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Abstract

The organic anion transporting polypeptide transporter OATP4A1, a member from the SLC 21-membrane transporter family may be important for tumor progression and the therapeutic response to chemotherapeutic drugs, due to its role in the transport of endogenous metabolites, xenobiotics and drugs. By transporting hormones, prostaglandins, cyclic nucleotides and chemotherapeutic agents into the cells, OATP4A1 influences the cellular levels of these compounds and their therapeutic efficacy.

Therefore, we investigated and defined the expression and potential prognostic value of OATP4A1 in 185 specimens from early colorectal cancer (UICC Stage I/II) and did comparative studies in 18 non-malignant diverticulitis samples.

OATP4A1 levels were evaluated by quantitative microscopic image analysis on paraffin-embedded tissue sections stained by immunohistochemistry (IHC). Quantification of the immunoreactive score (IRS) of OATP4A1 was done separately, on tumor cells, stroma and immune cells, using the HistoQuest® software. Cell identification was done automatically based on algorithms for cell identification from nuclei stained with hematoxylin.

OATP4A1 was detected in the membrane and cytosol of colon cancer and immune cells. A higher immunoreactive score (IRS calculated from the percentage of stained cells x staining intensity) was found in the cancer areas than in the normal mucosa in the adjacent non-cancerous tissue. However, highest OATP4A1 levels were observed in immune cells in the tumors from patients without an early relapse compared to patients with a relapse within five years. Similar values for OATP4A1 were observed in tumor and stroma cells, present at a higher number in tumors of patients with a better prognosis. In normal mucosa and in the mucosa of diverticulitis patients, OATP4A1 was largely absent.

To identify OATP4A1-positive cell types, double-immunofluorescence staining (IF) with appropriate cellular markers was done. OATP4A1 positive immune cells were identified in subpopulations of CD45⁺ leukocytes, CD3⁺ T- and CD20⁺ B-lymphocytes, monocytes, macrophages, dendritic cells and CD34⁺ precursor cells.

Abundant expression of OATP4A1 in cancer and immune cells in colonic disease led to the conclusion that by providing endogenous substrates (prostaglandins, cyclic

nucleotides or peptides) to cells, the transporter may contribute to cell proliferation and differentiation.

Abstract (German)

Transportproteine aus der Familie der Organischen Anion Transportierenden Polypeptide (OATP Familie, SLC21) regulieren den Transport und die Aufnahme unter anderem von Hormonen, Prostaglandinen, cyclischen Nukleotiden und verschiedenen Chemotherapeutika. Daher könnte OATP4A1 als Angriffspunkt für neue therapeutische Ansätze zur Therapie von Tumoren wichtig sein.

Die Expression von OATP4A1 wurde mittels quantitativer mikroskopischer Bildverarbeitungstechnik an Formaldehyd- fixierten und Paraffin-eingebetteten Gewebeproben erforscht. Dafür wurden Gewebeschnitte von 185 Patienten mit kolorektalem Karzinom (UICC Stadium I/II) untersucht und die Daten mit 18 Divertikulitispatienten verglichen.

Die Schnitte wurden mittels Immunhistochemie gefärbt und die Färbeintensität, wie auch der Prozentsatz an gefärbten Zellen mit dem Programm HistoQuest® ausgewertet, wobei zwischen Tumor-, Immun- und Stromazellen unterschieden wurde. OATP4A1 Membran- und Cytosolfärbung wurde sowohl in Tumorzellen, als auch in Immunzellen gefunden. Bei manchen Tumorpatienten war vereinzelt auch eine Kernfärbung zu sehen.

Ein höherer immunreaktiver Wert (immunoreactive score IRS) wurde bei Kolonkarzinompatienten, welche kein Rezidiv innerhalb von fünf Jahren erlitten, festgestellt, im Vergleich zu Patienten mit Rezidiv und normalen Epithelzellen. Die höchste Expression von OATP4A1 wurde in Immunzellen von Patienten mit einer besseren Prognose gefunden (kein Rezidiv innerhalb von 5 Jahren). Ähnliche Ergebnisse wurden auch für Tumor-, und Stromazellen beobachtet. Im Vergleich zu Epithelzellen in Divertikulitisproben zeigte sich nur eine sehr schwache Expression von OATP4A1.

Zur Identifizierung von Immunzellen, welche OATP4A1 exprimieren, wurden Doppelimmunfluoreszenzfärbungen mit geeigneten Markern durchgeführt. OATP4A1 positive Immunzellen wurden in Subpopulationen von CD45⁺ Leukozyten, CD3⁺ T- und CD20⁺ B-Lymphozyten, Monozyten, Makrophagen, dendritischen Zellen und CD34⁺ Vorläuferstammzellen gefunden.

Die erhöhte Expression von OATP4A1 sowohl in Tumor-, als auch in Immunzellen in Kolonkarzinompatienten weist darauf hin, dass die Aufnahme von endogenen

Substraten wie Prostaglandinen, cyclischem AMP, Peptiden oder Arzneistoffen, möglicherweise für Zellproliferation und das Tumorwachstum wichtig sein könnte.

1 Introduction

1.1 The Intestines

The intestines consist of the small intestine, large intestine, and rectum. The small intestine (small bowel) is divided into the duodenum, jejunum, and ileum in order to absorb most of the nutrients from the diet. The large intestine (colon) absorbs water from wastes and forms the stool, which is excreted through the rectum.

1.1.1 Anatomy and Physiology of the Small Intestine

The small intestine is divided into three sections, namely the duodenum which follows right after the pylorus sphincter of the stomach, the jejunum and ileum, which is the largest part of the intestine (approx. 2 m). All three segments have an enlarged intestinal surface formed by many folds, intestinal villi and microvilli in the mucosa (Welsch, 2006). The microvilli and the epithelial cells build the so-called brush border. They can be seen in the electron microscope, whereas they give a paintbrush-painted look of the surface in the light microscope.

Between the villi, crypts of Lieberkühn are localized, which secrete intestinal fluid. Apart from epithelial enterocytes and glandular epithelial cells (Goblet cells), which secrete gel forming mucins, enterochromaffin cells and Paneth cells are also present. The latter produce lysozymes and other enzymes and play a role in microbial processes in the intestine. Enteroendocrine cells are responsible for the secretion of hormones like secretin, cholecystokinin (CKK) and peptides (Tortora and Derrickson, 2008).

1-2 liters intestinal fluid containing water, mucus and pancreatic juices are produced on a daily basis to support the resorption of nutrients simultaneously.

Although the enzymatic digestion of nutrients already starts in the mouth by alpha-Amylase, the main digestion takes place in the stomach, where the nutrients are digested to the so called chyme. In the duodenum gastric juices, bile fluids and pancreatic fluids composed of trypsin, elastase, carboxypeptidase and lipase, merge seamlessly together.

All in all, chemical and mechanical processes of digestion influence and regulate the resorption of amino acids, monosaccharides and fatty acids through absorptive cells

which are connected to lymph vessels and blood circulation, to make the nutrients available for the body. The resorption of electrolytes is carried out by ion pumps. The intestinal Na^+/K^+ -ATPase is important for the transport of electrolytes, because it provides energy from the hydrolysis of ATP for a number of secondary active transporters.

The resorption is based on diffusion, facilitated diffusion, osmosis and active transport (Tortora and Derrickson, 2008).

1.1.2 Anatomy and Physiology of the Colon

The large intestine has a length of approximately 1.5 m and a diameter of 6.5 cm. It is mainly divided into four areas, the caecum, the colon with the colon ascendens, colon transversum, colon descendens and colon sigmoideum, the rectum and the anal canal (Tortora and Derrickson, 2008).

The colon wall is characterized by four layers, namely, mucosa, submucosa, muscularis and serosa. The cylindrical shaped epithelium of the mucosa contains mucus producing goblet cells and absorptive cells with small microvilli which mediate the absorption of water. Both cell types are localized in the intestinal glands, the so called crypts. In comparison to the small intestine there are no villi (Welsch, 2006).

One of the main functions of the colon is the absorption of water, vitamins and ions, such as potassium or sodium. The chyme is turned into feces by reducing the amount of water, which is transported into the colon, from 0.5-1.0 liters to 100-200 ml. The bacteria colonized in the intestinal flora ferment the last parts of carbohydrates and thereby release carbon dioxide and methane which may cause flatulence. Additionally proteins are degraded to amino acids.

As already described above, the goblet cells produce mucus to make the content of the colon lubricant. The peristaltic movements and waves move the undigested remains along the colon and through the contraction in the colon sigmoideum and rectum, the act of defecation gets initiated.

A fiber based diet accelerates the passage of the chyme and supports the intestinal motility.

Therefore, fiber rich food containing cellulose, lignin and pectin prevents the risk of obesity, diabetes, atherosclerosis, diverticulitis and colorectal cancer (Tortora and Derrickson, 2008).

1.1.3 The Immune System of the Large Intestine

The intestinal lumen is exposed to many potentially pathogenic microorganisms. Therefore, there is an effective immune system at the mucosal barrier provided by lymphocytes, macrophages and other cells of the immune system, the so-called gut-associated lymphoid tissue (GALT).

Lymphocytes in the GALT are organized in three structures.

Peyer's Patches (lymphoid follicles) which are found in the mucosa, reach into the submucosa of the small intestine. They are similar to lymph nodes and contain many B-lymphocytes. Smaller lymphoid nodules are also visible.

Lamina propria lymphocytes are B-cells that secrete Immunoglobulin A (IgA).

Intraepithelial lymphocytes are mainly found in the luminal epithelial cells of the Lamina propia at the basolateral side of the colonocytes. Macrophages are also located there.

In addition, microfold cells (M cells) are found in lymphoid follicles. They transport protein and peptide antigens to local dendritic cells and macrophages.

If dendritic cells and macrophages get antigens from M cells, they present them to T-cells. This causes IgA-secretion from plasma cells. Dendritic cells also collect luminal antigens from spaces located between epithelial cells.

Secretory IgA is transported into the lumen by epithelial cells where it prevents the adhesion and invasion of bacteria together with Immunoglobulin M (IgM). This mechanism serves as a protection against pathogens (Junqueira et al., 2005).

1.1.4 Tumor Immunology

The B and T-lymphocytes have a central function in the destruction of tumor cells. Above all, the natural killer cells and activated macrophages are able to react against cancerous cells without a specific stimulus.

In spite of this immunological vigilance the development of malignant tumors can occur, based on different mechanisms which are subject for discussion.

Tumor cells release tumor specific antigens in larger quantities, which interfere with the receptors of natural killer cells and cytotoxic cells, resulting in a lack of an immunological activation. This can also be the case if too little specific antigens are released by the cancerous cells, leading to an insufficient immune response.

Other factors are the absence of tumor recognizing genes, the age dependent decrease of powerful immune cells or the masking of tumor specific antigens by humoral antibodies (Thomas, 2006a).

1.2 Colon Cancer

1.2.1 Epidemiology

The prevalence of colon cancer is relatively high in the western civilization; especially in North America, Australia, Japan and Europe. These countries have the highest incidence rates. Colon cancer is one of the most frequently tumor related causes of death worldwide (Böcker et al., 2008).

After breast cancer in women and prostate cancer in men, and lung cancer, colon cancer is the most common leading cause for cancer related mortality. The incidence of colon cancer in males is higher than in women (Labianca et al., 2013; Vogl et al., 2014).

Growing incidence of colon cancer is related to changes in diet and lifestyle. A lack of physical exercise, obesity, smoking, and the consumption of too much alcohol, red meat and additionally, genetic predisposition promote the development of colon cancer.

However, the mortality rate has already decreased in some western countries, notably in the female population, probably due to fecal occult blood tests, endoscopic screenings leading to an early detection of precancerous lesions in the tissues and removal of polyps during a colonoscopy (Hutchison et al., 2013). From 1997 to 2007, the mortality rate in Europe has decreased by 8% in women and 6% in men per quinquennium (Bosetti et al., 2011; Labianca et al., 2013).

This phenomenon is also seen in Austria for the period from 1986 to 2011, where the incidence and the mortality rates of colon cancer have decreased not only in men, but also in women (Figure 1).

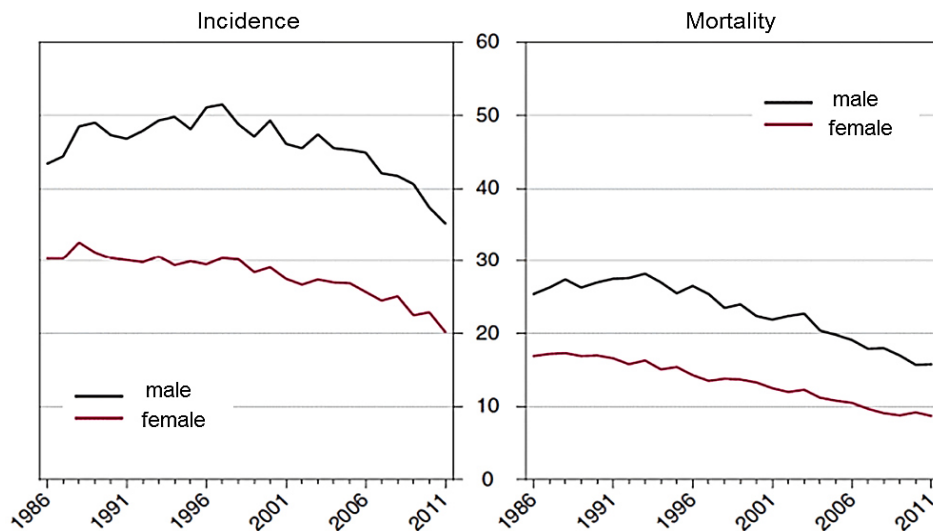


Fig. 1: Incidence and mortality of colon cancer in Austria

The incidence and mortality rates refer to 100,000 patients in Austria. A decrease in incidence and mortality is remarkable (Statistik Austria, 2013).

1.2.2 Etiology

The etiology of colon cancer covers different aspects and risk factors. The majority of patients suffering from colon cancer are aged over 65 years. Apart from a western life style including smoking habits and obesity, polyps, hereditary factors and inflammatory bowel diseases are seen as possible risk factors for the development of colon cancer.

Polyps and Adenoma-Carcinoma Sequence

Polyps are slow developing benign alterations in the mucosa of the large intestine. They often do not cause any symptoms, but are usually detected by traces of blood. Polyps are usually removed during a colonoscopy as they can become malignant (Tortora and Derrickson, 2008).

Therefore polyps and a positive family history of colorectal cancer or adenomas are seen as positive risk factors for colon cancer.

Inflammatory Bowel Diseases

The idiopathic inflammatory diseases ulcerative colitis and Crohn's disease increase the risk for colon cancer. An activation of the nuclear factor κ B (NF κ B) pathway may promote the secretion of proinflammatory cytokines. The resulting chronic inflammation with tissue repair is thereby a risk factor for the accumulation of cells

carrying mutations. This can lead to the malignant transformation of cells (Setia et al., 2014).

Hereditary Factors

Although most of the colon cancer cases are based on the development of a sporadic tumor, 5-10% of the patients show a genetic determination for colon cancer. The Lynch syndrome, also known as hereditary non-polyposis colorectal cancer (HNPCC) is the most frequent inherited cause for colon cancer. It is associated with mutations in the mismatch repair genes hMSH2 and hMLH1. A dysfunction in the process of replication and therefore also the promotion of other malign neoplasms is the consequence of this mutation (Hiddemann et al., 2004). In cancer patients, micro satellite instability, which consists of repetitive nucleotide sequences, is observed, whereas healthy patients do not show such instabilities (Wilmanns et al., 2000).

Patients with familial adenomatous polyposis coli (FAP) show a mutation in the adenomatous polyposis coli (APC) gene, a multifunctional tumor suppressor, localized in the region between the chromosomes 5q21 and 5q22. This results in a non-functioning APC gene leading to unregulated proliferation. An APC mutation is nearly 100% associated with colon cancer risk. (Nishisho et al., 1991; Nakamura et al., 1992; Vogelstein and Kinzler, 2004; Hutchison, 2013). Additionally, patients have a higher risk of malignancies in the oesophagus, stomach and duodenum (Hiddemann et al., 2004).

Single Nucleotide Polymorphisms (SNPs)

As defects in the DNA are related to cancer risk, single nucleotide polymorphisms (SNPs) leading to altered genotypes, have attracted attention. Risk factors for colon cancer are also genes, which mediate DNA repair mechanisms. Variations in the gene sequences may affect the mRNA levels and subsequently, also the protein expression (Duan et al., 2009; Naccarati et al., 2012).

For example, Chen et al. (2006) found an increased risk of colon cancer in the Taiwanese population because of frequent SNPs in the APC gene in this population.

1.2.3 Pathogenesis

Colon cancer is a malign epithelial tumor, derived from malignant transformation of colorectal mucosa cells with invasion into the submucosa or deeper mucosa layers (Böcker et al., 2008).

Approximately 90% of the malign tumors of the digestive tract are developed by the epithelial cells of the mucosa (Junqueira et al., 2005).

50% of the carcinomas are located in the rectum, 25% in the sigma and the other 25% affect the other part of the intestine (Thomas, 2006b).

Colorectal carcinomas often develop sporadically based on adenomas. Related risk factors like a positive family history, smoking, low fiber diet as well as chronic and inflammatory bowel diseases promote colon cancer.

Colorectal adenomas are benign tumors of the epithelial gland. They are classified as precancerous lesions and can progress to invasive adenocarcinomas according to the adenoma-carcinoma sequence. Four different types of adenomas are known, namely tubular, villous, tubularvillous and pseudopapillar adenomas.

In 20-25% of the cases, adenomas stay without clinical signs, but on account of the risk of them becoming malignant, they should be removed completely. If this is not possible during an endoscopic examination, the resection of the malignant part of the intestine is recommended. In particular, the villous type has the highest degeneration risk showing the lowest level of differentiation and the highest dysplasia rates (Böcker et al., 2008; Leischner, 2010).

1.2.4 TNM Classification

Among different staging systems of malign diseases, the TNM staging system is accepted worldwide for the classification of carcinomas and sarcomas. The rules and criteria of the TNM classification system are based on the convention of the Union Internationale Contre le Cancer (UICC) committees. According to the latest achievements and clinical trials, the data are updated regularly (Hiddemann et al., 2004).

Two different types of TNM classification are used.

- The clinical TNM (cTNM) system is based on the clinical results before therapy or operation.
- The pathologic-anatomic TNM classification (pTNM) is focused on the detailed examination of the surgical specimen.

The TNM system is mainly based on three components (TNM). T stands for the extent of the tumor. The absence or presence of lymph node metastases is characterized as component N and component M represents the absence or presence of distant metastases.

By adding numbers to these three components, the anatomic extent of the tumor is defined.

The definition of the different tumor stages is done on basis of prognostic data. For instance, not only the extent and localization of the primary tumor is defined, but also the size and infiltration depth (Hiddemann et al., 2004).

Tab. 1: TNM classification of colorectal cancer (National Cancer Institute)

T	Primary tumor
T0	No evidence for a primary tumor
Tis	Carcinoma in situ
T1	Infiltration of the submucosa
T2	Infiltration of the muscularis propria
T3	Infiltration of the subserosa or of the non-peritonealised pericolic or perirectal tissue
T4	Tumor invasion to other organs or tumor perforation of the visceral peritoneum
N	Regional lymph nodes
N0	No metastases in regional lymph nodes
N1	Metastases in 1-3 regional lymph nodes
N2	Metastases in more than 3 regional lymph nodes
M	Metastases
M0	No distant metastases
M1	Presence of distant metastases

Colorectal cancer is usually staged according to the precise TNM classification, but the so called Dukes classification has established itself as well for the staging of tumors (Wilmanns et al., 2000; Leischner, 2010).

Staging of Colon Cancer according to Dukes (National Cancer Institute)

Dukes A: The tumor is limited to mucosa and submucosa

Dukes B: The tumor already infiltrates all layers

Dukes C: Local lymph nodes metastases

Dukes D: Distant metastases

Tab. 2: Overview of TNM staging and Dukes (National Cancer Institute)

TNM stage	T	N	M	Dukes
Stage 0	Tis	N0	M0	
Stage I	T1	N0	M0	} A
	T2	N0	M0	
Stage II	T3	N0	M0	} B
	T4	N0	M0	
Stage III	all T stages	N1	M0	} C
	all T stages	N2	M0	
Stage IV	all T stages	all N stages	M1	

1.2.5 Diagnosis and Screening

As 30-40% of colon tumors get detected during an examination, especially through palpation of the rectum, it is an inherent part of tumor diagnosis (Leischner, 2010).

The fecal blood test is a routinized and fast, but yet not a sensitive test. 1.2% of asymptomatic patients show a positive fecal blood test allowing an early diagnosis of colon cancer (Wilmanns et al., 2000).

Endoscopy is one of the most important early diagnostic tools in the diagnosis of intestinal tumors. The American Cancer Society recommends undergoing a colonoscopy every 5 years for people over 50 years of age. The endoscopic examination is an invasive and specific method, requiring bowel preparation and often sedation of the patient. A big advantage of colonoscopy is that it is possible to collect biopsies, locate precancerous and cancerous lesions and remove tumor indicating polyps. The endoscopic examination can be done either for the whole colon (total colonoscopy) or for the sigmoid, where more than 35% of the tumors are located (Labianca et al., 2013). Total colonoscopy is associated with lower mortality rates in examined patients as compared to patients who have never undergone this examination (Nishihara et al., 2013).

The computer-assisted tomography (CT) methods for virtual colonoscopy permit the localization of the tumor (Labianca et al., 2013).

In the case of a confirmed diagnosis of colon cancer, further screening procedures are advised to determine the extent of the tumor. These include X-ray of the thorax, sonography especially of the small bowel, CT scans, determination of laboratory parameters and tumor markers such as lactate dehydrogenase (LDH), carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9). Although tumor markers are not specific for colon cancer, they are crucial for the follow-up. Elevated CEA values return to normal after a successful surgery. A persistent high level of CEA or a repeated increase after the removal of the tumor may indicate the appearance of a residual tumor or metastases. After a successful chemotherapy, the levels of tumor markers should decrease to normal values. If this is not the case, it might be a sign of chemoresistance (Wilmanns et al., 2000).

1.2.6 Symptoms

The number of symptoms is associated with the staging and localization of the tumor. The essence of the problem is that colon cancer often does not cause any specific symptoms at the beginning unless the tumor is of a large size. In many cases, patients only suffer from pain when the tumor has already advanced (Böcker et al., 2008; Laubert et al., 2010). The 5-year overall survival rates are lower in symptomatic patients than in asymptomatic patients (Laubert et al., 2010).

Rectal and distal sigmoid colon cancers often cause intestinal bleeding and together with the loss of fecal occult blood are primarily responsible for the appearance of anaemia, resulting in weakness, concentration deficits and iron deficiency. Furthermore, changes in bowel habits, tarry stools (melaena), abdominal pain and weight loss are also seen at advanced stages (Böcker et al., 2008; Labianca et al., 2013).

1.2.7 Prognosis

If the tumor is detected at an early stage, a cure is possible. Patients at stage I have a better survival rate than those at stage IV (93.2% vs. 8.1%) according to the American Joint Committee on Cancer (O'Connell et al., 2004). The prognosis is dependent on the tumor stage and the effectiveness of a successful surgery. Patients diagnosed with a T1 or T2 tumor stage and without an invasion of the lymph nodes show a good prognosis (with 90% 5-year overall survival rates) (Wilmanns et al., 2000).

Risk Assessment

After the diagnosis of colon cancer, examination and laboratory tests need to be performed to evaluate the risk of metastases. Above all, liver enzymes need to be examined. However, they may still be in the normal range despite the presence of metastases. For detection or exclusion of metastases a CT scan can be performed but its sensitivity is quite poor. Therefore, the magnetic resonance imaging is more appropriate in evaluating liver metastases.

The use of ultrasonography for examination of liver and abdomen is a routine procedure (Labianca et al., 2013).

1.2.8 Therapy

The first line therapy of colorectal cancer is the surgical removal of the tumor with a safety margin of 5 cm from the healthy tissue.

Isolated metastases in liver or lungs can also be removed surgically in addition to a chemotherapy treatment.

Radiotherapy is not a part of curative therapy anymore, but it may protect the cancer from invading other organs.

The implementation of adjuvant chemotherapy has been proved to be successful (Leischner, 2010).

5-Fluorouracil (5-FU)

Until now, 5-FU remains one of the key players in the first line treatment for advanced colorectal cancer. It increases the survival rate of patients by up to 12 months, while patients who do not receive any chemotherapy, only have a survival rate of approximately 8 months (Schrage, 2004).

5-FU is an antagonist of the pyrimidine bases, uracil and thymidine and causes the inhibition of DNA synthesis. 5-FU is activated and converted to 5-Fluorodesoxyuridine-triphosphate and 5-Fluorodesoxyuridine-monophosphate (5-dUMP). These two intermediates inhibit thymidilate synthase. The consequence is the blockage of DNA synthesis. Additionally, 5-dUMP gets incorporated in the RNA (Mutschler et al., 2008). 5-FU is given i.v. or as infusion in combination with the calcium folinate derivate Leucovorin (LV). In case of liver tumors, 5-FU is also given intra-arterial as a bolus. At stage III of colorectal cancer 5-FU is given together with Leucovorin for 6-8 months (Scheithauer et al., 2003).

5-FU has a half-life between 6 and 20 minutes and up to 20% of the dose is excreted renally (Mutschler et al., 2008). The side effects of 5-FU are cardiac toxicity, neurotoxicity and myelotoxic effects. In general, these side effects can be controlled. Unusual toxicity results in severe diarrhea, mucositis and strong leukopenia or thrombocytopenia. A dysfunction in the catabolism of 5-FU, caused by a defect in the enzyme dihydropyrimidine dehydrogenase, reduces the metabolic elimination of 5-FU, which subsequently leads to high plasma levels with higher toxicity and the risk of death. Modulators like calcium folinate and Methotrexate increase the effect of 5-FU, whereby the addition of Methotrexate is not common anymore due to its complex monitoring (Wilmanns et al., 2000; Hiddemann et al., 2004). Further indications for 5-FU therapy are cancers of the rectum, mamma, oesophagus, urinary bladder and liver (Mutschler et al., 2008).

Oxaliplatin

The platin derivatives Oxaliplatin, Carboplatin and Cisplatin are alkylating agents. They are applied in the chemotherapy of tumors of the colon, ovaries, cervix, endometrium, lungs and testes (Mutschler et al., 2008).

Oxaliplatin is not as nephrotoxic as Cisplatin, but it leads to neurotoxicity in the extremities (Hiddemann et al., 2004). In this case the therapy with Oxaliplatin has to be stopped. The combination of Oxaliplatin and 5-FU/LV (FOLFOX) was examined in the MOSAIC study (Andre et al., 2004). Under this treatment patients experienced a significant increased disease free survival rate with a decreased risk of recurrence compared to the control group which was treated with 5-FU/LV (Andre et al., 2009). Similar data were found in the NSABP C-07 trial. It could be demonstrated that the combination treatment with bolus 5-FU, LV and Oxaliplatin (FLOX) lead to a higher disease free survival time than the medication without Oxaliplatin (FULV) (Kuebler et al., 2007; Labianca et al., 2013).

Irinotecan

Irinotecan is an inhibitor of topoisomerase I and it is used for the treatment of colon cancer. Topoisomerase I is responsible for the DNA strand separation during replication. By inhibiting topoisomerase I, DNA strands break and cell death is the consequence.

Due to a higher activity of topoisomerases in many tumors, topoisomerase inhibitors have an important function as cytostatic drugs. Together with other chemotherapeutics, they act synergistically (Hiddemann et al., 2004).

Irinotecan is usually more effective than Topotecan and is given in combination with 5-FU for advanced colon cancer or cancer of the rectum. This combination is also given as second line therapy after the monotherapy with 5-FU fails.

Heavy diarrhea is a main side effect and often leads to the termination of the therapy. Further side effects are less severe and include increased secretion of saliva and lachrymal fluid (Mutschler et al., 2008).

Capecitabine

Capecitabine is a prodrug of 5-FU. After application of Capecitabine, the activation to 5-FU takes place in the liver. The thymidine phosphorylase, which is produced in a high amount by tumor cells, promotes the activation of Capecitabine to 5-FU.

The drug has a half-life of 40 minutes and is excreted renally.

Capecitabine is applied in the treatment of metastasizing colon cancer. Recent studies have shown that Capecitabine may have a better response to the therapy of colon cancer than 5-FU.

The XELOXA trial compared the treatment of Capecitabine and Oxaliplatin (XELOX) versus FU/LV in colon cancer patients. The outcome was, that the XELOX combination was found to be superior to 5-FU (Haller et al., 2011).

Monoclonal Antibodies

The successful therapy with tumor-specific monoclonal antibodies in different tumor types assumes the relevance of antibodies in mechanisms of tumor elimination (Hiddemann et al., 2004).

The vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) play a crucial role in tumor pathogenesis by inhibiting tumor growth (Schrug, 2004).

High expression levels of VEGF lead to neo-angiogenesis of the tumor and better supply of the tumor cells with blood and nutrients. Therefore, it leads to a higher risk of colorectal cancer progression and recurrence (Camus et al., 2009).

Bevacizumab

Bevacizumab, a humanized monoclonal antibody, binds to VEGF and prevents the new formation and regeneration of vessels and thereby the activation of VEGF. Consequently, tumor growth gets inhibited. Bevacizumab combined with 5-FU is applied in the treatment of metastasizing colon and rectum cancer. Further indications for Bevacizumab, together with Paclitaxel, is metastasizing breast cancer. Side effects, which are seen for Bevacizumab are stomatitis, hypertension, constipation, fever and dyspnoea (Mutschler et al., 2008).

Cetuximab

The chimeric monoclonal antibody Cetuximab interferes with the EGFR pathways, which are involved in the survival of cells and cell cycle. Cetuximab has an approximately 5 to 10 fold stronger affinity to the EGFR than natural ligands. By binding to EGFR, the receptor function gets lost. The consequence is the apoptosis of EGFR expressing tumor cells and the inhibition of neo-vascularization. The application of Cetuximab is indicated for advanced colon cancer after prior unsatisfactory treatment with Irinotecan.

Allergic reactions, dyspnoea and conjunctivitis are the most frequent side effects observed under the treatment with Cetuximab (Mutschler et al., 2008).

1.3 Colorectal Cancer Liver Metastases (CRCLM)

Metastases cause the death of nearly 90% of the patients suffering from a malign tumor. Therefore, metastasis is the central key feature of malignancy (Hiddemann et al., 2004).

The degree of metastasis is classified according to the TNM staging system (Böcker et al., 2008).

The process of metastasis relies on the separation of the individual tumor cells from the surrounding tissue of the primary tumor, the additional breakthrough of the basal membrane and the invasion into vessels. By attaching to host tissues and proliferating in different organs, the metastasis is completed (Hiddemann et al., 2004).

The most common pathways for metastasis are movements of cells via blood and lymphatic vessels. Due to their large pore structure, lymphatic vessels hardly show resistance against invading tumor cells. The cell flow towards the lymph nodes is driven by chemokines.

Lymphogenic metastasis is initiated by the migration of tumor cells of the primary tumor to the lymph nodes through afferent lymph paths.

In the hematogenic pathway, tumor cells travel to different organs through the blood flow. This type of metastasis occurs if tumor cells of the primary tumor directly infiltrate the blood vessels or efferent lymph ways. These types of metastases are usually distant metastases in peripheral organs.

If cells lose the contact to their environment, they normally become apoptotic. However, cancer cells activate anti-apoptosis mechanisms in order to survive in the circulation.

The most critical stage of metastasis is the growth into different tissues and different environments, respectively.

Some tumor cells can survive in tissues without proliferating immediately and form metastatic tumors (Wagener and Müller, 2010).

Since the venous blood from the intestinal tract enters the liver through the portal vein, carcinomas from the digestive tract usually induce the development of hepatic metastasis. Other adjacent organs, such as the stomach, uterus and urinary bladder are usually invaded by the tumors (Böcker et al., 2008).

From a histological point of view, distant metastases and the primary tumor have nearly the same histological pattern. For example, liver metastases of a colon carcinoma also show the gland structure of the primary tumor (Hiddemann et al., 2004).

1.4 Diverticulitis

Diverticulitis is an inflammation of diverticula in the colon. The stage of inflammation can be acute or chronic-recurrent.

1.4.1 Epidemiology

Diverticulitis is a wide ranging disease in the western population, especially in Europe, Australia and the USA. The vast majority of the patients, approximately 75-80% do not show any symptoms which leads to the difficulty to specify the incidence rate (Painter et al., 1971; Stollman et al., 2004). Thus, it is known that the reasons for the increased prevalence are age and life style dependent. Patients older than 70 years are mostly affected (60%); in comparison, diverticulitis is rare in patients younger than 40 years (10%) (Stollman and Raskin, 2004; Jacobs et al., 2007; Sheth et al., 2008).

Although the prognosis of patients who get cured from an acute diverticulitis attack is quite good (Rafferty et al., 2006), one third of the patients suffer from a recurrent attack within one year (Lamanna and Orsi, 1984; Stollman and Raskin, 1999; Rafferty et al., 2006).

1.4.2 Etiology

Diverticulitis is a frequent disease of the large intestine which is often characterized by abdominal pain and changes in stool habits, such as diarrhea or constipation. Biochemical alterations or infections are not found in these patients. Therefore, it is assumed that a disturbed motoric and sensory function is among the sources of this disease. No genetic predisposition has been found to be associated with diverticulitis. Furthermore, there is no evidence that smoking may contribute to the development of diverticulitis. However, in a Swedish cohort study, smokers still carried a higher risk

for a complicated form of diverticulitis (Welsch, 2006; Hjern et al., 2011; Andersen et al., 2012).

An interrelation between the appearance of diverticulitis and diverticular bleeding and physical activity was seen in obese patients with a BMI $\geq$ 30 and after the intake of acetaminophen or non-steroidal anti-inflammatory drugs (NSAIDs) (Aldoori et al., 1995; Strate et al., 2009; Andersen et al., 2012).

Intake of Steroids as a Risk Factor

The application of therapeutic drugs such as steroids and immunosuppressants is considered to be a crucial risk factor for sigmoid diverticulitis (Chapman et al., 2005; von Rahden et al., 2011). The glucocorticoid induced tumor necrosis factor receptor (GITR) is usually expressed by T-cells and its expression is activated by steroids (Nocentini et al., 1997; von Rahden et al., 2012). The group of von Rahden et al. (2012) observed that the expression of GITR in inflammatory diverticulitis samples was increased. It was concluded that activated CD68⁺ macrophages express GITR under inflammatory conditions, such as diverticulitis. The expression of GITR was significantly higher in cases of complicated diverticulitis ($p<0.0001$) than in non-complicated diverticulitis.

Similar data are reported by Chapman et al. (2005) showing a correlation between the perforation of a diverticulum and the mortality after a therapy with steroids ($p<0.001$ and $p=0.002$), suggesting that steroids are evident risk factors for diverticulitis.

Allergic Predisposition and Histamine

Another risk factor includes the possibility of an allergic predisposition and the expression of histamine and its receptors. Histamine is produced in the whole intestine, regulating intestinal motility. An excess of histamine is associated with abdominal changes such as diarrhea (Barbara et al., 2004; van Rahden et al., 2012). These findings were studied in more detail by van Rahden et al. (2012). The immunohistochemical staining revealed a higher expression of the histamine receptors H1R and H2R accompanied by a strong histamine expression in symptomatic diverticulitis as compared to normal colon tissue and non-complicated diverticulitis. The conclusion was that allergic disposition correlates with higher levels

of the histamine receptors H1R and H2R leading to a higher risk for complicated diverticulitis.

1.4.3 Pathogenesis

Diverticula mainly show a pathological sack-like bulge due to a weak point in the different layers of the colon. The mucosa and submucosa are especially affected (Andersen et al., 2012). Within the US and European population the majority of diverticula are localized in the distal colon. They usually have a diameter between 5 to 10 mm (Stollman and Raskin, 2004). The pathophysiology is not completely understood. However, it may be related to a higher intestinal pressure due to a fiber low diet and reduced colonic transit time of small stool volumes.

Although in the majority of the cases the disease remains without symptoms, a small group of patients (5%) develop a severe form with abdominal pain. In some cases, fistulas, abscesses and hemorrhage tendencies occur (Simpson et al., 2003).

1.4.4 Classification

In the German speaking countries the classification according to Hansen and Stock is used (Hansen and Stock, 1999).

In Anglo American countries, the Ambrosetti classification, which describes diverticulitis stages from Type 0 to Type IIA as a mild form, and from Type IIB to Type III as a severe one, is more commonly applied (Ambrosetti et al., 1997).

Tab. 3: Classification of different stages of diverticulitis

Hansen and Stock Classification (1999)		Ambrosetti Classification (1997)
Type of diverticulitis		Type of diverticulitis
Type 0	Asymptomatic diverticulitis	} Mild diverticulitis
Type I	Uncomplicated diverticulitis	
Type II divided into subgroups of	Complicated diverticulitis	
• Type IIA	Phlegmonous diverticulitis	
• Type IIB	Covered perforated diverticulitis	} Severe diverticulitis
• Type IIC	Free perforated diverticulitis	
Type III	Chronic recurrent diverticulitis	

Asymptomatic sigmoid diverticulitis (Type 0)

This Type of diverticulitis generally does not require surgery, because the risk for complicated diverticulitis is low (Hansen and Stock, 1999). The diagnosis is often an incidental finding during routine controls (Stollman and Raskin, 2004).

Uncomplicated diverticulitis (Type I)

This stage is characterized by a fast response to the conservative therapy and therefore a surgical intervention is often not necessary (Hansen and Stock, 1999).

Complicated diverticulitis (Type II)

The phlegmonous diverticulitis (Type IIA) is differently treated according to the patient.

A covered or free perforation as seen in Type IIB or IIC cases is treated surgically. In the histological picture, a high degree of inflammation is seen (Hansen and Stock, 1999).

Chronic recurrent diverticulitis (Type III)

The pathogenesis of Type III is usually treated by surgery (Hansen and Stock, 1999).

1.4.5 Diagnosis and Prognosis

As diverticulitis is an inflammatory disease, the evidence of inflammatory markers such as calprotectin and C-reactive protein (CRP) are considered to be common laboratory parameters (Tursi et al., 2008b). The CT scan of the abdomen and pelvis is among the most important diagnostic methods to find abscesses, perforations and to give an impression of the overall condition of the abdomen (Al-Sahaf et al., 2008; van Randen et al., 2009).

After medication with oral or intravenous antibiotics for acute diverticulitis, most of the patients get cured, revealing a good prognosis for patients who have suffered such an acute attack (Rafferty et al., 2006).

1.4.6 Therapy

Surgical therapy is a strategy to treat different types of diverticulitis. However, standardization has not been established yet.

The indication for sigmoid resection after a second attack has been abandoned by a majority of surgeons and doctors (Chapman et al., 2005; von Rahden et al., 2011).

An operation is often inevitable in the event of perforating sigmoid diverticulitis or in the case of a complicated sigmoid diverticulitis with fistulas and stenoses.

Surgery is not indicated for an asymptomatic sigmoid diverticulitis.

As new medical therapies and diagnostics are emerging, patients have additional therapy options at their disposal. These include fiber supplementation and the application of antibiotics such as rifaximin.

Fiber and Fiber Supplementation

It is a well-known fact that many diseases such as diverticulitis are related to diet and lifestyle issues, especially in industrialized countries (Painter and Burkitt, 1971). The theory that a fiber rich nutrition could be of benefit to patients with symptomatic diverticulitis was confirmed by a study performed in the USA (Aldoori et al., 1994). Small stool volumes, a slow motility and an altered colonic pressure due to a western associated diet may contribute to the symptoms. Treatment with wheat bran or methylcellulose as food supplements was effective in improving patients' symptoms. The outcome was superior to the treatment with placebo (Brodribb and Humphreys, 1976; Hodgson, 1977; Rocco et al., 2009). Although the use of indigestible food components may improve the symptoms of constipation, they may also cause flatulence and discomfort. To prevent these effects the intake of soluble fibers may be helpful.

In any case, the fact that a fiber rich diet constitutes an important part of daily nutrition is beyond dispute, but considering diverticulitis and its pathogenesis, current data do not show a significant improvement of the disease and its symptoms (Rocco et al., 2009).

Antibiotics

Rifaximin

Rifaximin with a pyridoimidazol structure belongs to the antibiotic drugs with a broad spectrum against many bacteria. It is effective against gram-positive and gram-negative bacteria as well as aerobic and anaerobic forms. Due to its chemical structure it hardly gets absorbed (Lamanna and Orsi, 1984).

Rifaximin affects the metabolic processes in the bacterial intestinal flora. According to the data of a multicentre trial, the group of Papi et al. (1992) firstly found a relationship between patients treated with rifaximin plus glucomannan or glucomannan alone. Patients receiving the antibiotic drug and the fiber diet showed a significant improvement of symptoms in comparison to patients only having a fiber based diet. The symptoms on a global score were reduced from 63.9-47.6% (Rocco et al., 2009).

Probiotics

As antibiotics are sometimes not fully effective or if patients suffer from too many side effects, probiotics are increasingly becoming a major focus in the treatment of diverticulitis. The application of probiotics in terms of rebuilding the mucosa in the intestine after an antibiotic treatment is widely accepted. As a result of inflammatory conditions, as seen in diverticulitis, the bacterial flora becomes disturbed. Probiotics as living organisms are able to normalize intestinal mucosa function by strengthening the immune system and inhibiting pathological processes. Probiotics have the benefit that they hardly interfere with the function of antibiotics and, therefore, rarely cause resistance to antibiotics (Gionchetti et al., 2002).

Fric and Zavoral (2003) have pointed out, that in patients suffering from an uncomplicated form of diverticulitis, treatment with the probiotic *Escherichia coli*, strain Nissle 1917, improved the symptoms. The patients experienced a longer remission-free time.

Similar data were found by Tursi et al. (2006) after *Lactobacillus casei* was used as probiotic, either as single treatment or together with mesalazine. Some patients received mesalazine alone. The combination of both, mesalazine and *Lactobacillus casei* was superior in therapy efficacy compared to single treatment. It increased the remission-free time.

Anti-inflammatory Agents

Mesalamine

In diverticulitis certain immune cells can be altered under inflammatory conditions. Indeed, lymphocytic infiltrates were found to be frequently observed in diverticulitis patients than in healthy controls (Tursi et al., 2008a). In addition, the bacterial flora

can change and this contributes to inflammation of intestinal mucosa. Cytokines trigger the development of diverticulitis (Tursi, 2007).

An appropriate treatment with 5-aminosalicylic acid or mesalamine as a single agent inhibits the process of inflammation. According to the data of a clinical trial performed by the group of Trepsi et al. (1999) diverticulitis patients treated with mesalazine experienced a longer remission-free period.

1.4.7 Diverticulitis and the Association with Colon Cancer

In a retrospective study by Gohil et al. (2012) it was revealed that diverticulitis patients who underwent a colonoscopy for the first time were found to have more polyps than patients without diverticulitis. The association between diverticulitis and the risk of polyps or even colon cancer was also revealed in data by Morini et al. (2002) documenting the higher incidence of adenomas in diverticulitis patients than in healthy controls. In contrast, Meurs-Szojda et al. (2008) found no correlation between diverticulitis and colon cancer.

There is evidence showing that the organic anion transporting polypeptides (OATPs), which are known to transport endogenous substrates, such as hormones, peptides and anticancer agents, change their pattern under inflammatory conditions. Studies have shown that OATPs can be up or down regulated in different types of cancer. An altered expression of OATPs was also seen for colon cancer patients.

1.5 Organic Anion Transporting Polypeptides (OATPs)

Drug transporters provide the transport of different substances into and from the cells. They allow the elimination of xenobiotics and thereby provide the detoxification of the body.

An important group of transporters include the organic anion transporting polypeptides (human: OATPs; rodents: Oatps), which are part of an expanding superfamily of the sodium independent transport systems. They are classified as the solute carrier family (SLCO) on the basis of their amino acid sequence identities (Hagenbuch and Gui, 2008). The SLCO family has twelve members, grouped in six families. Different hormones and growth factors modulate their expression (Koepsell et al., 2007; Wojtal et al., 2009).

The expression of OATP transporters is very heterogeneous not only in different species, e.g. humans and rodents, but also in the different tissues within a species. They are found in kidney, lung, liver, intestine and also in other tissues like heart, blood brain barrier, choroid plexus, placenta and testes (Tamai et al., 2000; Hagenbuch and Meier, 2004).

OATPs, like OATP1B1 and OATP1B3 are very tissue specific, as they only occur in healthy liver. They mediate the hepatic uptake of compounds (Hagenbuch and Gui, 2008; Roth et al., 2012). Unlike other OATPs, the liver specific OATP1B1 and OATP1B3 are regulated by the transcription factor, the liver enriched hepatocyte nuclear factor 1 α (HNF1 α) (Jung et al., 2001).

Besides OATP1B1 and OATP1B3, the transporters OATP1A2 and OATP2B1 are also well characterized. OATP2B1 and OATP1A2 are known to be essential mediators for the uptake of anionic drugs in the intestine (Kobayashi et al., 2003; Glaeserl et al., 2007).

Kraft et al. (2010) found high mRNA levels of OATP2A1 and OATP2B1 in ocular tissue. According to their data, the prostanoid latanoprost, which is used for glaucoma therapy, acts as a potent inhibitor of OATP2A1. OATP2A1 being responsible for the uptake of PGE₂ showed a high affinity for its substrate. OATP2A1 might even affect the clearance of prostanoids in eyes. These findings highlight the clinical potential of OATPs as mediating the uptake and clearance of essential natural ligands such as PGE₂.

The family OATP3 only contains the subfamily OATP3A1, which was found to mediate the uptake of estrone-3-sulfate. It was isolated from a human kidney (Tamai et al., 2000).

OATP4 has two subfamilies, namely OATP4A1 and OATP4C1. OATP4A1 was isolated from human kidney and brain (Fujiwara et al., 2001). Substrates, which are transported by OATP4C1, are for example cAMP and thyroid hormones (Mikkaichi et al., 2004).

There are data available for the OATP-families OATP1 to OATP4, but nevertheless little is known about the human OATP super-families 5 and 6 (Hagenbuch and Meier, 2003; König et al., 2006). OATP6A1 is mainly expressed in testes, but so far no data about its function are available (Suzuki et al., 2003; Hagenbuch and Meier, 2004).

OATPs are expressed at varying levels in different tissues. They can be up or down regulated in tumor tissue than in their non-malignant counterpart. For example, according to the work of Wlcek et al. (2008), OATP4A1 was up regulated in non-malignant breast cancer cell lines.

1.5.1 Nomenclature and Classification

Today, OATP nomenclature and classification are standardized. The implementation of a common nomenclature system according to the HUGO Gene Nomenclature Committee was done. This system, which shows similarities to the cytochrome P450 system is based on the amino acid sequence leading to the classification into families and subfamilies.

According to the amino acid sequence homology, OATPs are classified in the same family if members show at least 40% of amino acid identity or in the same subfamilies at least 60% of identity. The number is given due to the chronology of their identification. If there are same proteins in a subfamily, additional numbers will be given, e.g. OATP4A1. So far there are six subfamilies known for human OATPs (Hagenbuch and Meier, 2004).

The gene symbols, on the other hand are classified as *SLCO* for human and *S/co* for rodent members (Hagenbuch and Meier, 2004; Hagenbuch and Stieger, 2013).

Tab. 4: Overview of the human OATPs and their substrates

Superfamily	Subfamily	Substrates	References
OATP1	OATP1A2	Bilirubin PGE ₂	Briz et al. (2003) Kullak-Ublick et al. (2001)
	OATP1B1	Triiodothyronine (T3), Thyroxine (T4), Thromboxane B ₂	Abe et al. (1999)
	OATP1B3	T3, T4 Digoxin, Cholecystokinin 8	Abe et al. (1999) Ismair et al. (2001)
	OATP1C1	Estradiol-17 β -glucuronide, Estrone-3-sulfate, T3 Reverse-triiodothyronine (rT3)	Pizzagalli et al. (2002)
OATP2	OATP2A1	PGE ₂	Kraft et al. (2010)
	OATP2B1	PGE ₂	Tamai et al. (2000)
OATP3	OATP3A1	Estrone-3-sulfate	Tamai et al. (2000)
OATP4	OATP4A1	Estradiol-17 β -glucuronide, Estrone-3-sulfate, PGE ₂ T3, T4	Tamai et al. (2000) Fujiwara et al. (2001)
	OATP4C1	cAMP, T3, T4	Mikkaichi et al. (2004)

1.5.2 Structure

The organic anion transporting polypeptides consist of 643-722 amino acids. From the Hydrophobicity plots, it was calculated that OATPs have twelve transmembrane domains. The loops 2 and 5 contain putative N-glycosylation sites, which are important for membrane targeting and a proper function (Hagenbuch and Meier, 2004).

The appropriate function and expression is also influenced by the disulfide-bonded cysteine residues in the loop 5. Mutations in the cysteine molecules have the risk of promoting a dysfunction of the transporter (Haenggi et al., 2006; Hagenbuch and Gui, 2008).

Additionally, the function of the tryptophan residues in loop 3 and in the transmembrane domain 6 is not known yet, but they serve as a recognition site for OATPs (Hagenbuch and Meier, 2004). Figure 2 shows the predicted structure of the human OATP1B1.

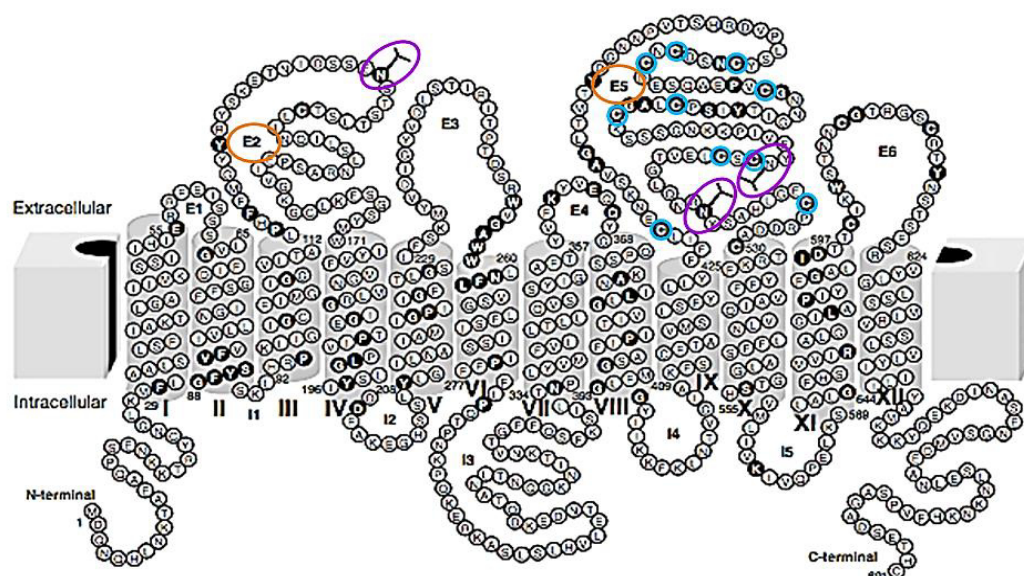


Fig. 2: Structure of OATPs

The structure of OATP1B1 is shown with 12 transmembrane domains (roman numerals). The loops in E2 and E5 are circled in orange and the N-glycosylation sites in purple. The cysteine molecules in loop 5 are labeled in blue (modified according to Hagenbuch and Gui, 2008).

1.5.3 Function

The driving forces for OATP dependent transport are not totally understood yet (Hagenbuch and Gui, 2008). The majority of the SLC transporters are responsible for the uptake of different substrates (Figure 3) (Nakanishi, 2007). The substrates usually have a molecular weight of more than 450 kDa and bind to plasma albumin. Additionally, they often have anionic amphipathic characteristics (Hagenbuch and Meier, 2004; Hagenbuch and Gui, 2008). OATPs mediate the sodium independent transport of different substrates including hormones, drugs, amino acids, peptides and xenobiotics (Wojtal et al., 2009). Common drugs, like statins and antihypertensive drugs, like angiotensin-converting enzyme (ACE) inhibitors are also important substrates (Hagenbuch and Meier, 2004; König et al., 2006; Hagenbuch and Gui, 2008; Fahrmayr et al., 2010). Changes in the pH range may influence the uptake of different agents and their affinity for these transporters (Kobayashi et al., 2003). The transport by OATPs can be stimulated or inhibited by herbal compounds, such as the flavonoids biochanin A or silymarin, as documented for the uptake of dehydroepiandrosteronsulphate (DHEAS) by OATP1B1 (Wang et al., 2005).

Anticancer drugs like Methotrexate, Docetaxel, Paclitaxel and Atrasentan are substrates for OATP1B1 and OATP1B3, respectively (Smith et al., 2005; Katz et al., 2006; Kindla et al., 2011).

OATP4A1 is expressed ubiquitously and its substrates include thyroid hormones (Fujiwara et al. 2001), benzylpenicillin, estradiol-17 β -glucuronide, estrone-3-sulfate and PGE₂ (Tamai et al., 2000). Figure 3 summarizes the main substrates mediated by OATP4A1.

The transport of thyroid hormones by OATP4A1 in the placenta is discussed as OATP4A1 may be a mediator for the supply of the fetus with thyroid hormones (Hagenbuch, 2007).

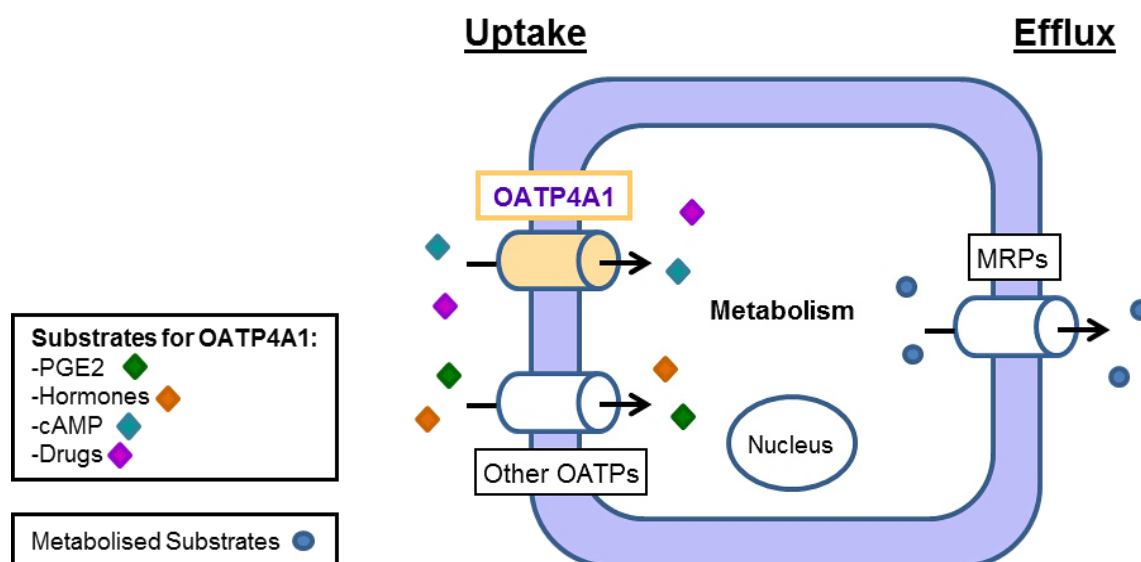


Fig. 3: Uptake, metabolism and efflux of OATP4A1

Various important substrates such as PGE₂, cAMP, drugs and hormones are transported by OATP4A1. MRPs=Multi Drug Resistant Transporters

1.5.4 Altered Expression of OATPs in Cancer

It is assumed that some OATPs provide cells in the colon with different substrates, e.g. PGE₂. In inflammatory diseases or in cancer, this OATP can be down-regulated and other OATPs cannot compensate the loss of that specific function. This can be seen on the basis of findings on the liver specific transporters OATP1B3 and OATP1B1.

The altered expression of OATP1B3 and OATP1B1 under malignant diseases, such as cancer is well studied. OATP1B3 protein levels were found to be altered in hepatocellular cancer (Obaidat et al., 2012). The expression of these transporters is also altered in other cancers, e.g. ovarian cancer (Svoboda et al., 2011).

In addition, OATP1B3 was found to be increased in pancreatitis and cancer of the pancreas leading to the conclusion that OATP1B3 might act as a potential tumor marker for diagnostic methods in an early stage pancreatic cancer (Hays et al., 2013).

Similarly to the OATPs of family 1 in cancer, the testes specific OATP6A1 was documented to be expressed in lung and cerebral tumors (Buxhofer-Ausch V. et al., 2013). Furthermore, Kleberg et al. (2012) found increased levels of OATP4A1 and also OATP2B1 in colonic epithelial cells from colorectal neoplasia patients.

As cancers like ovarian or breast cancer are often hormone dependent types of cancer it should be taken into consideration that hormones like estrogen sulfate and –glucuronide, which are also transported by different OATPs, may be targeted for cancer therapy (Kindla et al., 2011).

1.5.5 OATP4A1 and Colon Cancer

The family of OATP4 consists of the proteins OATP4A1 and OATP4C1. OATP4A1 is expressed ubiquitously and consists of 722 amino acids with a mass of about 65 kDa. It was first isolated from human brain and kidney (Tamai et al., 2000; Fujiwara et al., 2001; Sato et al., 2003; Hagenbuch, 2007). The localization of its gene, SLCO4A1 is on chromosome 20q13.33 (Tamai et al., 2000; Fujiwara et al., 2001; Hagenbuch and Gui, 2008).

OATP4A1 is found in organs like liver, heart, pancreas and placenta (Fujiwara et al., 2001).

Previous studies have shown that OATP4A1 is expressed in colon cancer, but it has been reported that mRNA levels of OATP4A1 also increase in inflammatory bowel diseases as compared to healthy colon specimens (Wojtal et al., 2009). An up-regulation of OATP4A1 in colon cancer specimens was further documented by Ancona et al. (2006). This is important, as inflammatory diseases of the colon are predicted to be a risk factor for the development of colon cancer.

1.5.6 Expression of OATPs in Immune Cells

The identification of the type of immune cells that express OATPs is very important, as the immune system is always involved in pathological processes.

Previously, some transporters have been identified in immune cells. For example, the Equilibrative Nucleoside Transporter 1 (ENT1) is one of those well-known transporters, which are responsible for the uptake of different drugs in immune cells. It also shows a high activity (Minuesa et al., 2009). Other transporters like the Organic Cation/Carnitine Transporters (OCTN), in specific OCTN1 and OCTN2 were detected in CD68⁺ macrophages, but were absent in CD20⁺ B-cells (Peltekova et al., 2004).

Also the expression of OATPs in different immune cells was reported. A high expression of OATP4A1 was found in monocytes, macrophages and mature dendritic cells. However, OATP2B1 was not found in monocytes and no expression of OATP1A2 and OATP1B1 could be detected for these cells (Bleasby et al., 2006; Janneh et al., 2008; Skazik et al., 2008).

2 Aim of the Study

Previous studies have shown that OATPs are gaining wide acceptance for their important role in tumor progression based on their ability to mediate the uptake of xenobiotics and drugs. Since certain drugs are able to inhibit OATP function, they might change the efficacy of therapeutic drugs.

Different transporters, e.g. some OATPs show an altered expression in cancer as compared to healthy tissue and the pattern of OATPs in different organs and cells is no longer maintained. OATPs contribute to the uptake of endogenous substrates such as hormones, prostaglandins, peptides and chemotherapeutic agents. Tumor cells may use the activity of OATPs for their own survival as they get supply of important substrates through the transporters. Altered expression of these transporters may have a significant role in tumor progression. Therefore, the understanding of the OATP pattern in cancer may lead to a promising approach for improving cancer chemotherapy.

This thesis aims to reveal the distribution of OATP4A1 in colon cancer specimens in comparison to its distribution in non-malignant diverticulitis samples. It will be evaluated whether OATP4A1 may have a prognostic power to predict a better outcome for patients with early stage colon cancer.

To study this approach, paraffin-embedded tumor sections from patients suffering from colon cancer (UICC stage I/II) and samples from non-malignant diverticulitis will be investigated for OATP4A1 by immunohistochemical staining and evaluation of stained proteins using the automated microscope TissueFAXS®. The quantitative analysis will be performed with the HistoQuest® image analysis program. The results of OATP4A1 in cancerous and non-cancerous samples will be compared with clinical data. Additionally, for the identification of OATP4A1-positive cell types, double-immunofluorescence staining (IF) with appropriate cellular markers will be carried out.

The results from this thesis aim to provide an insight into the importance of OATP4A1 in colon cancer progression. This may be pivotal in predicting the outcome of certain cancer chemotherapies.

3 Material and Methods

3.1 Patients

Colonic tissue samples were obtained from 185 patients, who had been diagnosed with colon cancer and from 18 patients with diverticulitis, undergoing a surgery at the hospital “Donauspital, Sozialmedizinisches Zentrum Wien” in Vienna, Austria.

The colon cancer tissue micro arrays (TMAs) were provided by the Department of Pathology of the same hospital and included 185 patients.

Table 5 gives an overview of the most important clinical parameters of the colon cancer patients.

Ethical considerations: Informed consent was given by all patients. The study was approved by the Ethical committee of the institutions.

3.1.1 Colon Cancer Patients

Tab. 5: Clinical parameters of colon cancer patients

Characteristics	Patients (n)
Female	80
Male	105
UICC Stage I	68
UICC Stage II	117
Relapse $\leq$ 5 years	44
Non Relapse $\leq$ 5 years	141
Localization of Primum: - colon - rectum	123 62
Localization of 1. Relapse: - local - peripheral - local and peripheral	10 169 6

3.1.2 Colorectal Cancer Liver Metastases Patients

For evaluation of mRNA levels of OATP4A1 in CRCLM patients, 22 samples were kindly provided by the hospital Franz-Josef in Vienna. Eight samples included normal-appearing liver, adjacent to the tumor. 14 samples were obtained from colon cancer metastases patients.

3.2 Indirect Immunohistochemistry

The immunohistochemistry (IHC) is an essential technique in diagnostics, clinical and basic research. It allows the localization of proteins in specific cells. The IHC is based on the specific antibody reaction with the epitope, which is a specific antibody binding site, localized on the surface of the antigen. The immunohistochemical staining can be performed on fresh or frozen tissue samples and on formalin-fixed and paraffin-embedded tissue sections, which are used in the pathological examinations. For visualization of the antibody binding, a direct or indirect method is used (Hermey et al., 2010; Lloyd, 2010).

Tissue samples are incubated with the primary antibody before the secondary antibody is added. To make the binding reaction visible, the secondary antibody is coupled to an enzyme, usually to horse radish peroxidase (HRP). By adding an adequate substrate, e.g. 3,3'-Diaminobenzidine (DAB) and hydrogen peroxide (H_2O_2), the substrate converts into a brown precipitate. To avoid unspecific reactions from peroxidase in organelles, the tissue samples are usually pretreated with hydrogen peroxide (H_2O_2) to block endogenous peroxidase.

During the fixation of the tissue samples the epitope binding sites often get masked so that the binding of the antibody is inhibited. To break these crossed-linked bonds, an antigen retrieval, which is based on enzymatic reactions or heat treatment, is crucial. Suitable buffers such as citrate or EDTA can be used as antigen retrieval solutions. The heating source can be a microwave, a pressure cooker or a steamer (Hermey, et al., 2010).

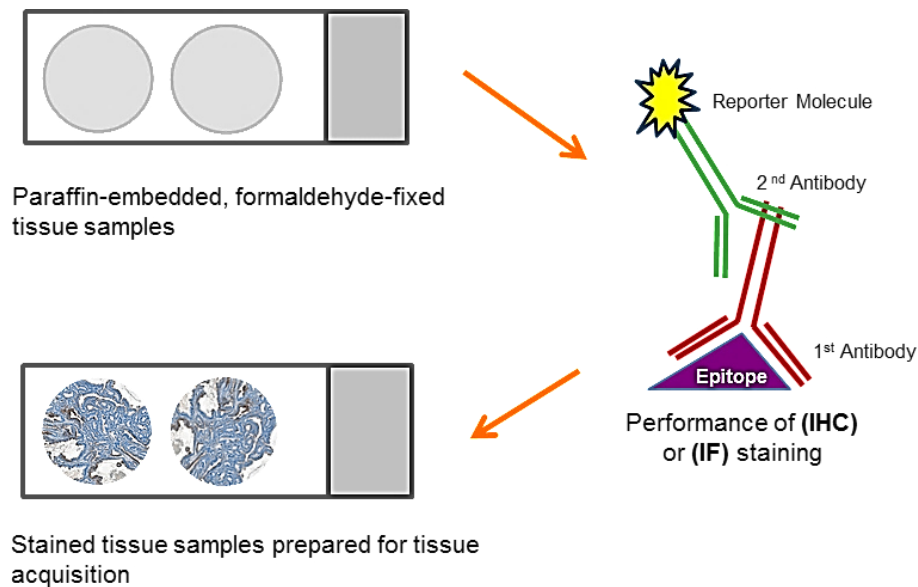


Fig. 4: Immunohistochemical staining procedure

IHC, as well as IF are based on the binding of two different antibodies. The primary antibody is OATP4A1. The secondary antibody is coupled to an enzyme e.g. horse radish peroxidase.

In the indirect immunohistochemistry experiments, the antibody anti-OATP4A1 was used for the staining of OATP4A1.

Tissues:

- Colon tissue samples from diverticulitis patients
- Tissue Micro Arrays (TMAs) from colon cancer patients (UICC Stage I/II)

Reagents:

- Xylol (Merck, Darmstadt, Germany)
- Ethanol (Merck, Darmstadt, Germany)
- Deparaffinisation and Pretreatment Epitope Retrieval Solution buffer pH9 (DEPP-9 Buffer, Eubio, Vienna, Austria)
- 1x Phosphate Buffered Saline (PBS) pH=7.4
- 1x PBS + 0.1% Tween
- Tween®20 for electrophoresis (Sigma-Aldrich Chemie GmbH, Steinheim, Germany)
- Dako Pen (Dako Cytomation, Denmark)
- 10% BSA (Bovine Serum Albumin)/PBS Blocking buffer (PAN Biotech, Aidenbach, Germany)

- Ultravision LP Detection System: HRP Polymer & DAB Plus Chromogen (Thermo Scientific, Fremont, USA)
- Chem Mate™ Hematoxylin (Dako Cytomation, Denmark)
- Distilled water
- Fluoromount-G (Southern Biotech, Alabama, USA)

Antibodies:

- Primary antibody: polyclonal rabbit anti-human anti-OATP4A1 (Lot:R31219 Atlas Antibodies; Stockholm, Sweden)

Equipment:

- Microwave
- Humidified chamber
- Cover glass 24x50 mm Borosilicate Glass, Thickness No. 1 (VWR International, Radnor, Pennsylvania)
- Microscope Zeiss AXIO Imager Z1 (Zeiss, Jena, Germany)
- TissueFAXS® (TissueGnostics GmbH, Vienna, Austria)

Fabrication of 10x PBS:

10x PBS – phosphate buffered saline (pH 7.4)	
sodium chloride (NaCl)*	80 g
potassium chloride (KCl)*	2 g
disodium hydrogen phosphate (Na ₂ HPO ₄ ·2H ₂ O)*	14.4 g
potassium dihydrogen phosphate (KH ₂ PO ₄)*	2 g

*Commercial Source: Merck, Darmstadt, Germany

The above listed reagents were dissolved in distilled water, filled up to one liter and adjusted to a pH range of 7.4.

Procedure for Indirect Immunohistochemical Staining

In the first step, paraffin-embedded tissues were given into xylol and thereafter into ethanol (100%) for 3 minutes for deparaffinization. For antigen retrieval, slides were put into the deparaffinization and pretreatment epitope retrieval solution buffer pH 9 (DEPP buffer), which was diluted in a ratio of 1:20 with distilled water. Afterwards, the samples were heated in the microwave for 10 minutes. The following cooling period was for 25 minutes. The samples were washed for 2x5 minutes in phosphate buffered saline pH 7.4 (PBS) and surrounded with a Dako Pen, which has a lipophilic character so that the tissues were prepared for the application of reagents. The process continued with the washing in PBS/Tween 0.1% for 3x3 minutes. For the blocking of unspecific binding sides in the tissue, Ultra Vision Block was applied on the tissues for 7 minutes. At the same time the antibody was diluted in 10% bovine serum albumin (BSA) in PBS. The slides were covered with the antibody solution and then incubated in a humidified chamber over night or for 1 hour depending on the antibody (Table 6).

Thereafter, washing with PBS/Tween 0.1% for 3x3 minutes was done. Afterwards, the detection system was applied. The tissues on the slides were covered by an antibody enhancer solution containing IgG against the first antibody to enhance the specific antibody binding. After 10 minutes, washing with PBS/Tween 0.1% for 3x3 minutes was carried out. The photosensitive substrate horse radish peroxidase (HRP) was applied on the tissues for 15 minutes before being washed in PBS/Tween 0.1% for 3x3 minutes.

For detection, one drop of 3,3'-Diaminobenzidine (DAB) was added to 2 ml DAB plus substrate and mixed. Due to the catalytic reaction of peroxidase with the DAB solution, a brown staining of the positive antibody-antigen reaction became visible. To obtain optimal results for OATP4A1, the DAB solution was kept for 11 minutes on the slides. To stop the reaction, slides were dipped into distilled water and washed four times with water. Tissue sections were counterstained with hematoxylin for one minute for visualization of the cell nuclei. Afterwards, slides were washed with tap water for three times. At the end, all samples were embedded in the mounting medium Fluoromount. Images were taken of all samples using the TissueFAXS® microscope system.

3.3 Indirect Immunofluorescence

In the indirect immunofluorescence the secondary antibody, which is conjugated with a fluorochrome is applied, instead of the application of a HRP-labeled IgG. The advantage of this method is that double staining of different tissues with antibodies from different species, conjugated with selected fluorochromes, can be performed simultaneously.

Tissues:

- Colon tissues from diverticulitis patients
- TMAs from colon cancer specimens

Reagents:

- Xylol (Merck, Darmstadt, Germany)
- Ethanol (Merck, Darmstadt, Germany)
- DEPP-9 Buffer (Eubio, Vienna, Austria)
- 1x PBS
- 1x PBS + 0.2% Tween
- 1x PBS + 0.05% Tween
- Tween®20 for electrophoresis (Sigma-Aldrich Chemie GmbH, Steinheim, Germany)
- EDTA buffer (pH 9)
- Citrate buffer (pH 6)
- Proteinase K (Sigma-Aldrich Chemie GmbH, Steinheim, Germany)
- Dako Pen (Dako Cytomation, Denmark)
- 5% Fetal calf serum (FCS) (PAN Biotech, Aidenbach, Germany)
- 0.1% BSA/PBS (PAN Biotech, Aidenbach, Germany)
- 4',6-Diamidine-2'-phenylindole, dihydrochloride (DAPI; Roche Diagnostics GmbH; Mannheim; Germany)
- Distilled water
- Fluoromount-G (Southern Biotech, Alabama, USA)

Antibodies:

- Primary antibody: anti-OATP4A1 (Lot:R31219 Atlas Antibodies; Stockholm, Sweden)
- Secondary antibody: see Table 7

Equipment:

- Microwave
- Steam cooker (Braun, Multigourmet Type 3-216)
- Incubator
- Humidified chamber
- Centrifuge (Eppendorf AG, Hamburg, Germany)
- Cover glass 24x50 mm Borosilicate Glass, Thickness No. 1 (VWR International, Radnor, Pennsylvania)
- Microscope Zeiss AXIO Imager Z1
- TissueFAXS® (TissueGnostics, Vienna, Austria)

Procedure for Indirect Immunofluorescence Staining

The first steps of the procedure in immunofluorescence double staining are the deparaffinization, rehydration and heat induced antigen retrieval. Depending on the marker, different buffers were used (Table 6). If DEPP buffer was used, then the slides were first put into xylol and ethanol (100%) for 3 minutes each. Thereafter, slides were submerged in the DEPP buffer and heated for 10 minutes for final antigen retrieval.

If citrate buffer pH 6, ethylene-diamine-tetra acetic acid (EDTA) pH 9 or proteinase K solution were used, then the samples were put in an oven (60°) for 25 minutes to melt the paraffin. For rehydration, the slides were first incubated for 3x5 minutes in xylol followed by 5 minutes incubations in ethanol at declining concentrations starting with 100-30%. Afterwards, the tissue sections were heated in the hot buffer for 20 minutes. At that point, the process was the same for all antigens. After washing the slides for 2x5 minutes in PBS, tissues were marked with a Dako Pen. For permeabilization of the cell membranes, tissue sections were put into PBS/0.2% Tween for 5 minutes and then incubated for 30 minutes with 5% fetal calf serum (FCS)/PBS to block unspecific bindings. In the meantime the antibody dilution for the first antibody was prepared. In the case of double staining experiments, both primary

antibodies, e.g. the anti-OATP4A1- and the anti-CD20 antibody were diluted in 0.1% BSA/PBS. The mixture was added immediately onto the tissues on the slides, after the blocking procedure. After an adequate incubation time (Table 6), slides were washed for 3x10 minutes in PBS/0.05% Tween. To make the antigen binding visible, a secondary antibody, with a fluorochrome was added. As secondary antibodies anti-mouse Alexa Fluor 568 and anti-rabbit Alexa Fluor 488 antibodies (diluted 1:2000 in PBS) were applied onto the samples. The incubation lasted for 1 hour. To end that step, washing with PBS/0.05% Tween was done for 3x10 minutes. For nuclei visualization, a counterstaining with 4',6-Diamidine-2'-phenylindole, dihydrochloride (DAPI) in a dilution of 1:000 dilution in PBS was applied for 10 minutes. After washing for 3x10 minutes with PBS and twice with distilled water, the tissue sections were embedded in the mounting medium Fluoromount.

For a successful staining with the anti-CD3 antibody, the protocol had to be modified. The steps for deparaffinization and rehydration were done as described above. Due to the fact that proteinase K had been proven to be the best reagent for antigen unmasking, slides were submerged in prewarmed (37°C) proteinase K solution for 5 minutes. Afterwards, the tissue samples were put into cold PBS (4°C) to stop the enzymatic reaction. From that point on, the protocol was continued as described above.

Negative IgG controls were done by removing the primary antibody or using non-immunogenic IgG instead. Otherwise, staining experiments were performed under the same conditions as described for other tissue samples.

The Tables 6 and 7 summarize the primary and secondary antibodies, the optimal working dilutions, incubation times and the accurate buffers for antigen retrieval.

Tab. 6: Overview of primary antibodies

Primary Antibodies	Dilution	Host	Commercial Source	Incubation time	Buffer
Anti – OATP4A1	1:200	Rabbit	Atlas Antibodies (LotNo:R31219)	1h/Over night	
Anti-CD3	1:20	Rat	Serotec MCA 1477	Over night	Proteinase K
Anti-CD20	1:50	Mouse	Thermo Scientific (AB-1, L26)	Over night	DEPP
Anti-CD34	1:100	Mouse	Abcam (ab8536)	1 hour	DEPP
Anti-CD45	1:200	Mouse	Abcam (MEM-28 ab8216)	1 hour	DEPP
Anti-CD68	1:300	Mouse	Thermo Scientific (MS-397-PO)	1 hour	DEPP, EDTA
Anti-CD83	1:20	Mouse	Abcam (ab49324)	1 hour	EDTA pH 9
Anti-CD141	1:20	Mouse	Acris Antibodies (LotNo:201112)	1 hour	Citrate pH 6
Anti-CD163	1:100	Mouse	Abcam (ab15676)	Over night	DEPP
Negative Controls					
IgG mouse	1:2000	Mouse	Invitrogen Prod.Code:10400C	1 hour	DEPP
IgG rabbit	1:200	Rabbit	Abcam (ab27478)	1 hour	DEPP

Tab. 7: Overview of secondary antibodies

Secondary Antibodies	Dilution	Host	Commercial Source
Anti-Mouse Alexa Fluor 568	1:2000	Goat	Invitrogen*
Anti-Rabbit Alexa Fluor 488	1:2000	Goat	Invitrogen*
Anti-Rat Alexa Fluor 568	1:2000	Goat	Invitrogen*
Streptavidin Alexa Fluor 568	1:2000	Goat	Invitrogen*
*Life Technologies, Carlsbad, California, USA			

3.4 Acquisition of Microscopic Images with the TissueFAXS®

After immunohistochemical or immunofluorescence staining the tissue samples were analyzed with the TissueFAXS® (TissueGnostics GmbH, Vienna, Austria). This is a combined system consisting of a high quality microscope (AXIO Imager Zeiss 1, Jena, Germany), a customized hardware and an adequate scanning software to provide high resolution images (Figure 5).

The automated scanning system allows consecutively scanning of eight slides. The wide range of objectives (2.5x, 5x, 10x, 20x, 40x, 63x) permits the inspection of cell and tissue structures at different magnifications.

Before starting the acquisition it is important to get an overview of the whole slide by starting the preview. This allows selecting regions of interests (ROIs), which can be marked afterwards.

It is possible to choose specific settings e.g. for the different channels (Dapi, TexasRed, GFP), saturation, exposure time, condenser or the intensity of the UV light. The images have to be brought into the focus before every image acquisition, which delivers an overall picture of the specimen. Desired images can be viewed and then exported in forms of field of views (FOVs) for image analysis. Those exported images not only provide the images in the single channel view, but also merged images of the samples. This is a necessary tool to confirm or exclude co-localization of proteins in double immunofluorescence staining experiments.

For the identification of immune cells, double immunofluorescence staining with different cell markers and OATP4A1 was performed. The secondary antibodies were either coupled to Alexa Fluor 568 or Alexa Fluor 486, giving a green or red staining in the image. To ensure that the positive staining with antibodies was not caused by unspecific bindings, negative controls were used to confirm the staining results in the different channels.

The data generated by the TissueFAXS® system provide virtual images. These were used to analyze the samples afterwards with the HistoQuest® software.



Fig. 5: TissueFAXS® (TissueGnostics, Vienna, Austria)

The automated microscopy based system allows image scanning for further quantitative analysis.

3.5 Analysis with the HistoQuest® Software

The HistoQuest® software (version 3.5.3.0173 and 4.0.4.150) distributed by TissueGnostics (GmbH Vienna, Austria), uses different algorithms for quantitative analysis of cells in a tissue stained by immunohistochemistry. This software allows the user to obtain statistic reports of the analyzed images.

Furthermore, it measures the intensity of the nuclei, determined by its blue staining through hematoxylin. The brown staining of OATP4A1 positive cells resulting from immunohistochemical staining with an anti-OATP4A1 antibody was also measured.

The images acquired with the automatic microscopy based TissueFAXS® system, were used to create a new project for each sample in the HistoQuest® program. An illumination correction was done to improve the quality of the staining. The nuclei were set as master in order to define cells.

Regions of interests (ROIs) were defined and then attributed to groups. For non-malignant diverticulitis tissue samples, groups for normal mucosa (=epithelial cells), immune and stroma cells were created. The colon cancer samples were subcategorized into tumor, immune cells, stroma and adjacent mucosa.

Cell identification was done by algorithms based on nuclei staining with hematoxylin (one blue stained nucleus corresponds to one cell).

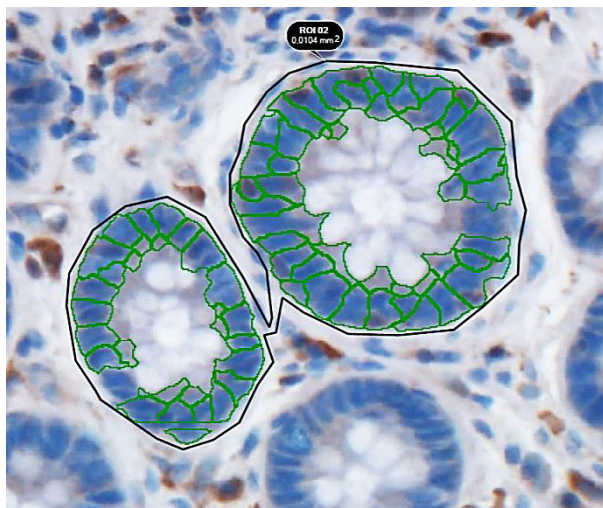


Fig. 6: Nuclei detection with HistoQuest®
Cells in the crypts are detected by their blue nuclei and are labeled in green.

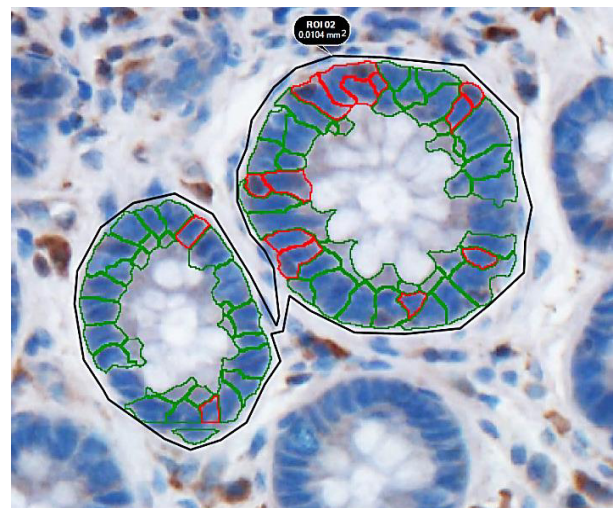


Fig. 7: OATP4A1 detection with HistoQuest®
OATP4A1 positive cells are detected due to their brown staining. They are labeled in red. The black line surrounds the region of interest.

Figure 6 gives an overview of a diverticulitis sample where the blue nuclei were identified (each nucleus is labeled in green).

The measurement of the intensity of brown-stained immunoreactive OATP4A1 gives the amount of OATP4A1 in the cells. The software recognizes cells positive for OATP4A1, which are outlined in red (Figure 7). The region of interest is surrounded by a black line.

For the identification of OATP4A1 positive cells and to avoid false-positive results, threshold values were set by preparing threshold levels for the staining (Figure 8). Although there is no standardization, the user can use the Backward- function in the program to edit the threshold and to control the parameters. This functions permits the user to exclude false positive cells and to extend the threshold to obtain the positive cells only. By setting threshold values, the so-called Cut Off divides the Histogram into four quadrants. The upper right (UR) area is crucial, because it contains OATP4A1-positive cells.

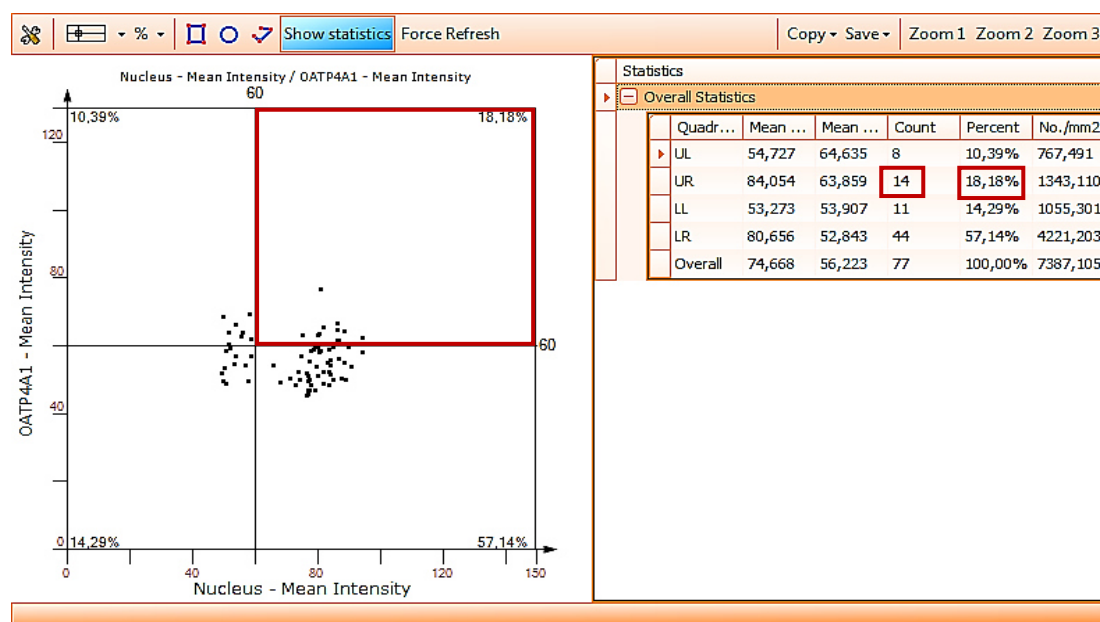


Fig. 8: Scattergram from the data analyzed from a diverticulitis specimen

The scattergram consists of four quadrants. The positive cells are located in the upper right (UR) quadrant (labelled in red). The statistics displayed next to the scattergram show the positive cells (as count) and percentage.

Moreover, Figure 8 shows a scattergram together with the statistics for the calculation of the percentage and the staining intensity of OATP4A1-positive cells in a diverticulitis sample.

It has to be noted that the user can define the area on the tissue samples, which should be analyzed.

After the quantitative analysis was done, the data were exported to an Excel file for further statistical evaluation.

The data for positive OATP4A1 cells were expressed as immunoreactive score (IRS), which was calculated from the percentage of positive cells multiplied with the intensity of the brown staining. The brown staining resulted from a positive OATP4A1 staining in the tissue samples. IRS values were calculated for colon cancer and for diverticulitis patients separately.

Statistical Evaluation, Documentation and Analysis of Data

Statistical analysis was carried out using the GraphPadPrism5[®] software (GraphPadPrism[®] Inc., La Jolla, CA, USA). Therefore, colon cancer patients were divided into two groups. Relapse (n=44) and non-relapse patients (n=141) were compared. Non-malignant samples were obtained from 18 diverticulitis samples in order to perform valuable comparisons with a control group. Data from patients of the different groups were analyzed statistically using the One-way Anova analysis followed by the analysis of variance. The One-way Anova, Dunn's Multiple Comparison or Mann-Whitney-U-test were performed to determine significant differences between the groups ($p < 0.05$ was considered as statistically significant).

3.6 StrataQuest[®] as a New Approach to Quantitative Analysis

The newly developed software StrataQuest[®] which is based on HistoQuest[®] has new functions to do a better automated analysis of tissue regions. StrataQuest[®] works with different algorithms to define different layers on the tissue. By measuring the density of the cells in a structure, e.g. glands, these structures can be recognized automatically.

Six colon cancer TMAs and six non-malignant diverticulitis samples were analyzed with StrataQuest[®].

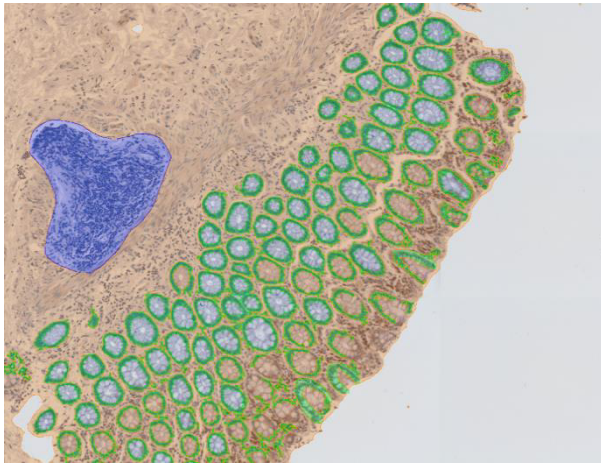


Fig. 9: Automated analysis in a diverticulitis specimen with StrataQuest®

Detection was done automatically using StrataQuest®. The crypts are highlighted in green and the immune cluster in blue. The stroma is shown in orange.

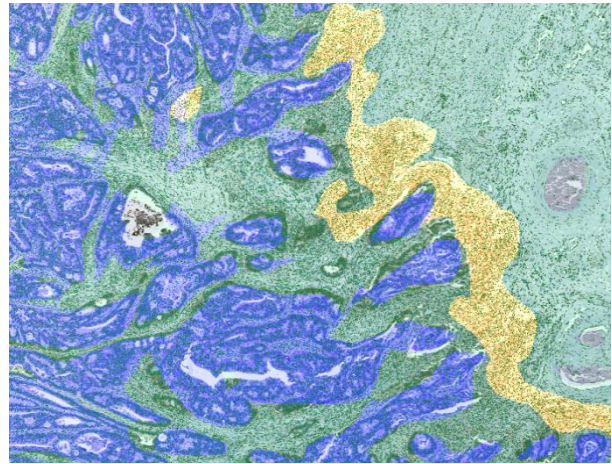


Fig. 10: Analysis in a colon cancer specimen with StrataQuest®

Manual workflow was regarded for a successful analysis. Tumor regions are labeled in blue, stroma is indicated in green and immune clusters in yellow.

As StrataQuest® is still in development, profile settings were not done for all samples and the software was not used for quantitative analysis. However, after further development, a more precise analysis and less analysis time (because the ROIs will not to be outlined manually anymore), will be of benefit in the future.

3.7 Cell Culture

C205 Human Colon Cancer Cell Line

Cells from the C205 cell line were cultured and used for cell staining.

Reagents:

- 10x Trypsin-EDTA suspension (Sigma-Aldrich Chemie GmbH, Steinheim, Germany)
- 1x PBS* pH 7.4
- FBS*
- RPMI 1640 medium (Sigma Aldrich, Buchs, Switzerland)
- Ethanol*

*Commercial source: Merck, Darmstadt, Germany

The C205 cells were kept frozen at -80°C. After a thawing procedure, cells were transferred into a 10 ml tube with fresh medium and then centrifuged. Then the supernatant was removed and the resulting pellet was suspended in fresh medium to be transferred into a 75 cm² flask.

The cells were grown at 37°C in 95% humidified air with 5% CO₂ in RPMI 1640 medium with 10% fetal bovine serum (FBS).

3.7.1 Cell Fixation

Reagents and Material

- Well plate
- 1x PBS
- 4 % (w/v) PFA (Sigma-Aldrich Chemie GmbH, Steinheim, Germany)
- NH₄Cl (Ammonium chloride) (Merck, Darmstadt, Germany)
- Triton X 100 (Merck, Darmstadt, Germany)
- 0.1% and 1% BSA in PBS
- 0.1 %BSA in PBS
- DAPI (Roche Diagnostics GmbH; Mannheim; Germany)
- Primary antibody: anti-OATP4A1 (Atlas Antibodies, Stockholm, Sweden)
- Secondary antibody: anti-rabbit Alexa Fluor 568 (Invitrogen Life Technologies, Carlsbad, California, USA)
- Fluoromount (Southern Biotech, Alabama, USA)

C205 cells were splitted and cell suspensions were transferred into a 96 well plate. Then the cells were washed for 2x5 minutes in PBS. For fixation, incubation with 4% (w/v) PFA in PBS was done for 15 minutes. After washing with PBS, 50 mM NH₄Cl was added for 15 minutes. After its removal, the cells were covered with PBS and put at 4°C.

3.7.2 Cell Staining

For permeabilization and blocking, cells grown on cover slips were incubated in 0.5% (v/v) Triton X 100 and 1% BSA in PBS for 1 hour. The antibody anti-OATP4A1 was diluted 1:200 in 0.1% BSA in PBS and added to the cover slips for an hour. Afterwards washing with PBS was performed four times for 10 minutes.

The secondary antibody Alexa Fluor 488 was diluted 1:2000 in PBS and remained 30 minutes on the cover slips for incubation. Again washing in PBS was carried out twice for 10 minutes.

For nuclei staining, the cover slips were covered with a 1:1000 DAPI solution in PBS for 15 minutes. After washing for 2x5 minutes with distilled water, the cover slips were mounted by adding one drop of Fluoromount.

Images were taken from the AXIOPLAN Fluorescence Microscope (Zeiss, Jena, Germany).

3.8 Quantitative Real Time TaqMan® PCR

The Polymerase chain reaction (PCR) permits to detect smallest amounts of DNA.

The PCR cycle is based on three steps; denaturation, annealing and elongation. The process of heat denaturation leads to the formation of a single stranded DNA. For the subsequently annealing procedure, the temperature has to be decreased so that the primers can anneal. Afterwards, the temperature is increased again so that the heat resistant polymerase (e.g. Taq polymerase) can generate the complementary strand at the 3'-OH end of the primer.

The replication of a DNA fragment is repeated (approx. for 30 cycles), to achieve the amplification of the desired fragments.

In the real time PCR, the formation of the amplified fragment is determined during the reaction, e.g. by an increase of the fluorescence, generated through a cleavage of the reporter dye from the primer. The values for quantity are expressed as Ct values (Kück, 2005).

The mRNA levels of samples from CRCLM patients were evaluated by quantitative real time TaqMan® PCR. The samples had already undergone RNA isolation and reverse transcription. The thereby obtained cDNA was used for the following PCR procedure.

Reagents and Devices

- GIBCO distilled RNase/DNase free water*
- 2x TaqMan Gene Expression Master Mix*
- TaqMan primer/probes*:
OATP4A1 (Atlas Antibodies, Stockholm, Sweden)
- MicroAmp 96-well Reaction Plate*
- MicroAmp Clear Adhesive Film*
- 7900HT-Fast Real Time PCR System with SDS 2.4 Software*
- geNorm Reference (house-keeping) gene selection kit (Primer Design, Southhampton, UK)
- Safe-seal tips (Biozym Scientific GmbH, Oldendorf, Germany)

* Commercial Source: Life technologies, Carlsbad, California, USA

Procedure

The first step was to transfer 2 µl of cDNA to a 96- well plate where the Mastermix was added to the solution. The PCR was performed in duplicates and then the reaction was started in the cycler. The different steps of the PCR cycle are characterized by three steps. The procedure gets started with the denaturation at 92°C-96°C, followed by the process of annealing at 50°C-65°C and the elongation at 68°C-72°C.

Mastermix for one well:

Material	Amount
TaqMan® Primer	1 µl
2x TaqMan® Gene Expression MasterMix	10 µl
RNA free H ₂ O	7 µl
Sum	18 µl

After the reaction was completed, the data from the SDS software were transferred to the Excel program for the calculation of the $2^{-\Delta\Delta CT}$ values and for further data analysis.

4 Results

4.1 OATP4A1 in C205 Colon Cancer Cells

Experiments in C205 cells were done to elucidate whether OATP4A1 is expressed by colon carcinoma cell lines. As shown in Figure 11, immunoreactive OATP4A1 was detected after C205 cells were stained with an antibody against OATP4A1. The staining was performed with immunofluorescence.

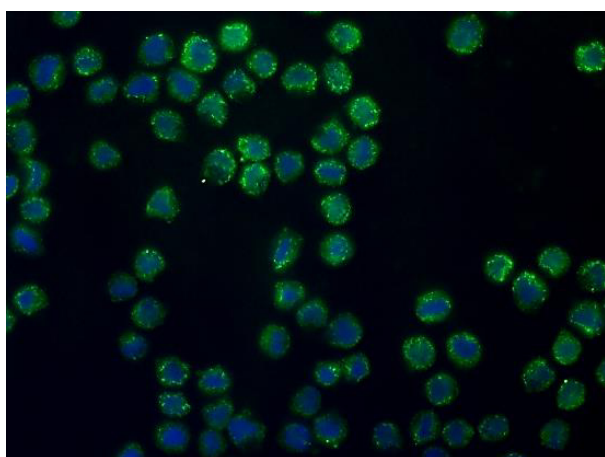


Fig. 11: Staining of OATP4A1 in C205 cells
The blue nuclei were stained by DAPI. OATP4A1 was detected by immunofluorescence staining (green staining).

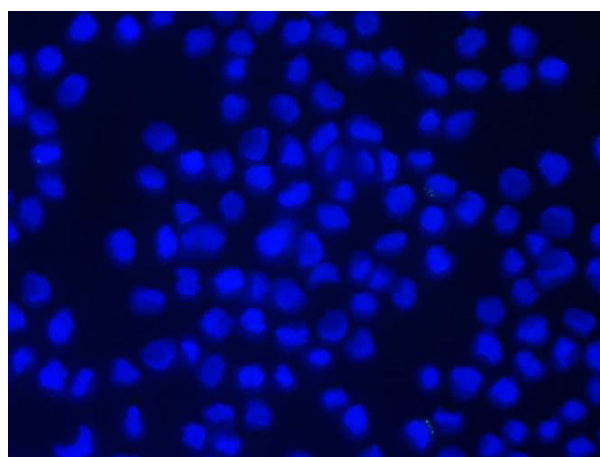


Fig. 12: C205 Cells-Negative control
The antibody against OATP4A1 was omitted. No green staining is seen using an Alexa Fluor 486 labeled secondary antibody, only. Nuclei were stained with DAPI.

High levels of OATP4A1, predominantly at the plasma membrane were visible in nearly all the tumor cells as shown in Figure 11.

The negative control in Figure 12 confirms that OATP4A1 is present in the C205 cells. There is no background, nor any unspecific staining visible, which could lead to misinterpretation or false-positive results.

4.2 Staining of OATP4A1 in Colon Cancer Specimens

4.2.1 Histological Observations

For quantitative analysis of OATP4A1 positive cells in colon cancer, Tissue Micro Arrays (TMAs) from colon cancer specimens were stained by immunohistochemistry. The TMAs were taken from the tumor center. As this study was focused on the OATP4A1 expression in the tumor, sections from the tumor were chosen.

In order not to receive false positive results, negative controls were carried out. The staining for OATP4A1 was performed with an anti-OATP4A1 antibody. In the negative control, non-immunogenic IgG was used, as seen in Figure 13. The negative control does not give any positive brown staining; only the blue hematoxylin staining of the nuclei is visible.

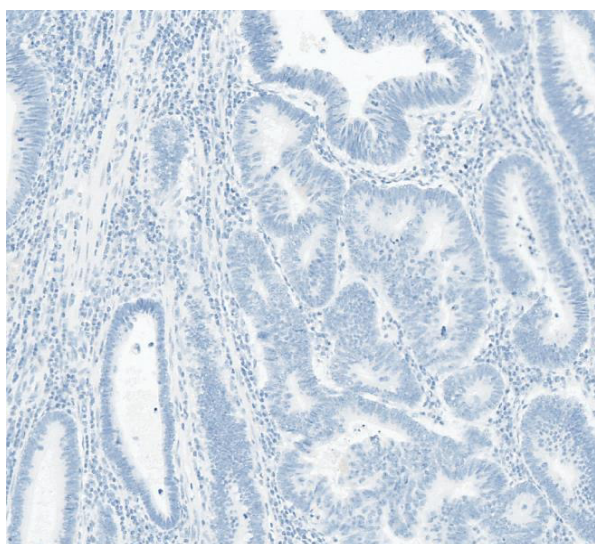


Fig. 13: IgG Negative control in a colon cancer specimen (*patient 4065*)

The negative control was carried out with an IgG mouse antibody in a colon cancer sample. Nuclei staining with hematoxylin.

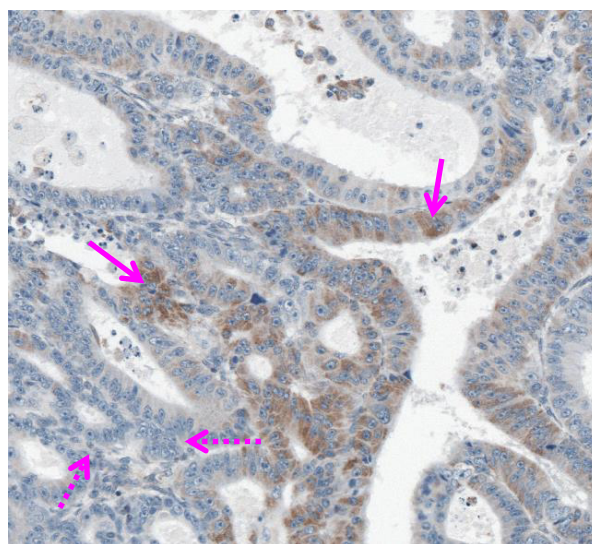


Fig. 14: Partial positive staining of OATP4A1 in a colon cancer sample (*patient 6701*)

Dotted arrows indicate a negative staining of OATP4A1. Some tumor cells express OATP4A1 (arrows).

The intensity of the brown OATP4A1 staining indicates the cellular levels of the transporter. A very low or a moderate positive staining of OATP4A1 in the tumor cells is shown in Figure 14. However, the majority of the colon cancer cells showed a very intensive positive brown staining of OATP4A1 as given in Figure 15. In general, there were less OATP4A1-positive stroma cells than tumor cells (Figure 15), but it must be noted that quantitatively seen, there is a higher number of epithelial or tumor cells within the tissue.

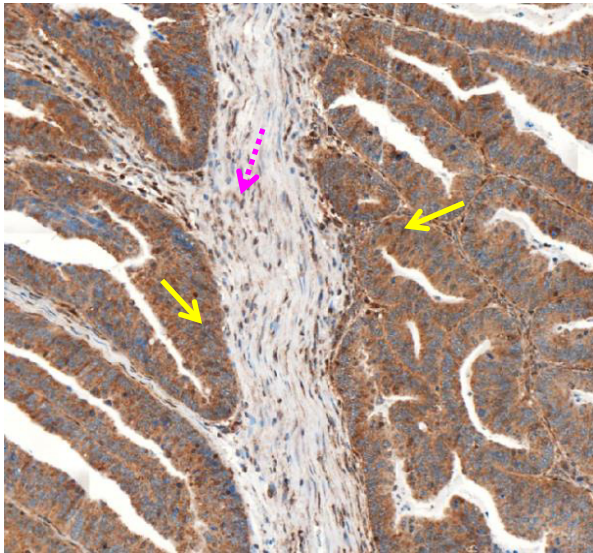


Fig. 15: OATP4A1 in tumor and stroma cells in a colon cancer specimen (*patient 1525*)
In the tumor cells an intensive brown staining of OATP4A1 can be seen (yellow arrows). Also the stroma cells are positive for OATP4A1 (pink dotted arrow).

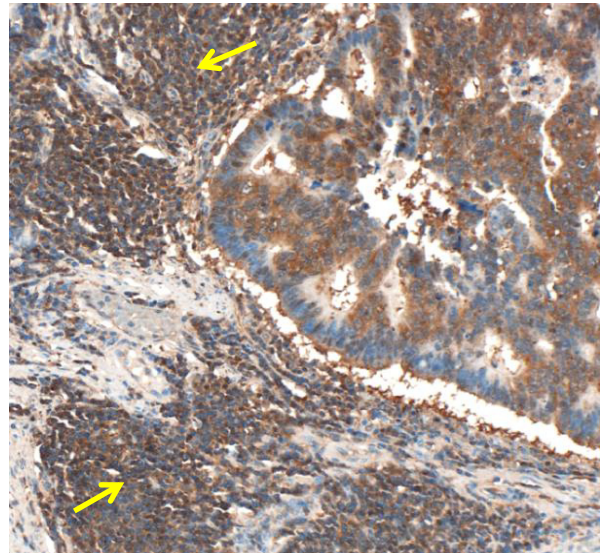


Fig. 16: OATP4A1 positive immune cells in a colon cancer specimen (*patient 4065*)
A high density of immune cells surrounding the tumor cells is visible (arrows). There is an intensive brown staining of OATP4A1 in these cells.

Immune cells, which were found in a high density, especially near the tumor, showed an intensive positive staining for OATP4A1 (Figure 16).

4.2.2 Quantitative Analysis of OATP4A1 in Colon Cancer Specimens

The data for the quantitative analysis were assessed using the TissueFAXS® system and the HistoQuest® software. This software calculates the intensity of the staining and the percent of positive cells in an area. The product of the staining and the percent of positive cells gives the immunoreactive score (IRS) which was calculated for tumor, immune and stroma cells separately.

From 185 colon cancer patients, 44 patients suffered an early relapse (within five years). 141 patients had a relapse-free five year survival. For statistical analysis the IRS values for the relapse group were compared with the non-relapse group using the Mann-Whitney-U-test.

OATP4A1 in Tumor Cells

Figure 17 compares the OATP4A1 levels in the tumor cells in colon cancer patients with an early relapse versus non-relapse patients. The non-relapse group shows higher IRS values ($IRS=4524\pm2813$) than the relapse cohort ($IRS=3468\pm2851$).

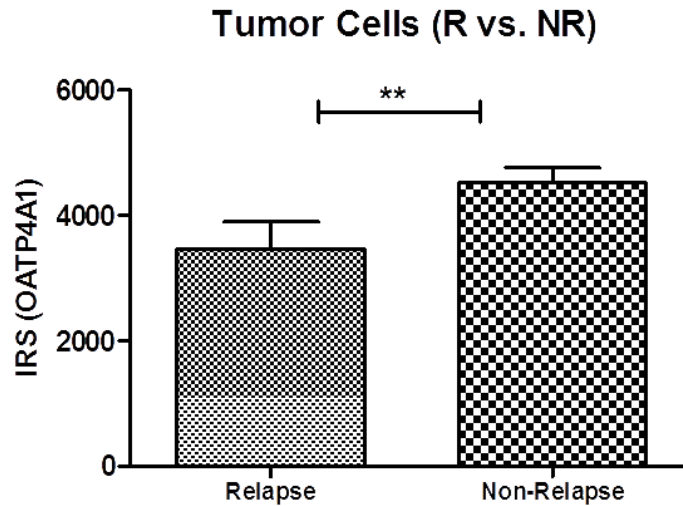


Fig. 17: IRS values of OATP4A1 in tumor cells of relapse (R) and non-relapse (NR) patients

Non-relapse (NR) patients show higher IRS values compared to relapse (R) patients. Both groups were compared using the Mann-Whitney-U-test, **p=0.001.

OATP4A1 in Immune Cells

Immune cells play a central role in cancer environment. The IRS values for immune cells of relapse and non-relapse patients were calculated (Figure 18). Non-relapse patients showed increased IRS values than relapse patients (IRS=5588±2752 vs. 4021±3023).

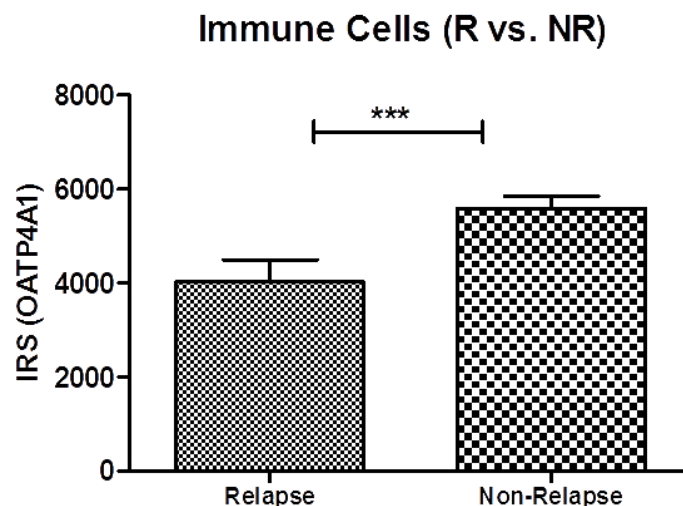


Fig. 18: IRS values of OATP4A1 in immune cells of relapse (R) and non-relapse (NR) patients

Non-relapse (NR) patients show higher IRS values for positive OATP4A1 immune cells than relapse (R) patients using the Mann-Whitney-U-test, ***p=0.0001.

OATP4A1 in Stroma Cells

From all the cancer tissue samples, which were stained for immunohistochemistry, using an anti-OATP4A1-antibody, it was observed that stroma cells had the poorest OATP4A1 expression, resulting in low IRS values compared to tumor or immune cells.

Similar to the results of the tumor cells, higher IRS values were seen in stroma cells of non-relapse colon cancer patients than in relapse patients (IRS=1126±1126 vs. 629±785).

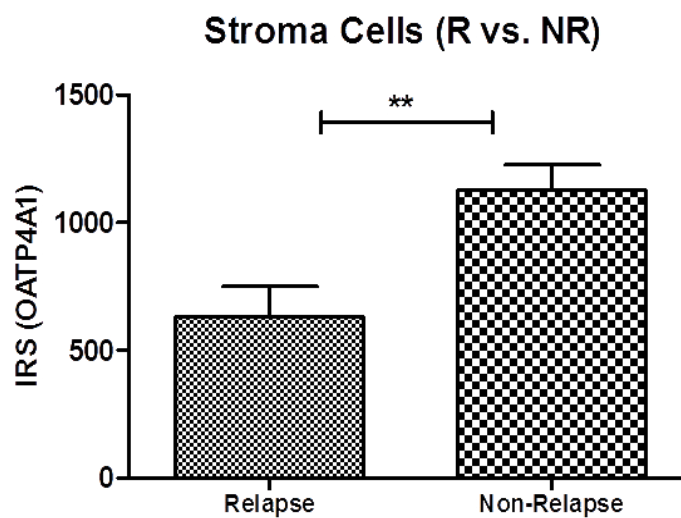


Fig. 19: IRS values of OATP4A1 in stroma cells of relapse (R) and non-relapse (NR) patients
Non-relapse patients show higher IRS values for OATP4A1 in stroma cells than relapse patients, **p=0.001 (Mann-Whitney-U-test).

Table 8 gives an overview of the compared groups and their statistical values, e.g. median, mean and standard deviation (SD).

Tab. 8: Statistical summary of relapse (R) and non-relapse (NR) patients

	Tumor Cells (R)	Tumor Cells (NR)	Immune Cells (R)	Immune Cells (NR)	Stroma Cells (R)	Stroma Cells (NR)
N	44	136	40	115	43	128
Minimum	26	5.2	268	611	8.8	11
Median	2679	4304	3151	5615	263	711
Maximum	16050	15454	16434	13682	3011	5562
Mean	3468	4524	4021	5588	629	1126
SD	2851	2813	3023	2752	785	1126
St. Error	430	241	478	257	120	99

Summary

185 colon cancer patients were stained for OATP4A1. Tumor, immune and stroma cells of patients with and without an early relapse within five years were compared, using the Mann-Whitney-U-test, with a confidence interval of 95%. Not only in tumor cells, but also in immune and stroma cells of non-relapse patients, higher IRS values for OATP4A1 were detected, compared to patients with a relapse. In this context it is positive to note, that immune cells showed the highest significance (** $p=0.001$).

4.3 Results Colorectal Cancer Liver Metastases (CRCLM)

4.3.1 Histological Observations

In the case of colorectal cancer liver metastases (CRCLM) patients, the colon metastases nearly have the same morphology as in colon cancer tissue.

Similar to colon cancer, the amount of immune cells at the tumor border was very high. The noticeable dark brown staining, as a result of a positive OATP4A1 staining in metastatic samples (Figure 20), was similar to the staining intensity, which was observed for colon cancer specimens. Compared to the expression of OATP4A1 in colon cancer epithelial cells, the expression in colon metastases was similar. Conversely, the strong absence of OATP4A1 positive cells in liver tissue seemed to be very characteristic for this type of specimen. The light colored liver tissue in the lower Figure hardly shows a positive staining for OATP4A1.

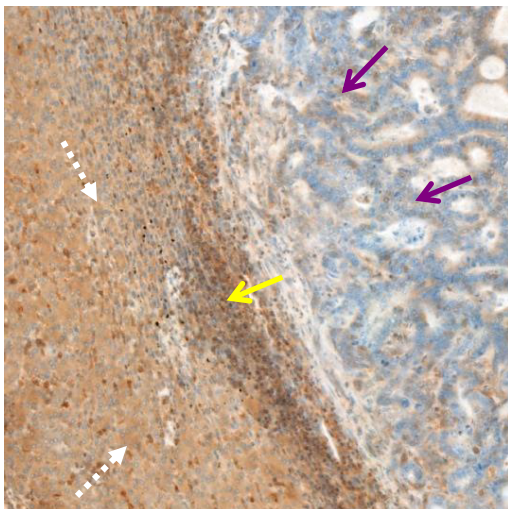


Fig. 20: Colon metastases in liver tissue (patient 2594)

The brown staining is the result of a positive OATP4A1 staining in colon cancer cells (purple arrows). Clusters of immune cells (yellow arrow), which also express OATP4A1, basically appear at the border of liver tissue (dotted arrows) which is negative for OATP4A1.

Due to the few numbers of paraffin-embedded tissue samples from CRCLM patients, no quantitative analysis for IRS values of OATP4A1, nor statistics were possible to calculate.

4.4 Staining of OATP4A1 in Diverticulitis Specimens

4.4.1 Histological Observations

Diverticulitis belongs to inflammatory bowel diseases. Immune cells may change their pattern under inflammatory conditions.

OATP4A1-positive immune cells primarily appeared in the stroma or in small populations also defined as immune clusters as seen in Figure 22. Due to the high density of immune cells gathered at one point, the neighbored stroma (dotted arrows in Figure 22) often showed a higher infiltration of immune cells.

Patients suffering from diverticulitis largely do not show histological changes in colon morphology. Figure 21 undoubtedly accentuates this fact and at the same time reveals the detail that OATP4A1-positive cells were nearly absent in colon mucosa (epithelial) cells of diverticulitis patients.

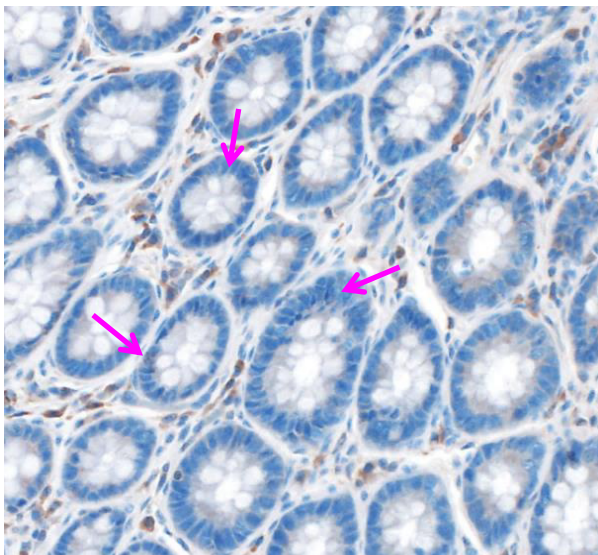


Fig. 21: OATP4A1-positive epithelial cells in a diverticulitis specimen (*patient 448*)

The epithelial cells of patients suffering from diverticulitis hardly show a positive staining of OATP4A1.

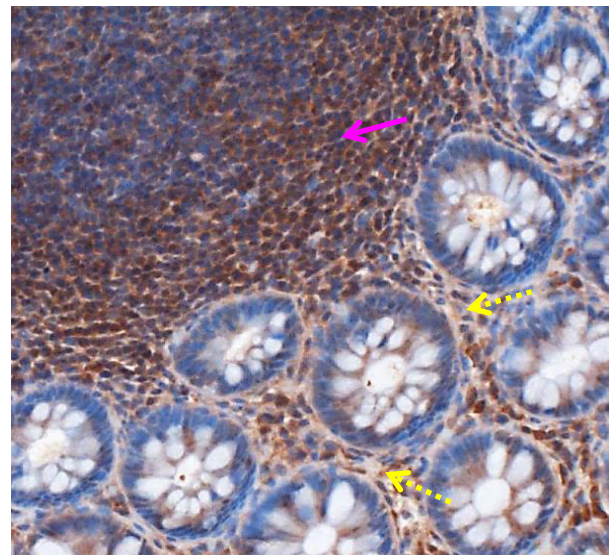


Fig. 22: OATP4A1-positive immune and stroma cells in a diverticulitis specimen (*patient 21938*)

Not only in tumor, but also in non-malignant tissue, immune cells (arrow) show high levels of OATP4A1. A high density of positive stroma cells is also seen (dotted arrows).

4.4.2 Quantitative Analysis of OATP4A1 in Diverticulitis Specimens

To compare colon cancer specimens with a control group, 18 diverticulitis tissue samples were stained for OATP4A1 and analyzed. Statistical analysis was carried out with the One-way Anova and the Dunn's Multiple Comparison test.

The data revealed, that while the expression of OATP4A1 in epithelial cells was not very high ($IRS=546\pm717$), immune cells, however, showed the highest IRS values ($IRS=4131\pm2272$) in this tissue. This information is consistent with the previous histological observations, where immune cells showed the strongest staining for OATP4A1 (Figure 22).

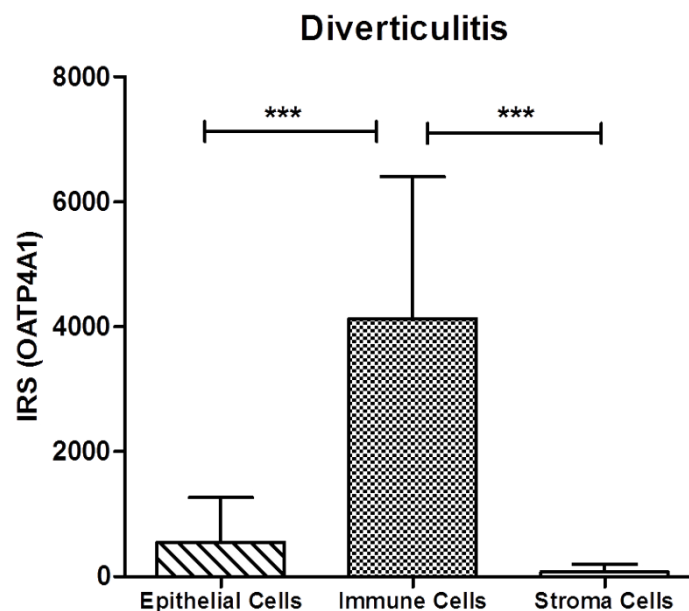


Fig. 23: IRS values of OATP4A1 in diverticulitis patients

The IRS values for OATP4A1 were calculated for mucosa cells (=epithelial cells), immune and stroma cells, separately. Statistical analysis was performed with the One-Way Anova and Dunn's Multiple Comparison test, *** $p=0.001$.

A significant difference was seen between the IRS values of epithelial cells and immune cells in diverticulitis patients ($IRS=546\pm717$ vs. 4131 ± 2272). A similar expression was observed for stroma and immune cells ($IRS=77\pm119$ vs. 4131 ± 2272).

Tab. 9: Summary of OATP4A1 levels (IRS) in epithelial, immune and stroma cells in diverticulitis

	Epithelial Cells	Immune Cells	Stroma Cells
N	18	18	18
Minimum	0	953	0
Median	303	3638	38
Maximum	2795	10744	510
Mean	546	4131	77
SD	717	2272	119
St. Error	169	536	28

4.5 OATP4A1 in Colon Cancer and Adjacent Non-Cancerous Mucosa

4.5.1 Histological Observations

There is evidence showing that in cancer specimens the adjacent mucosa does not show physiological pattern anymore, but pathological one. Although the morphology in the adjacent mucosa might be similar to that of healthy tissue, the function often gets lost or at least changes. These observations were also made in the colon cancer samples, which were analyzed and characterized within this thesis.

Figure 24 is an example for the above mentioned characteristics. The image was taken from a colon cancer sample stained for OATP4A1. Tumor and tumor border are divided by stroma. The adjacent mucosa strongly differs from other parts of the tissue because of its histological structure; but at the same time there is also a clear and significant positive staining of OATP4A1 in the cancer area. This is in agreement with the fact, that the adjacent mucosa has a different morphology with an altered function. Furthermore, the morphological changes of the crypts are remarkable. The crypts lose their normal structure and turn into malignant cells without a clear morphological structure. This is marked in dotted arrows in Figure 24. As already observed in other cancer samples, the stroma usually contains OATP4A1 positive immune cells. At some parts of the stroma, an invasion of immune cells is visible (also indicated by arrows in Figure 24).

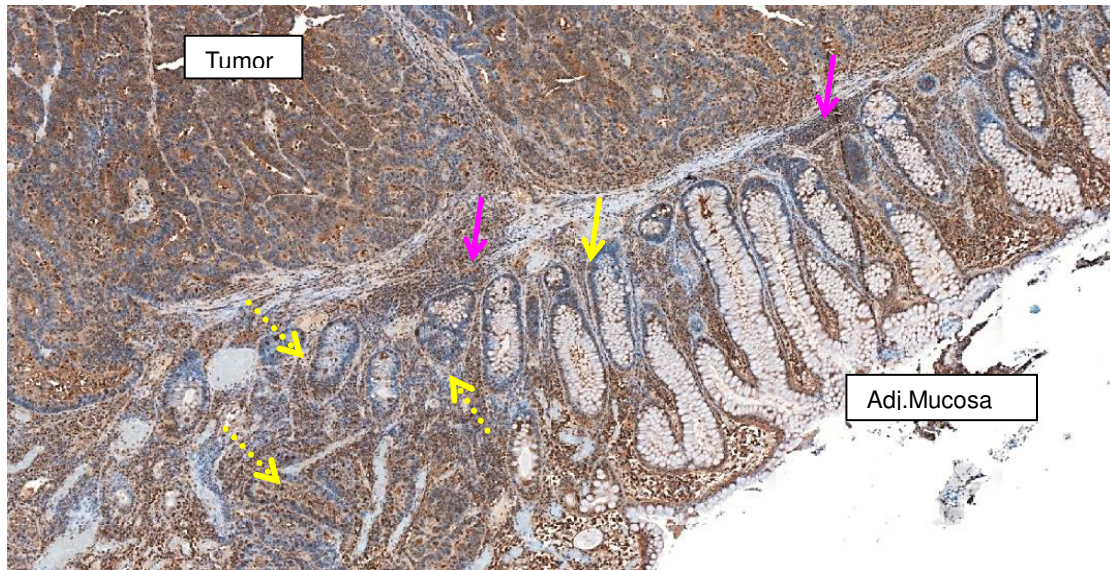


Fig. 24: Overview of a colon cancer specimen with the adjacent non-cancerous mucosa - IHC staining of OATP4A1 (patient 14034)

The overview shows a separation of tumor and non-malignant adjacent mucosa by the stroma, which clearly defines the border. Immune clusters invading the stroma are remarkable (pink arrows). In the adjacent mucosa the crypts often show a healthy morphological structure (yellow arrow) which usually turn into malignant tissue (dotted arrows).

4.5.2 Quantitative Analysis of OATP4A1 in Adjacent Mucosa

In some of the colon cancer samples, the adjacent non-malignant mucosa was visible. Therefore, normal looking mucosa cells were compared to tumor cells.

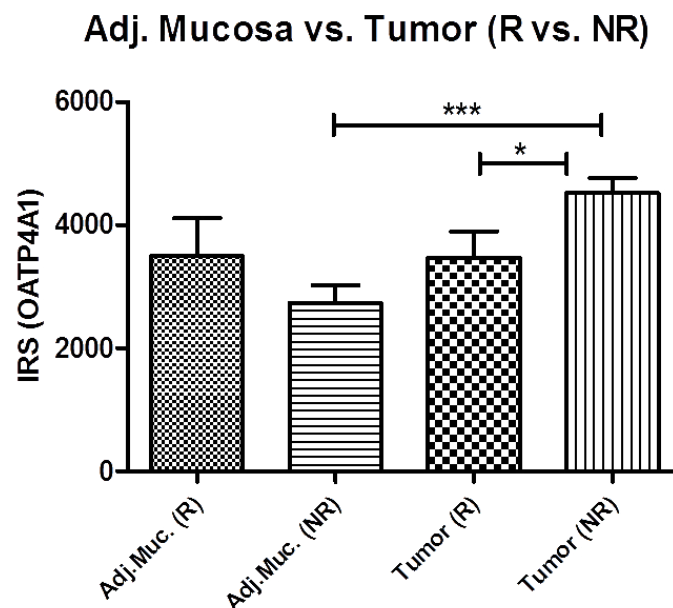


Fig. 25: IRS values of OATP4A1 in tumor cells compared to adjacent mucosa cells

The IRS values for OATP4A1 were calculated for adjacent mucosa cells of relapse (R) and non-relapse (NR) patients and for tumor cells, separately. Statistical analysis was performed with the One-Way Anova and Dunn's Multiple Comparison test, *** $p=0.001$, * $p=0.05$.

Data given in chapter 4.2.2 revealed that colon cancer cells of patients without an early relapse (non-relapse patients) showed increased levels of OATP4A1 in different cell types. This was calculated by using the Mann-Whitney-U-test ($p=0.001$). These findings were supported (Figure 25) by the calculation of the IRS values using the One-way Anova and Dunn's Multiple comparison tests ($p=0.05$).

It is remarkable that the adjacent mucosa cells of non-relapse patients showed a significant difference in the immunoreactive score as compared to cancer cells of the non-relapse patients ($IRS=2732\pm1763$ vs. 4524 ± 2813).

Tab. 10: Statistical summary of adjacent mucosa and tumor cells

	Adjacent Mucosa (R)	Adjacent Mucosa (NR)	Tumor (R)	Tumor (NR)
N	7	36	44	136
Minimum	1276	11	26	5.2
Median	3964	2725	2679	4304
Maximum	5601	8402	16050	15454
Mean	3499	2732	3468	4524
SD	1622	1763	2851	2813
St. Error	613	294	430	241
(R=Relapse, NR=Non-Relapse)				

4.6 Comparison of Colon Cancer and Diverticulitis Specimens

Not only in diverticulitis specimens, but also in cancer samples, membrane and cytosol staining was visible. In some cancer specimens, also a positive OATP4A1 nuclei staining was found.

It is obvious that the morphological and histological structure of non-malignant diverticulitis and colon cancer differ from each other. So does the OATP4A1 expression in epithelial, immune and stroma cells. As the expression of different transporters and, in general, the whole environment of the cells change under malignant pattern, the function is often disturbed.

To understand the distribution of OATP4A1 in different tissues, diverticulitis patients were compared with relapse and non-relapse colon cancer patients (Figure 26).

The expression of OATP4A1 in Tumor Cells vs. Epithelial Cells in Diverticulitis

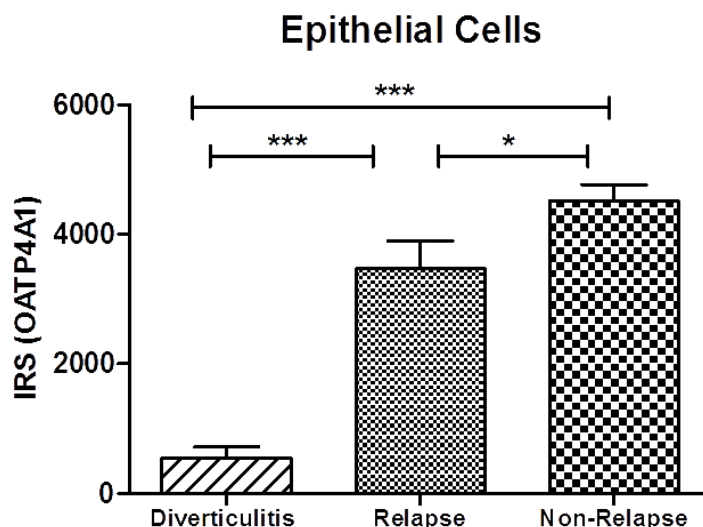


Fig. 26: IRS values of OATP4A1 in tumor cells compared to epithelial cells in diverticulitis patients

Tumor cells of relapse and non-relapse patients were compared with diverticulitis patients. Statistical analysis was performed with the One-Way Anova and Dunn's Multiple Comparison test, *** $p=0.001$, * $p=0.05$.

A decisive difference in the expression of OATP4A1 in epithelial cells was found in colon cancer compared to diverticulitis patients. Epithelial cells in diverticulitis patients showed lower IRS values than relapse (IRS=546 $\pm$ 717 vs. 3468 $\pm$ 2851) and non-relapse patients (IRS=546 $\pm$ 717 vs. 4524 $\pm$ 2813). Both, samples from relapse and non-relapse patients showed significant different pattern when compared to diverticulitis patients (*** $p=0.001$, each).

The expression of OATP4A1 in Immune Cells in Cancer vs. Immune Cells in Diverticulitis

As diverticulitis is an inflammatory disease, the abundant presence of immune cells is not very surprising (Figure 27). There was no significant difference seen in the IRS values for diverticulitis patients compared to cancer patients. Patients with a better prognosis had higher IRS values of OATP4A1 than relapse patients. These findings are in consent to the results given in chapter 4.2.2.

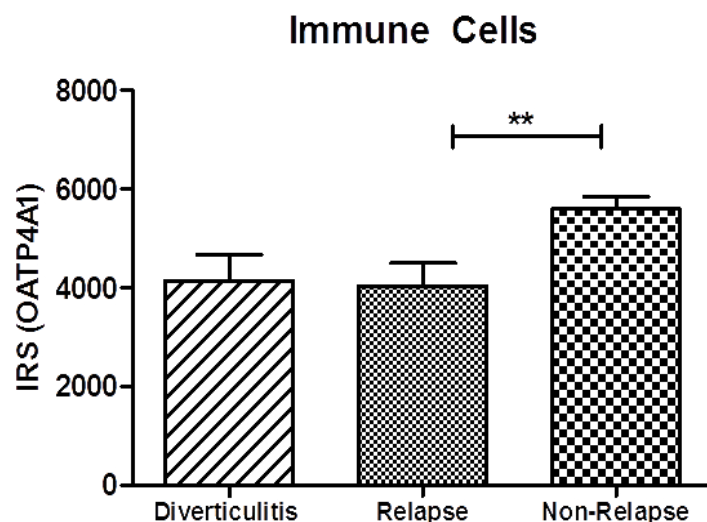


Fig. 27: IRS values of OATP4A1 in immune cells of colon cancer and diverticulitis patients
Only relapse and non-relapse patients show a significant difference of OATP4A1 levels. Statistical evaluation was performed using the One-way Anova and Dunn's Multiple Comparison test, **p=0.01.

The expression of OATP4A1 in Stroma Cells in Cancer vs. Stroma Cells in Diverticulitis

Stroma cells in cancer specimens of non-relapse patients showed higher levels of OATP4A1 than relapse patients. The lowest IRS values for OATP4A1 positive cells were found in stroma cells in diverticulitis specimens. These data were calculated and compared with clinical data using the One-Way Anova and Dunn's Multiple Comparison test.

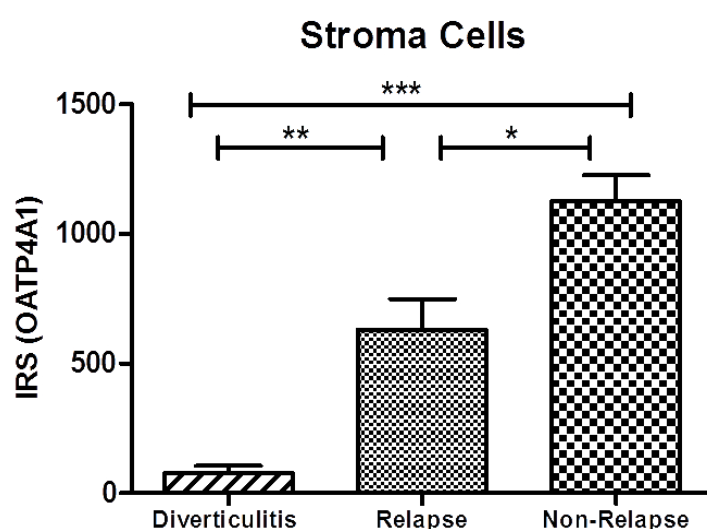


Fig. 28: IRS values of OATP4A1 in stroma cells of colon cancer and diverticulitis patients
Stroma cells in samples from relapse and non-relapse patients were compared with diverticulitis patients. Statistical analysis was performed with the One-Way Anova and Dunn's Multiple Comparison test, ***p=0.001, **p=0.01, *p=0.05.

Significant differences were observed for the IRS values of OATP4A1 in samples from diverticulitis and non-relapse colon cancer patients ($p=0.001$). When OATP4A1 IRS values for diverticulitis patients were compared to those of relapse patients, there was still a remarkable significance visible ($p=0.01$).

Summary

The statistical evaluation of tumor, immune and stroma cells was done separately using the Mann-Whitney-U, One-Way Anova and Dunn's Multiple Comparison test. Tumor, immune and stroma cells of non-relapse patients showed a higher expression of OATP4A1 than relapse patients. No significant difference was found for OATP4A1 levels in immune cells of colon cancer patients compared to diverticulitis patients.

4.7 Results CRCLM

Metastases in liver are very common if patients suffer from colon cancer. So far, data from immunohistochemistry revealed an altered pattern of OATP4A1 in colon cancer tissue samples. Therefore, we investigated OATP4A1 mRNA levels in samples from colon cancer liver metastases.

Despite individual variations between the patients, a tendency for higher mRNA levels was found in colorectal cancer metastases. Liver tissue samples from the adjacent normal looking tissue were used as controls.

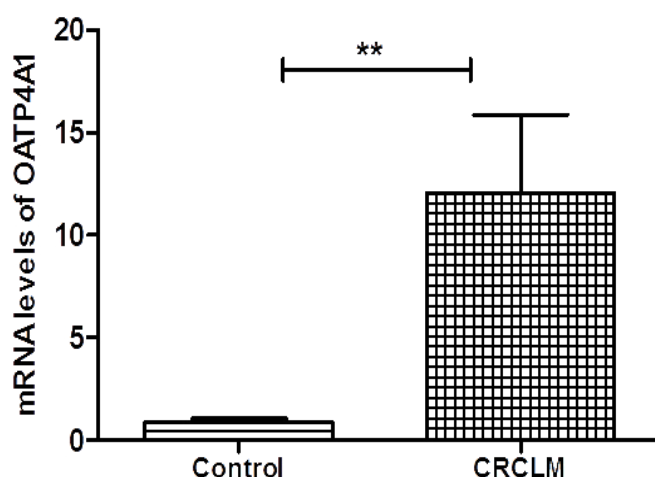


Fig. 29: mRNA expression of OATP4A1 in CRCLM

Normal adjacent liver samples were compared with CRCLM specimens. The metastatic samples show higher mRNA levels of OATP4A1 than the control group, $**p=0.001$ (Mann-Whitney-U-test).

CRCLM patients showed higher mRNA levels of OATP4A1 than the control group. Statistical evaluation was performed using the Mann-Whitney-U-test ($p=0.001$).

4.8 Identification and Localization of OATP4A1 in Diverticulitis and Cancer Specimens

To identify and localize the immune cells expressing OATP4A1, double IF staining was performed in colon cancer and diverticulitis specimens.

The surface markers anti-CD3, anti-CD20, anti-CD34, anti-CD45, anti-CD68, anti-CD83, anti-CD141 and anti-CD163 were labeled with the fluorochrome Alexa Fluor 568 to result in a red staining. The antibody anti-OATP4A1 and a secondary antibody conjugated with the fluorochrome Alexa Fluor 488, resulted in a green staining. In the case of positive double staining, the overlap of the colors red and green resulted as a yellow or orange staining, whereby the resulting color depended on intensity of the green or red staining.

Tab. 11: Identification of the double (OATP4A1, cell marker) positive cells in tissue samples of diverticulitis and colon cancer patients

Double staining	Marker for	Diverticulitis	Colon Cancer
CD3 + OATP4A1	T-lymphocytes	pos.	-
CD20 + OATP4A1	B-lymphocytes	pos.	pos.
CD34 + OATP4A1	Precursor Cells	pos.	pos.*
CD45 + OATP4A1	Leukocytes	pos.	pos.
CD68 + OATP4A1	Macrophages	pos.	<i>neg.</i>
CD83 + OATP4A1	Dendritic Cells	pos.	<i>neg.</i>
CD141 + OATP4A1	Dendritic Cells	pos.	pos.*
CD163 + OATP4A1	Macrophages, Monocytes	<i>neg.</i>	<i>neg.</i>
* Refers to the non-malignant adjacent border/cells in cancerous tissue.			

Due to changes in the environment, immune cells in non-malignant tissue might differ from those in cancer. Therefore, the double immunofluorescence staining was not only performed in diverticulitis, but also in cancer specimens. Table 11 gives an overview of double positive cells in colon cancer and diverticulitis specimens. It must be noted that not every positive double staining experiment in non-malignant samples revealed the same results as for cancer specimens. The double staining

experiments for CD68 (macrophages) and CD83 (dendritic cells) with OATP4A1 were only positive in diverticulitis specimens and not in cancer samples.

4.8.1 Identification of OATP4A1 in CD45-positive Leukocytes

The function of leukocytes is the defense against infectious pathogens. The population of leukocytes comprises among other cells, neutrophilic, eosinophilic and basophilic granulocytes (Junqueira et al., 2005). Due to the fact that the concentration of leukocytes increases during infection, fever, stress or in areas of tissue necrosis, OATP4A1 may play a central role in these cells. Therefore, CD45 leukocytes were investigated for their ability to express OATP4A1.

Double IF staining of OATP4A1 and CD45 clearly revealed that leukocytes express OATP4A1. It is evident that the staining with the anti-CD45 antibody detects the majority of immune cell subtypes. However, the double positive staining predominantly was found in cells in immune cell clusters, as seen in Figure 31. The equivalent IHC staining of CD45 in the tissue of the same patient endures the high quantity of CD45⁺ leukocytes (Figure 32). Double positive cells were also frequently found in the stroma between the epithelial cells. This pattern can be observed in Figure 30. In summary, the expression of OATP4A1 positive CD45⁺ leukocytes in cancer and non-malignant tissue showed a similar pattern.

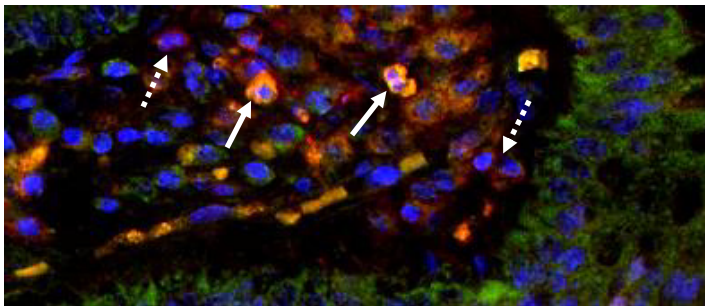


Fig. 30: Detection of CD45 and OATP4A1 by IF staining in a diverticulitis section (patient 15695)

The orange overlay is the result of a positive staining of OATP4A1 (green) and CD45 positive leukocytes (red) as indicated by arrows. CD45 positive cells without OATP4A1 appear in a red color (dotted arrows). Nuclei were stained blue by DAPI

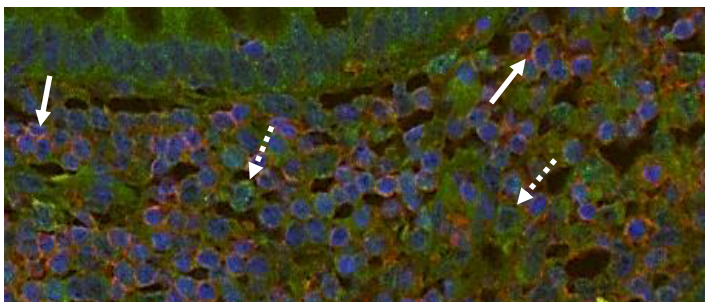


Fig. 31: Detection of CD45 and OATP4A1 by IF staining in a cancer specimen (patient 25155)

From the leukocytes in a cluster, the majority shows a positive staining of OATP4A1 (orange-red color, indicated by arrows). Single OATP4A1 positive cells appear in a green staining only (dotted arrows).

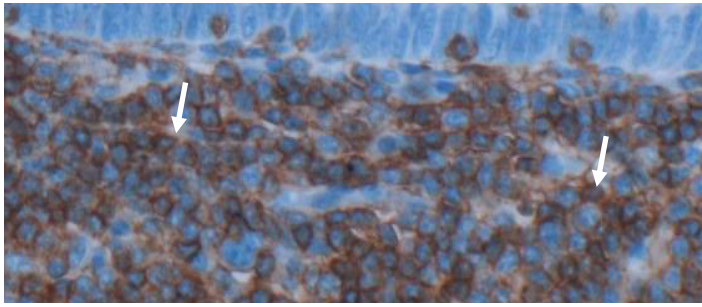


Fig. 32: Detection of CD45 positive leukocytes in IHC experiments; positive staining in a cancer sample (patient 25155)

The brown membrane staining is clearly seen for CD45 positive leukocytes (arrows).

4.8.2 Identification of OATP4A1 in CD20-positive B-Lymphocytes

CD20 is a 33-37 kDa membrane associated and non-glycosylated phosphoprotein and acts as a surface marker. It is expressed on B-lymphocytes, especially in the mature forms (Cragg et al., 2005).

Lymphocytes are mononuclear cells and thereby important for the immune defense (Junqueira et al., 2005).

Using surface protein markers, two types of lymphocytes can be classified; namely the B-lymphocytes which carry immunoglobulins on their surface and T-lymphocytes. The precursor cells of T-cells leave the thymus and after final maturation and differentiation, they circulate in lymphoid organs. They do not have immunoglobulins on their surface (Junqueira and Mescher, 2010). Natural killer cells (NK), which are part of the innate immune response (similar as granulocytes and macrophages) neither carry B, nor T-cell markers. They need the assistance of the antigen presenting cells for stimulation. B-cells need the T-helper cells for activation.

As B-lymphocytes have a crucial impact on anti-tumor responses, CD20 was a suitable marker for the detection of B-lymphocytes. Therefore, double IF staining was performed in malignant and non-malignant tissue samples to identify OATP4A1 positive B-cells.

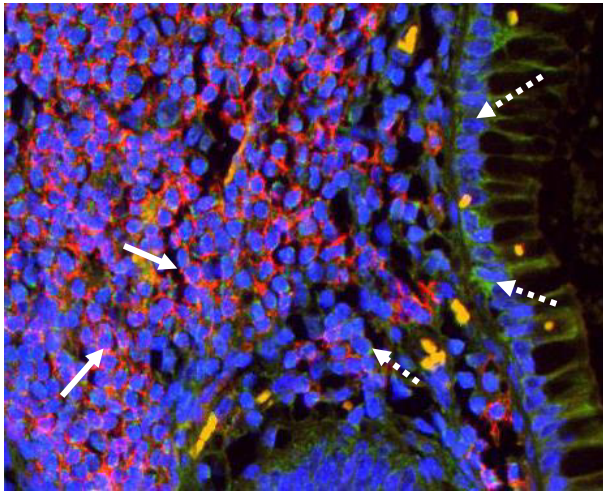


Fig. 33: Detection of CD20 and OATP4A1 by IF staining in a diverticulitis section (patient 2527)

This Figure shows the positive double staining of CD20 and OATP4A1 in immune cells. CD20 positive B-cells (arrows) often appear in clusters, as seen above. But not all B-cells are positive for OATP4A1. The adjacent crypts (dotted arrows) and some immune cells in stroma are OATP4A1 positive.

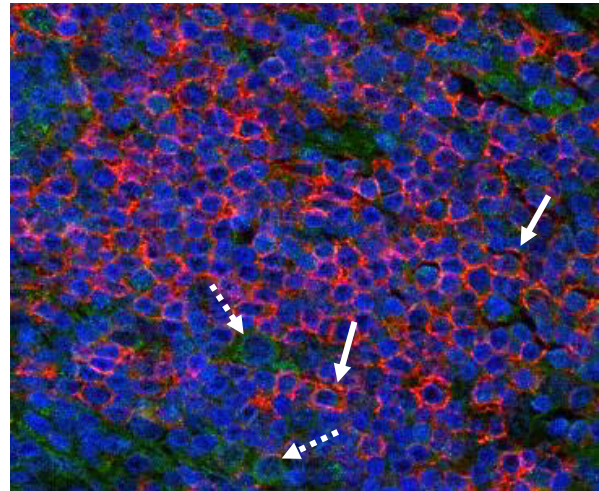


Fig. 34: Detection of CD20 and OATP4A1 by IF staining in a colon cancer section (patient 25155)

As seen in this image, an immune cluster with B-cells express OATP4A1 (white arrows). Not every B cell is OATP4A1 positive (dotted arrows).

Having a closer look at the pattern of CD20 positive B-lymphocytes in colon cancer and diverticulitis specimens, the B-cells mainly appeared as immune colonies or clusters of different sizes.

The Figures 33 and 34 illustrate the overlay of OATP41 positive CD20⁺ B-cells in diverticulitis and colon cancer tissue, respectively. Scattered OATP4A1 positive cells were also seen in both tissues.

As already described, B-cells mainly appear in clusters, but they can also appear in smaller density in the stroma. Compared to other markers, which were stained together with OATP4A1 (for the identification of OATP4A1 positive immune cells), the CD20⁺ B-lymphocytes in addition to CD45-positive leukocytes, showed a higher amount of OATP4A1 in the examined samples.

4.8.3 Identification of OATP4A1 in CD3-positive T-Lymphocytes

In mice experiments, lymphocytes, which are able to secrete cytotoxic perforin, play an important role in cancer immunology. It has been reported that lymphocytes have anti-tumoral effects (Smyth et al., 2000) and conclusive reports have shown that T-cells, which infiltrate the tumor, correlate with a better survival of patients with colorectal cancer (Baier et al., 1998; Camus et al., 2009).

As T-lymphocytes are important key players in immune response and activation, it was absolutely essential to find out, whether OATP4A1 is expressed in a certain population of immune cells.

Double IF staining of OATP4A1 and CD3 was performed on diverticulitis samples. The staining of OATP4A1 was positive for few cells, but the majority was only CD3-positive. CD3⁺ T-cells, which were positive for OATP4A1, were very rare, as indicated in Figure 35. CD3/OATP4A1 positive cells were predominantly detected in the stroma. CD3 positive cells without OATP4A1 staining were seen more often.

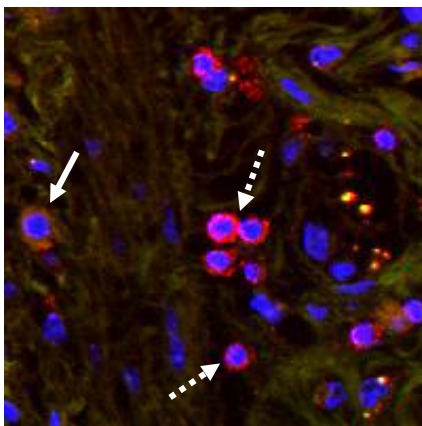


Fig. 35: Detection of CD3 and OATP4A1 by IF staining in a diverticulitis specimen (patient 16106)

Positive CD3 cells are mainly localized in the stroma (dotted arrows). The rare appearance of CD3/OATP4A1 double positive cells is indicated by an arrow.

4.8.4 Identification of OATP4A1 in CD83-positive Dendritic Cells

Dendritic cells play an important role in cancer therapy because of their key function as powerful antigen-presenting cells. Their presence may correlate with a better prognosis for patients with colorectal cancer.

Interestingly, dendritic cells are more frequently localized near the tumor and mainly occur in earlier stages of cancer (Zlobec and Lugli, 2009).

The double immunofluorescence staining for OATP4A1 and CD83 was done on tissue samples from colon cancer and diverticulitis patients.

First, single CD83 IHC staining was performed in diverticulitis and cancer sections. Surprisingly, the amount of CD83-positive dendritic cells was very modest, in cancer tissue even more than in non-malignant tissue.

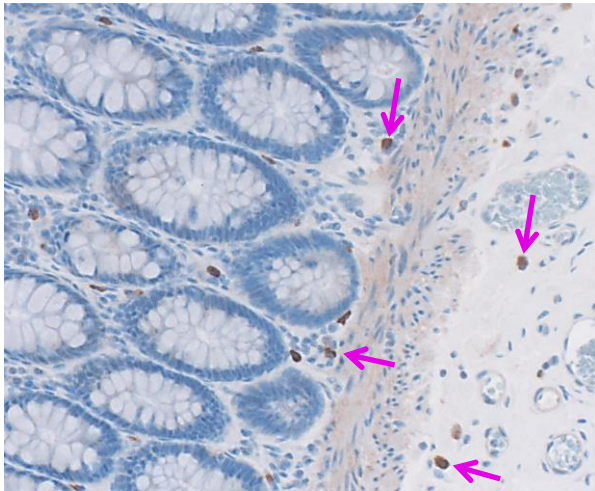


Fig. 36: IHC staining of CD83 in a diverticulitis section (patient 9938)

A brown staining of CD83 positive dendritic cells is shown. Nuclei are stained blue by hematoxylin.

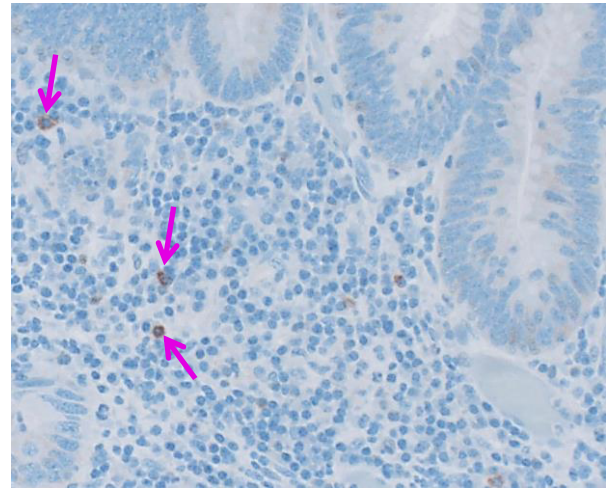


Fig. 37: IHC staining of CD83 in a cancer section (patient 25155)

A brown membrane staining of CD83 positive dendritic cells in the stroma is visible.

Positive dendritic cells were predominantly located in stroma between the crypts as shown in tissue samples of diverticulitis and cancer patients (Figures 36 and 37).

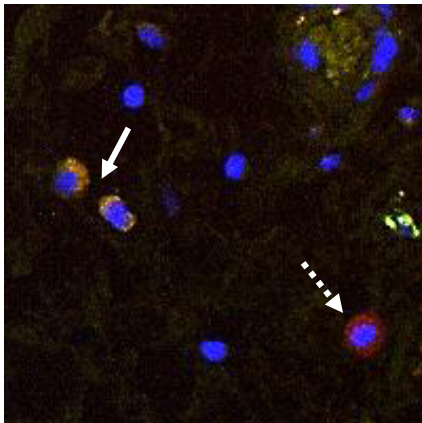


Fig. 38: Detection of CD83 and OATP4A1 by double IF staining in a diverticulitis tissue sample (patient 20279)

Besides double positive cells, single positive dendritic cells were also detected, primarily in stroma.

Figure 38 shows the double staining of CD83/OATP4A1 in a diverticulitis tissue. Compared to CD20 or CD45 positive cells, the amount of CD83 cells, which were positive for OATP4A1, was much lower. CD83 positive dendritic cells were seen predominately in stroma of diverticulitis and colon cancer tissue samples.

However, in cancer specimens, CD83⁺ cells, positive for OATP4A1, were absent. These findings match with the immunohistochemical staining of CD83 in a cancer sample. The IHC staining of CD83 positive cells demonstrates the low expression of CD83 positive cells in a cancer specimen (Figure 37). The absence of CD83 dendritic and OATP4A1-positive cells was confirmed by double IF staining.

4.8.5 Identification of OATP4A1 in CD34-positive Precursor Cells

CD34, a marker for endothelial and hematopoietic precursor stem cells, was stained together with OATP4A1. A positive double staining was found in diverticulitis and colon cancer specimens. In the diverticulitis samples (Figure 39), the overlay of the red CD34⁺ and green positive OATP4A1 staining is clearly visible. Figure 40 confirms the OATP4A1 staining in the same patient (assessed by immunohistochemistry).

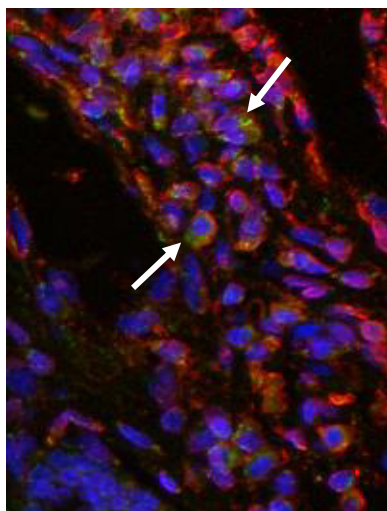


Fig. 39: Detection of CD34 and OATP4A1 by IF staining in a diverticulitis section (patient 24511)

The overlay of the red and green staining can be seen clearly (white arrows).

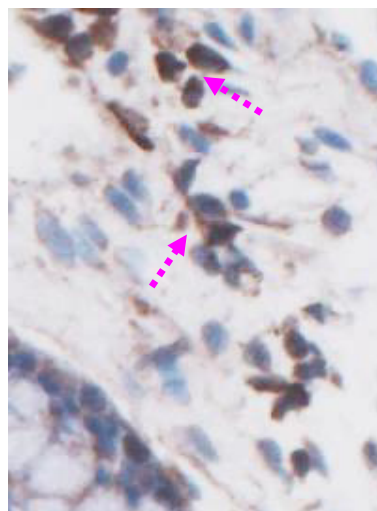


Fig. 40: IHC staining of OATP4A1 positive cells in a diverticulitis section (patient 24511)

The brown staining is the result of a positive OATP4A1 membrane staining (arrows).

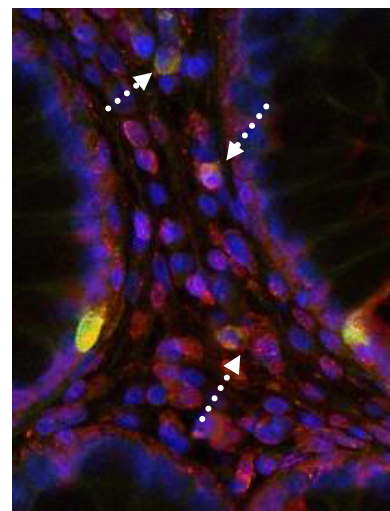


Fig. 41: Detection of CD34 and OATP4A1 by IF staining in a colon cancer sample (patient 9890)

The merged image shows positive CD34/OATP4A1 cells in the tumor adjacent tissue (dotted arrows).

CD34⁺ positive precursor cells, which also express OATP4A1, were found in cancer and diverticulitis specimens. These cells were predominantly located in the tumor adjacent tissue or stroma respectively as depicted in Figure 41. There is evidence showing that CD34-positive precursor cells in cancer tissue may lose the ability to express OATP4A1.

4.8.6 Identification of OATP4A1 in CD141-positive Dendritic Cells

As stated earlier, dendritic cells are significant for the function of the immune system. Using double immunofluorescence staining of CD141 and OATP4A1, it could be shown that CD141-positive dendritic cells express OATP4A1. A staining of the endothelial cells lining the blood vessels was seen quite often in the tissue samples of colon cancer and diverticulitis. Figure 42 indicates a positive endothelial staining of

CD141/OATP4A1. The immunohistochemical staining of OATP4A1 done on a sample from the same patient confirms these findings (Figure 43).

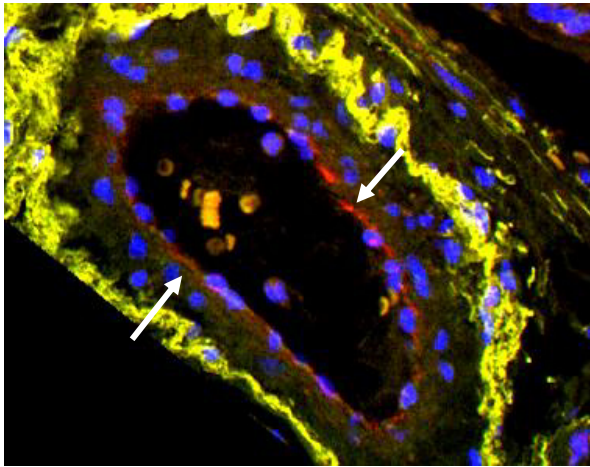


Fig. 42: Detection of CD141 and OATP4A1 by IF staining in a diverticulitis section (*patient 16106*)

This Figure shows the positive double staining of OATP4A1 and CD141. CD141, a marker for endothelial and dendritic cells, is clearly seen as positive staining in red (white arrows).

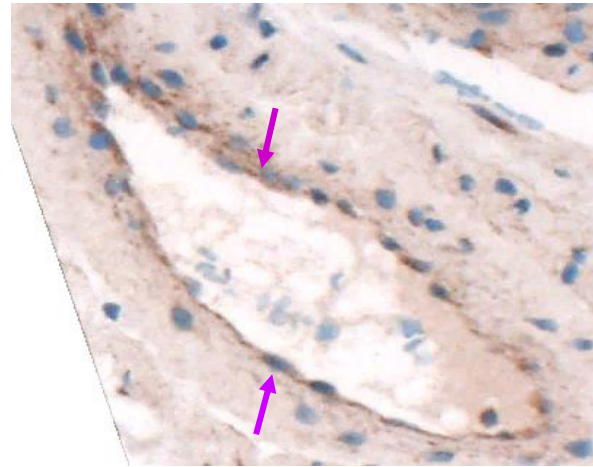


Fig. 43: IHC staining of OATP4A1 positive cells in a diverticulitis section (*patient 16106*)
OATP4A1-positive endothelial staining is visible (indicated by colored arrows).

Interestingly, similar to the data for CD34⁺ positive precursor cells, the double positive staining with CD141 and OATP4A1 in cancer specimens was mainly found in the tumor adjacent normal tissue as seen in Figure 44. The overlay shows an orange staining of cells, resulting from the red staining (CD141⁺ dendritic cells) and the green staining (OATP4A1).

In addition, the immunohistochemical staining of OATP4A1 cells on a specimen from the same patient confirms the expression of OATP4A1 (Figure 45).

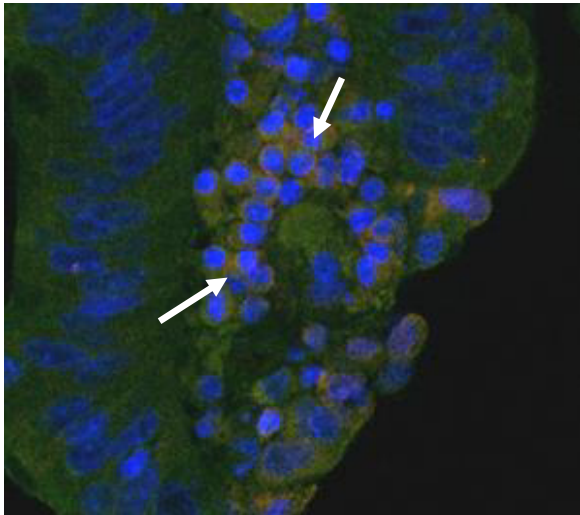


Fig. 44: Detection of CD141 and OATP4A1 by IF staining in a colon cancer specimen (patient 25155)

The orange-brown overlay (arrows) is the result of a positive staining of CD141 dendritic cells (red) and positive OATP4A1 cells (green).

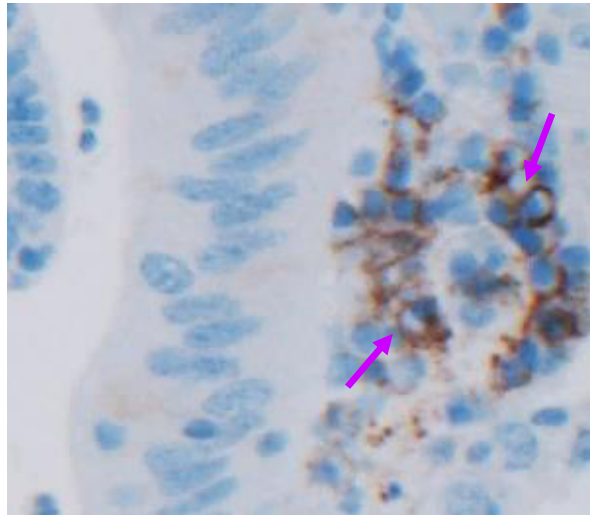


Fig. 45: IHC staining of OATP4A1 in a colon cancer specimen (patient 25155)

The brown staining is the result of a positive OATP4A1 staining

4.8.7 Identification of OATP4A1 in CD68-positive Macrophages

Macrophages develop in the bone marrow and their main function is the phagocytosis of harmful particles (e.g. dead cells, cell debris, bacteria and other particles). Therefore, they are important in inflammation and cancer. Due to their localization in the connective tissue, they are called histiocytes and hepatic macrophages are called Kupffer cells (Junqueira et al., 2005).

Similar to granulocytes, monocytes are transported from the bone marrow into the tissues. In certain tissues, e.g. the liver, the alveoli in the lungs or lymphatic tissues, monocytes differentiate to macrophages (Junqueira et al., 2005). Growth factors secreted from monocytes, e.g. epidermal growth factor (EGF) receptor ligands, scatter factor/hepatocyte growth factor (HGF) directly operate on tumor cells and induce proliferation and migration (Wagener and Müller, 2010).

CD68 is a marker for macrophages and also present on monocyte subpopulations. The marker CD163 identifies activated macrophages.

Patients with diverticular disease often show different inflammatory infiltrates in the colon, where many activated CD68⁺ macrophages are present. Interestingly, this kind of inflammation is also seen after previous therapy with antibiotics (Ambrosetti et al., 1997).

After performing a double IF staining of CD68 and OATP4A1 in diverticulitis specimens, it could be shown that the amount of positive CD68⁺ macrophages was remarkably high. Normally, the density of macrophages in colon cancer specimens is lower. As seen in Figure 46, positive CD68⁺ macrophages (red staining) express OATP4A1 (green staining). Additionally, to confirm these findings, an IHC staining of OATP4A1 was performed on a diverticulitis sample from the same patient (Figure 47).

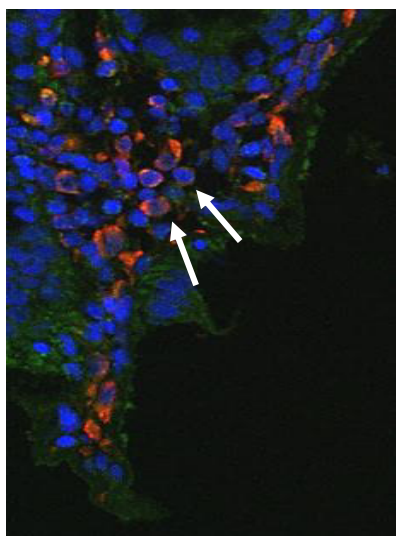


Fig. 46: Detection of CD68 and OATP4A1 by IF staining in a diverticulitis section (*patient 21938*)

CD68 positive macrophages (red staining) express OATP4A1 (green staining). The overlay is orange-red (arrows).

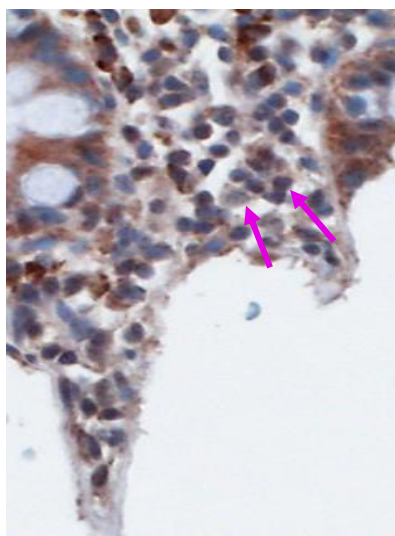


Fig. 47: IHC staining of OATP4A1-positive cells in a diverticulitis section (*patient 21938*)

The brown membrane staining is the result of a positive OATP4A1 staining (arrows).

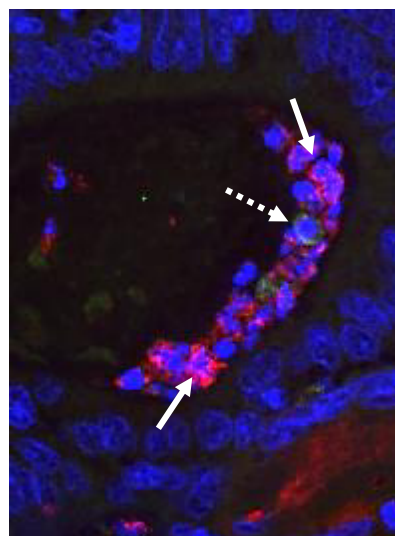


Fig. 48: Detection of CD68 and OATP4A1 by IF staining in a colon cancer section (*patient 25155*)

CD68 positive macrophages result in a pink staining (arrows). OATP4A1 is absent in the double staining. Isolated OATP4A1 positive cells are seen (dotted arrow).

In cancer specimens a positive staining of CD68⁺ macrophages and OATP4A1 could not be detected (Figure 48).

CD68-positive macrophages, which were found in cancer specimens, were mainly located in the stroma and blood vessels. In the same tissue, OATP4A1 single positive cells were also visible.

4.8.8 Identification of OATP4A1 in CD163-positive Macrophages

From the population of CD68⁺ macrophages, particularly, activated CD163⁺ macrophages contribute to inflammation in specimens of diverticulitis patients (von Rahden et al., 2011).

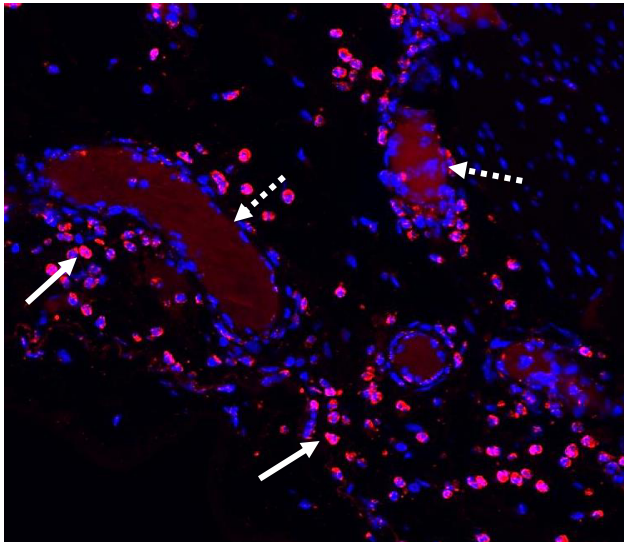


Fig. 49: Detection of CD163 and OATP4A1 by IF staining in a diverticulitis tissue section (patient 24511)

CD163 positive cells resulting in a pink staining (white arrows) are predominantly seen around blood vessels (dotted arrows). CD163 positive cells do not express OATP4A1.

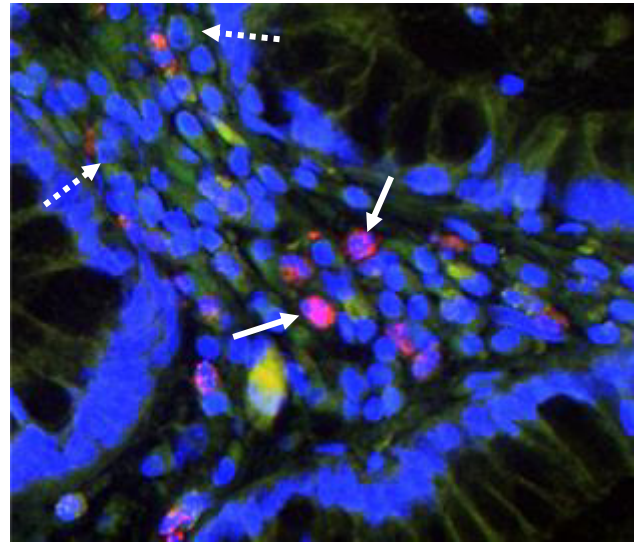


Fig. 50: Detection of CD68 and OATP4A1 by IF staining in a colon cancer tissue section (patient 25155)

Similar to diverticulitis patients, CD163 (white arrows) positive macrophages do not express OATP4A1. Isolated OATP4A1 positive cells are also visible (dotted arrows).

The marker CD163 was used to identify activated macrophages, but this population did not show a positive result for OATP4A1, neither in cancer nor in diverticulitis specimens. This leads to the conclusion that not all types of macrophages express OATP4A1.

Not only in diverticulitis tissue samples, as seen in Figure 49, but also in colon cancer samples, which were stained for CD163 and OATP4A1 (Figure 50), a high density of activated macrophages (CD163⁺) around blood vessels was found. However, in epithelial cells of the crypts or tumor cells only a few macrophages were visible, which were negative for OATP4A1.

5 Discussion

Results from different laboratories have shown that OATPs are gaining wide acceptance for their important role in cancer chemotherapy and tumor progression based on their ability to mediate the uptake of xenobiotics and drugs. Since certain drugs are able to inhibit OATP function, they may influence the efficacy of therapeutic drugs.

To obtain more information about mechanisms active in colon cancer progression, the purpose of this study was to clarify the distribution of OATP4A1-positive cells in colon cancer specimens from 185 patients with and without an early relapse. The data were compared with non-malignant diverticulitis samples.

The main finding of this study was that tumor, immune and stroma cells of non-relapse patients showed higher IRS values for OATP4A1 compared to relapse patients. OATP4A1 seems to be up-regulated in patients with a better prognosis as already shown in a previous pilot study (Zotter, 2012). Moreover, mRNA levels of OATP4A1 were increased in colon metastases in liver as compared to normal liver. Due to the fact that mRNA expression analysis does not give any information about the expression of OATP4A1 in different cell types, immunohistochemistry studies were also done on the tissue sections. These studies revealed that OATP4A1 is highly present in tumor cells and immune cells while its expression in normal mucosa and stroma is low. The expression of OATP4A1 in non-malignant mucosa, even in diverticulitis colon showing signs of inflammation, is low, but OATP4A1 is abundantly present in immune cells. Significantly higher expressions of OATP4A1 were seen in immune cells in non-malignant diverticulitis tissue compared to epithelial cells and stroma by quantitative microscopic image analysis.

As the immune system is always involved in pathological processes in the colon, the identification of the type of immune cells that express OATP4A1 is very important.

By double immunofluorescence staining experiments, OATP4A1 was found in CD20⁺ B-cells and CD45⁺ leukocytes in non-malignant diverticulitis and cancer specimens. Both types of tissues showed a higher amount of double positive CD20/OATP4A1 and CD45/OATP4A1 cells than other immune cells. Interestingly, CD68⁺ macrophages, which express OATP4A1 were detected in samples from diverticulitis patients, but not in those from colon cancer. Also CD83⁺ and CD141⁺ dendritic cells were positive for OATP4A1 in diverticulitis specimens only. The immune cells in

cancer may lose their function of expressing OATP4A1. These data are consistent with previous findings on OATP4A1 mRNA in macrophages and dendritic cells (Bleasby et al., 2006; Janneh et al., 2008; Skazik et al., 2008).

Similar to macrophages, CD34⁺ positive endothelial and monocyte precursor cells, as well as CD83⁺ and CD141⁺ dendritic cells were also positive for OATP4A1 in diverticulitis samples only. Interestingly, in cancer samples CD34⁺, CD83⁺ and CD141-positive immune cells were mainly found in the adjacent tumor mucosa, leading to the conclusion that specific subtypes of immune cells may lose the function of expressing OATP4A1 following malignant transformation leading to colon cancer. Otherwise, CD163⁺ macrophages did not show any positive staining for OATP4A1, neither in diverticulitis nor in cancer specimens.

Nevertheless, the results on the expression of OATP4A1 in inflammatory cells, suggests a role of this OATP in inflammatory colon disease and colon cancer. Previous studies of Wojtal et al. (2009) have unambiguously shown that certain transporters of the OATP family, such as OATP4A1, may contribute to inflammatory bowel diseases including Crohn's disease and ulcerative colitis. Thereby, the stage of inflammation may even affect the function of the transporters. They found that in Crohn's disease and also in ulcerative colitis, the mRNA levels of OATP4A1 were increased compared to healthy colon specimens. Besides peptide transporters and equilibrative nucleoside transporters, significant higher levels of these OATPs were observed when the paired biopsies of ulcerative colitis patients were compared and analyzed (Wojtal et al. 2009).

These results lead to the conclusion that inflammation may induce OATP4A1 mRNA levels in colon. However, studies in our laboratory showed that OATP4A1 mRNA levels in the metastatic cell line C205 indeed changed in response to inflammatory mediators. Treatment with interleukin-6 and tumor necrosis factor alpha however lead to a reduction of OATP4A1 mRNA levels in this metastatic colon cancer cell line (Katic, 2014). Therefore, no direct correlation with inflammation in tissues could be found. We also found that mRNA levels of OATP4A1 were increased in colon metastases in liver; here too the reason for the induction remains unclear.

In conclusion, high levels of OATP4A1 in immune cells in tumor samples from colon cancer patients with a better prognosis and in diverticulitis patients suggest that the transporter may play an important role in activating the immune system.

OATP4A1 could mediate the uptake of endogenous substrates, such as prostaglandins, cyclic nucleotides or peptides. These compounds could be important for the activation of specific cell populations in malignant and non-malignant diseases of the colon.

Furthermore, OATP4A1 positive immune cells were identified in subpopulations of CD45⁺ lymphocytes, CD3⁺ T-cells, CD20⁺ B-lymphocytes, CD68⁺ macrophages, CD83⁺, CD141⁺ dendritic cells and CD34⁺ precursor cells.

Whether OATP4A1 may be targeted to prevent an early recurrence of colon cancer still requires further investigation.

6 Summary

So far there are no appropriate biomarkers or other prognostics tools available to reliably predict colon cancer progression.

Therefore, the organic anion transporting polypeptides (OATPs) may be a target. OATPs are transmembrane proteins with six families and subfamilies. The uptake of endogenous substrates is sodium-independent. OATPs mediate the uptake of different hormones, cyclic nucleotides, peptides, prostaglandins, xenobiotics and drugs. It is known that drugs such as Methotrexate or Irinotecan are also substrates for OATPs.

OATPs continue to attract more and more attention. It has become increasingly apparent that the expression of these transporters plays an important role in cells and different tissues, particularly on a cellular level. Expression of these transporters can be very tissue specific and may even change in cancer. Therefore, OATPs may act as a predictive factor for the progression of different tumors including colorectal cancer. Previous studies have shown increased mRNA levels of OATP4A1 in colon cancer patients compared to healthy controls.

This work has elucidated the expression of OATP4A1 in tissue sections from 185 colon cancer patients, suffering from colon cancer UICC stage I and II, with and without an early recurrence within 5 years. Paraffin-embedded tissues were stained by immunohistochemistry. To facilitate a comparison with non-malignant colon tissues, OATP4A1 staining was also performed on diverticulitis specimens. Following an image acquisition with the TissueFAXS[®] microscopy-based system, the evaluation of the cellular levels was done by using the HistoQuest[®] program. Statistical analysis was performed with the GraphPadPrism[®] software. The immunoreactive score (IRS), calculated by the percentage and the mean intensity of OATP4A1-positive cells, revealed different patterns of OATP4A1 in tumor, stroma and immune cells. Comparative analyses were also done for diverticulitis patients.

For colon cancer patients, patients were divided into two groups. Patients without an early relapse (n=141) were compared to patients with a relapse (n=44) within 5 years. Immune cells of patients with a better prognosis (no relapse within 5 years) showed higher IRS values for OATP4A1 when compared to relapse patients (IRS=5588±2752 vs. 4021±3023, p=0.0001). Tumor cells of colon cancer patients

without a relapse compared to those who relapsed, also showed significantly higher levels of this transporter (IRS=4524±2813 vs. IRS=3468±2851, $p=0.001$). Similar significant values were found for stroma cells in non-relapse versus relapse patients (IRS=1126±1126 vs. 629±785, $p=0.001$).

A statistical evaluation was also performed for non-malignant diverticulitis samples. In diverticulitis samples, epithelial and stroma cells showed the lowest amount of OATP4A1 positive cells, when compared to colon cancer. Surprisingly, OATP4A1 IRS values of immune cells in diverticulitis did not show any significant difference to those in colon cancer patients.

For the identification of immune cells which express OATP4A1, double IF staining was performed. CD20⁺ B- and CD3⁺ T cells, CD45⁺ leukocytes, CD68⁺ macrophages, CD83⁺ and CD141⁺ dendritic cells were identified as OATP4A1 positive in colon cancer and diverticulitis specimens. CD83⁺ and CD141⁺ dendritic cells, and CD34⁺ precursor cells in the tumor adjacent border in colon cancer tissue samples were positive for OATP4A1.

CD163⁺ macrophages were negative for OATP4A1 in diverticulitis and in colon cancer tissues.

Additionally, in patients with colon metastases higher OATP4A1 mRNA levels were found than in the control group.

Current knowledge does not permit a complete understanding of OATPs and their potential role in tumor and cell proliferation, but a correlation between OATP4A1 with clinical parameters showed that there is a better prognosis for colon cancer patients with higher levels of OATP4A1.

The data obtained from this thesis should contribute to a better understanding of this significant transporter from the SLCO family in the near future.

7 Abbreviations

A

ABC transporters	ATP-binding cassette transporters
APC	Adenomatous polyposis coli

B

BSA	Bovine serum albumin
-----	----------------------

C

CA 19-9	Carbohydrate antigen 19-9
CD	Cluster of differentiation
CEA	Carcino embryonic antigen
CKK	Cholecystokinin
CRCLM	Colorectal cancer liver metastases
CRP	C-reactive protein
CT	Computer-assisted tomography

D

DAB	3,3'-Diaminobenzidine
DAPI	4',6-Diamidine-2'-phenylindole, dihydrochloride
DEPP	Deparaffinisation and pretreatment epitope retrieval solution
DHEAS	Dehydroepiandrosteronsulphate
5-dUMP	5-Fluordesoyuridine-monophosphate

E

EDTA	Ethylene-diamine-tetra acetic acid
EGFR	Epidermal growth factor receptor
ENT1	Equilibrative nucleoside transporter 1

F

FAP	Familial adenomatous polyposis coli
FBS	Fetal bovine serum
FCS	Fetal calf serum
FOVs	Field of views
5-FU	5-Fluorouracil

G

GALT	Gut-associated lymphoid tissue
GFP	Green fluorescent protein
GITR	Glucocorticoid induced tumor necrosis factor receptor

H

H1R	Histamine 1 receptor
H2R	Histamine 2 receptor

H ₂ O ₂	Hydrogen peroxide
HGF	Hepatocyte growth factor
HNF1 α	Hepatocyte nuclear factor 1 α
HNPCC	Hereditary non-polyposis colorectal cancer
HRP	Horse radish peroxidase
HUGO	Human genome organization
I	
IF	Immunofluorescence
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IL	Interleukin
IRS	Immunoreactive score
L	
LDH	Lactate dehydrogenase
LV	Leucovorin
M	
M cells	Microfold cells
N	
NF κ B	Nuclear factor κ B
NSAIDs	Non-steroidal anti-inflammatory drugs
O	
OATP(s)	Organic anion transporting polypeptide(s)
OCTN	Organic cation/carnitine transporters
P	
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
R	
ROIs	Region of interests
S	
SLCO family	Solute carrier organic anion transporter family
SNPs	Single nucleotide polymorphisms
T	
T3	Triiodothyronine
T4	Thyroxine
TMA _s	Tissue micro arrays

TNF α	Tumor necrosis factor alpha
TNF β	Tumor necrosis factor beta
U	
UICC	Union Internationale Contre le Cancer
UR	Upper right
V	
VEGF	Vascular endothelial growth factor

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10 Literature

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Work experience

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