections of stained cells treated with both interleukin 6 (100ng/ml) and tumor necrosis factor α (10ng/ml) for 6, 12 and 24 hours vs. untreated cells**

The immunofluorescence stained cells were scanned with TissueFAXS®. The nucleus is blue according to the DAPI staining. The membrane protein OATP4A1 forms the green edge around the nucleus.

### 4.4. Indirect Immunohistochemistry staining of colorectal cancer tissue

15 samples of malignant diverticulitis tissues were stained with an antibody against vimentin as a marker for cells of mesenchymal origin. The virtual images were generated after scanning of the stained tissue slides with TissueFAXS® program. The acquired data was quantitatively analysed using the HistoQuest® software.

Because tumor cells are negative for vimentin, only stroma and immune cells were analysed. The staining intensity of the immune cells and stroma cells is similar. (Figure 25)
Figure 35: The intensity of stained samples with Vimentin

*Categorization of tissues in tumor, stroma and immune cells.*

Figure 36 shows that the percentage of immune cells stained was about 86% whereas only 44% of the stroma cells were stained.

Figure 36: The percentage of stained samples with Vimentin

*Categorization of tissues in tumor, stroma and immune cells.*
The immunoreactive score of stroma and immune cells was calculated. Figure 37 illustrates how IRS values in immune cells are higher due to the higher percentage of stained immune cells. The IRS values for immune cells are twice as much as for stroma cells.

![IRS values of stained samples with Vimentin](image)

**Figure 37: IRS values of stained samples with Vimentin**

*IRS = Intensity x Percentage*

The first observed feature in Figure 38 is the negative vimentin staining of tumour cells. It is also noticed in the following illustration that the staining intensity of immune cells and stroma is nearly the same. Additionally, colorectal tumour cells are tightly surrounded by a great amount of immune cells. The staining pattern doesnot differ between tissues from patients Stage I or II.
Figure 38: Staining differences of tumor, immune and stroma cells in human CRC tissue samples

A: immune cells, B: Tumor, C: Stroma
5. Discussion

5.1. Role of inflammatory mediators in CRC

It is well established that inflammation processes are linked to tumorigenesis. Cancer initiation as well as promotion, malignant transformation, invasion and metastasis are mostly affected by inflammatory responses. Apart from the decisive role at all stages of cancer development, inflammatory mechanisms influence the response to therapy by augmenting or decreasing the therapeutic effects. The direction is dependent of the expression and activation of certain immune mediators, modulators and cell types. [113]

In CRC associated with the inflammatory bowel disease, called colitis-associated cancer, and in the sporadic CRC, the colon tissue is infiltrated by immune cells. The inflammatory microenvironment plays a role either as tumor growth and survival factor or it can have an antitumoral effect. The production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) by activated inflammatory cells causes DNA damage and mutations. It can lead to the inactivation of the mismatch repair enzymes. [114]

Oncogenic transcription factors and signalling pathways are important in cancer development. The activation of NF-kB transcription factors influences the carcinogenesis by stimulating cell proliferation and decreasing cell death. Cell invasion and metastasis are promoted by NF-kB activation as well. Cytokines, such as TNF, IL-6 and IL-1, trigger tumor growth while other cytokines, like IL-10 and TGF-β can prevent colorectal cancer development. Patients with inflammatory bowel diseases are at high risk to develop colitis-associated cancer. Tumorigenesis is often linked to the presence of proinflammatory cytokines. [114]

The focus of the thesis was to study the influence of TNF-α and IL-6 on the expression of OATP4A1 in HT-29 cells. Moreover, the presence of immune cells in malignant diverticulitis tissue samples was also investigated.
5.1.1. Interleukin-6 (IL-6)
The proinflammatory cytokine IL-6 is regarded as promotor of colorectal tumor growth. Its serum levels are correlated with the cancer stage, the survival rate and metastasis estimating its role as prognostic factor. The multifunctional protein participates in many physiological and pathological functions leading to cell growth, differentiation, cell motility and angiogenesis. In the colon cancer cell line Colo 205, IL-6 stimulates the phosphorylation of the transcription factor STAT3 mediating the immune response and oncogenesis. It was observed that IL-6 serum levels are higher in colorectal cancer patients than in normal individuals. However, these patients show a reduced overall survival rate. [115],[116],[117],[118]

The experiments demonstrate a higher expression of OATP4A1 mRNA after addition of IL-6 to the HT-29 cells. The stimulation of the protein expression after 12 and 24 hours of treatment was only seen in some experiments. An increase of the expression of OATP1B3 mRNA is observed as well.

In most experiments, a higher expression of OATP4A1 on the mRNA- and protein level after treatment with IL-6 was noticed. These observations point out the important role of the inflammatory mediator concerning the expression of OATPs in HT-29 cells. A possible effect to the increased expression of OATP4A1 in colorectal cancer is that there could be an increase of the uptake of drugs into cancer cells. However, further investigations are required to confirm the results. For a better understanding of the role of the upregulation of OATP4A1 caused by IL-6 on the outcomes of the therapy, more studies are necessary.

5.1.2. Tumor necrosis factor - α (TNF- α)
The pro-inflammatory cytokine is involved in physiologic and pathologic processes similar as seen for IL-6. It is regarded as endogenous tumor promotor due to its involvement in chronic inflammatory diseases, which subsequently may lead to the proliferation and spread of cancer. While TNF-α stimulates the growth of fibroblasts and other angiogenic factors, it is a crucial factor at NK-cell-mediated killing of tumor cells. This makes its function in cancer not easily understandable. A poor prognosis is linked to TNF-α production by cancer. [119],[120]
In this study an elevated expression of OATP4A1 mRNA was seen in HT-29 cells after addition of TNF-α. Analogue to the results of IL-6, the expression rate is variable. An increase in the expression of OATP1B3 is also observed on mRNA level. However, in the immunofluorescence a conclusion about the expression pattern of OATP4A1 was not possible to evaluate because of the inconsistent growth pattern of the cells and the high staining background colour.

In summary, elevated concentrations of OATP4A1 in HT-29 cells are observed on mRNA- level as well as on protein level. The results refer to the significant role of TNF-α on the expression mode of OATPs in HT-29 cells. Nevertheless, further studies are needed to elucidate the upregulation of the transport protein OATP4A1 -caused by of TNF-α- and its consequences on a given therapy.

5.1.3. Effect of IL-6 and TNF-α on OATP expression
The highest expression of OATP4A1 mRNA was found after addition of both inflammatory mediators, IL-6 and TNF-α to HT-29 cells. This may relate to the additive effect of both cytokines on the growth of HT-29 cells. A strong increase of OATP1B3 mRNA expression was observed after adding IL-6 and TNF-α to the cells indicating the strong influence of inflammatory mediators on this protein. However, additional studies are required to get a better knowledge of the role of both cytokines on OATP expression which may affect colorectal cancer initiation, progression, and metastasis. This would allow to consider these mediators as therapeutic targets.

5.2. Role of anti-inflammatory agents in CRC therapy
The upregulation of inflammatory mediators in colorectal cancer make them new targets for the development of new drugs. Antagonists of inflammatory mediators are known to be beneficial in cancer prevention. For patients with chronic inflammatory bowel disease, anti-inflammatory agents may serve as preventive agents. They could contribute to the reduction of the CRC incidence. However, it is questionable whether cytokine antagonists would be sufficient for colon cancer therapy, particularly if they are applied in as a monotherapy. A therapeutic potential of inhibitors of pro-inflammatory cytokines is seen in adjuvant therapy regimes. For example, the
antibodies bevacizumab or cetuximab which are combined with the various treatment regimes.

Another consequential aspect would be to consider the effect of inhibitors of pro-inflammatory agents on the expression of OATP4A1. If OATP4A1 is responsible for the uptake of endogenous compounds e.g. steroid hormones which could influence tumor growth, than suppression of the expression of the transporter could be beneficial. However, if OATP4A1 might contribute to the uptake of anticancer drugs into the tumor, blocking the expression of the protein would suppress the efficacy of a certain regimen. Therefore further studies are need to elucidate the role of the transporter in colon cancer in order to prove high suitability for a therapeutic invasion.
List of Figures

Figure 1: Anatomy of the colon ................................................................. 1
Figure 2: The Histology of the colonic wall ........................................... 2
Figure 3: The number of estimated new cancer cases and death worldwide, 2008... 3
Figure 4: Key molecular events associated with the chromosomal instability pathway ........................................................................................................ 6
Figure 5: Main events involved in the initiation of CRC with MSI .............. 7
Figure 6: Old nomenclature system of the human and rodent OATP/Oatp Family .. 26
Figure 7: New nomenclature of the OATP/Oatp superfamily ................. 26
Figure 8: Cell count Report ........................................................................ 37
Figure 9: The conversion of mRNA to cDNA by Reverse Transcription .... 42
Figure 10: The PCR growth curve ............................................................. 44
Figure 11: Fluorescence detection through the TaqMan Method .......... 45
Figure 12: Workstation consisting of the TissueFAXS® System and TissueQuest® Software (TissueGnostics GmbH, Vienna, Austria) .......... 48
Figure 13: Schematic principle of Indirect Immunofluorescence........... 50
Figure 14: mRNA-Expression of OATP4A1 in the HT29- cancer cell-line .... 53
Figure 15: mRNA-Expression of OATP4A1 in the HT29- cancer cell-line .... 53
Figure 16: mRNA-Expression of OATP4A1 in the HT29- cancer cell-line .... 54
Figure 17: mRNA-Expression of OATP4A1 in the HT29- cancer cell-line .... 55
Figure 18: mRNA-Expression of OATP4A1 in the HT29- cancer cell-line .... 55
Figure 19: mRNA-Expression of OATP4A1 in the HT29- cancer cell-line .... 56
Figure 20: mRNA-Expression of OATP4A1 in the HT29- cancer cell-line .... 57
Figure 21: mRNA-Expression of OATP4A1 in the HT29- cancer cell-line .... 57
Figure 22: mRNA-Expression of OATP4A1 in the HT29- cancer cell-line .... 58
Figure 23: mRNA-Expression of OATP1B3 in the HT29- cancer cell-line .... 59
Figure 24: mRNA-Expression of OATP1B3 in the HT29- cancer cell-line .... 60
Figure 25: mRNA-Expression of OATP1B3 in the HT29- cancer cell-line .... 60
Figure 26: The intensity of OATP4A1 stained cells after interleukin 6 treatment .... 62
Figure 27: Percentage rate of HT29-cells with the protein OATP4A1 after interleukin 6 treatment ................................................................. 62
Figure 28: selected image sections of stained cells treated with interleukin 6 (100ng/ml) for 6, 12 and 24 hours vs. untreated cells .................................................. 63
Figure 29: The intensity of OATP4A1 stained cells after tumor necrosis factor α treatment ........................................................................................................................................................................ 64
Figure 30: Percentage rate of HT29-cells with the protein OATP4A1 after tumor necrosis factor α treatment ........................................................................................................................................................................ 64
Figure 31: selected image sections of stained cells treated with tumor necrosis factor α (10ng/ml) for 6, 12 and 24 hours vs. untreated cells .................................................. 65
Figure 32: The intensity of OATP4A1 stained cells after combination treatment consisting of interleukin 6 and tumor necrosis factor α treatment ........................................................................................................................................................................ 66
Figure 33: Percentage rate of HT29-cells with the protein OATP4A1 after combination treatment consisting of interleukin 6 and tumor necrosis factor α treatment ........................................................................................................................................................................ 66
Figure 34: selected image sections of stained cells treated with both interleukin 6 (100ng/ml) and tumor necrosis factor α (10ng/ml) for 6, 12 and 24 hours vs. untreated cells ........................................................................................................................................................................ 67
Figure 35: The intensity of stained samples with Vimentin ........................................ 68
Figure 36: The percentage of stained samples with Vimentin ................................... 68
Figure 37: IRS values of stained samples with Vimentin .......................................... 69
Figure 38: Staining differences of tumor, immune and stroma cells in human CRC tissue samples ........................................................................................................................................................................ 70
List of Tables

**Table 1:** The TNM staging system for CRC classification-7th edition ...................... 19
**Table 2:** The staging system due to Duke ........................................................................ 20
**Table 3:** A combined classification scheme based on the TNM- & Duke staging system ................................................................................................................................. 20
**Table 4:** Statistical 5-years survival rate for patients due to their clinical and pathological stage .......................................................................................................................... 21
**Table 5:** List of OATPs with their organ distribution and substrates ............................ 27
**Table 6:** Scheme of Chamber slides ................................................................................. 39
**Table 7:** The composition of the Master Mix ................................................................... 41
**Table 8:** The composition of Mastermix for 1 well ......................................................... 45
**Table 9:** List of Antibodies used for immunofluorescence staining of cells ............... 47
**Table 10:** Settings used for the scanning ........................................................................... 48
**Table 11:** Specific settings for DAPI and GFP used while scanning .............................. 49
References


