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ABBREVIATION

WHO	World Health Organization
EOC	epithelial ovarian cancer
FIGO	International Federation of Gynecology and Obstetrics
GOG	Gynecology Oncology Group
OSE	ovarian surface epithelium
EGF	epidermal growth factor
TGFα	transforming growth factor alpha
TGFβ	transforming growth factor beta
NF-κB	nuclear factor-κB
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)
NO	nitric oxide
HIF1α	hypoxia-inducible factor 1α
COX2	cyclooxygenase 2
TNF	tumor necrosis factor
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-3	Interleukin-3
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-18	Interleukin-18
IL-23	Interleukin-23
IL-1RA	Interleukin-1 receptor antagonist
INF-γ	Interferon-γ
MMP	matrix metalloproteinases
M-CSF	macrophage colony stimulating factor

ABBREVIATION: Continuation

GM-CSF	granulocyte-macrophage colony-stimulating factor
iNOS	inducible nitric oxide synthase
ROS	reactive oxygen species
ROI	reactive oxygen intermediates
TAMs	tumor-associated macrophages
MCP	monocyte chemotactic protein
CSF	colony stimulating factor
VEGF	vascular endothelial growth factor
Tregs	regulatory T cells
Th1	T helper cells 1
Th2	T helper cells 2
LPS	lipoploysaccharide
MTC	macrophage-mediated tumor cytotoxicity
ADCC	antibody-dependent cellular cytotoxicity
MIF	migration inhibitory factor
uPA	urokinase-type plasminogen activator
FGF	fibroblast growth factor
PDGF	platelet-derived growth factor
ECM	extracellular matrix
CD68	cluster of differentiation 68
CD163	cluster of differentiation 163
EDTA	ethylendiaminetetraacetic acid
PBS	phosphate buffered saline
FCS	fetal calf serum
BSA	N,O-bis(trimethylsilyl)acetamide
DAB	3,3-Diaminobenzidine
IHC	immunohistochemistry
IF	immunofluorescence

TABLE OF CONTENT

ABSTRACT

Ovarian carcinoma is the fifth leading cause of cancer-related death and the ninth most common cancer among women thereby, remaining the deadliest gynecological malignancy in the western world. The common therapy for ovarian carcinoma is still the standard platinum-based therapy and further personalized therapeutic strategies were not yet defined. Several epidemiologic studies have shown that inflammation plays a crucial role in initiation and progression of epithelial ovarian cancer. A novel approach for understanding ovarian carcinoma tumerogenesis is thus attributed to assessment of local immune response.

In the current study the microscopy-based TissueFAXS system was used to assess the immunological imprint in ovarian carcinoma specimens. Being part of a large-scale-project, the current work deals with the qualitative and quantitative analysis of the macrophage/monocyte lineage. Examination included tissue samples from patients with different stages of primary serous and endometrioid carcinomas stained with the anit-CD68 antibody. Paraffin-embedded sections were stained using immunohistochemical approach and acquired using the microscopy-based TissueFAXS system. Patient-specific localization and organization pattern as well as intra- and inter-patient variability in the extent of CD68-positive macrophages found in tumor and stroma tissue was observed. As determined by anti-CD163 staining, part of the CD68-positive population showed the phenotype of pro-tumoral, M2 macrophages. Next, using the HistoQuest software an algorithm was established which allows quantitative analysis of the CD68-positive population. Thereby different regions of interest (ROIs) were defined manually, attributing to tumor anatomy and the accumulation pattern of positively stained cells. As results, data sets representing the percentage of CD68-positive cells quantified in the region of interest were exported and incorporated into SPSS database containing the corresponding patient data. As outlook, staining-derived data sets will be used for alignment with clinicopathological parameters and prognostic modelling. The herein described strategy represents an innovative approach toward patients' stratification for prognostic and predictive monitoring and might open new directions for therapeutic interventions.

ABSTRACT (GERMAN)

Ovarialkarzinom ist die fünfthäufigste Ursache bei krebsbedingten Todesfällen und die neunthäufigste Krebserkrankung bei Frauen. Ovarialkarzinom ist damit die tödlichste gynäkologische Erkrankung der westlichen Welt. Die übliche Therapie für Ovarialkarzinom ist immer noch die platinumbasierende Chemotherapie, da andere, personalisierte Therapieansätze noch nicht gefunden worden sind. Verschiedene epidemiologische Studien haben gezeigt, dass Entzündung eine entscheidende Rolle bei der Initiation und der Progression des epithelialen Ovarialkarzinomen spielt. Die Begutachtung des lokalen "immunologischen Imprints" stellt somit einen neuen Ansatz für ein verbessertes Verständnis des Prozesses der Tumorgenese des Ovarialkarzinoms dar.

In der vorliegenden Studie wurde das Mikropskopie-basierte TissueFAXS System zur Untersuchung des immunologischen Imprints in Ovarialkarzinomgewebeproben herangezogen. Als Teil eines Großprojektes, beschäftigt sich diese Arbeit mit der qualitativen und quantitativen Analyse des Makrophagen-/Monozyten Populaton. Die Untersuchung beinhaltet Gewebeproben von Patientinnen mit serösen und endometrioiden Karzinomen in verschiedenen Stadien, welche mit dem anti-CD68 Antikörper gefärbt wurden. Die in Paraffin eingebetteten Schnitte wurden immunohistochemisch gefärbt und mit Hilfe des Mikroskopie-basiertem TissueFAXS System aufgenommen. Dabei konnten patientenspezifische Lokalisations- und Organisationsmustern, sowie Intra- und Interpatientenvariabilität in der Ausdehnung der CD68 positiven Makrophagen im Tumor- und Stromagewebe beobachtet werden. Wie mittels anti-CD163 Färbung festgestellt, zeigte ein Teil der CD68-positiven Population den Phänotyp von pro-tumor, so genannte M2 Makrophagen. Als nächstes wurde die HistoQuest Software in den Prozess miteinbezogen, um einen Algorithmus zu etablieren, der eine quantitative Analyse von CD68-positiven Makrophagen erlaubt. Dabei wurden verschiedene Regionen von Interesse (ROI) manuell definiert, entsprechend der vorliegenden Tumoranatomie und des Auftretens von Ansammlungsmustern der positiv gefärbten Zellen. Im letzten Schritt wurden die Datensätze, die den prozentualen Anteil von positiv quantifizierten CD68-Zellen innerhalb der Regionen von Interesse beschreiben, exportiert und in die SPSS Datenbank, welche bereits die dazugehörigen Patientendaten enthält, eingefügt.

In den nachfolgenden Untersuchungen sollen diese Datensätze zur Korrelation mit klinisch-pathologischen Parametern verwendet werden, um somit prognostische Modelle erstellen zu können. Die hier beschrieben Vorgehensweise repräsentiert einen neuen, innovativen Ansatz für die Patientenstratifizierung im Hinblick auf prognostische und präventive Kontrolluntersuchungen und weist mögliche neue Ansätze für therapeutische Maßnahmen auf.

1. INTRODUCTION

1.1 Ovarian Carcinoma

1.1.1 Epidemiology

40 years after the declaration on the "war against cancer" ovarian cancer is still the fifth leading cause of cancer-related deaths; the ninth most common cancer among women thereby, remaining the deadliest gynecological malignancy in the western world. Ovarian cancer makes up three percent of all cancers in women, whereby the Caucasian women are stronger affected than African-American women.
In Austria malignant ovarian carcinoma is the eighth most common cancer among women and accounts for around 4% of the tumors in Austrian women. In 2010, 675 women were diagnosed an ovarian cancer (9 of 100000) and 438 (5 of 100000) died. In the last ten years the rate of incidences has decreased by 29% and so is mortality rate by 28% for ovarian cancer, up to an age of 75, it is 0,9%. This means one in 111 women will have ovarian cancer in their lifetime. [65,66]

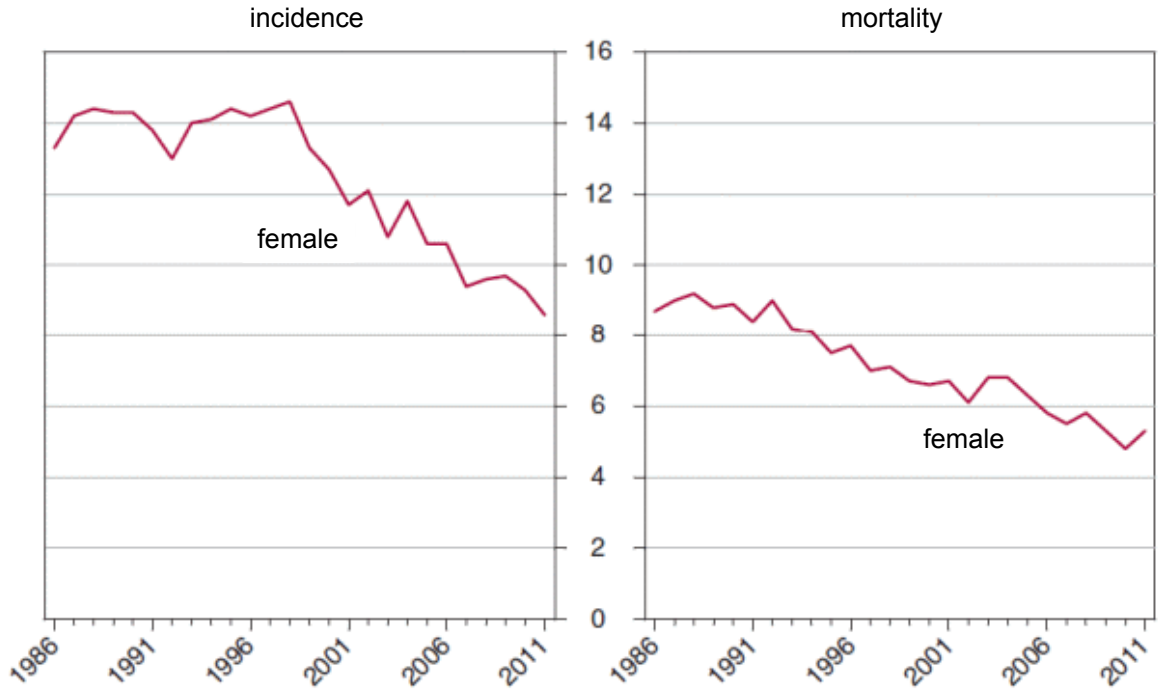

Figure 1: Incidence of ovarian cancer during the time period:
age standardized per 100,000 inhabitants [66]

1.1.2 Classification

The ovary is made up of many different tissues (i.e. germplasma, epithel and connective tissue). The different tissue types are responsible for the function-specific processes in the ovary. Due to the heterogeneity of the tissues comprising the ovary, the ovarian carcinomas can be derived in many different types of tumors that show a variety of structures and original tissues.

The WHO sub-divided ovarian tumors into four main categories according to their tissue of origin:

- epithelial ovarian cancer
- germ cell tumors
- gonadal stromal tumors
- metastatic neoplasm's

Over 85% of the malignant ovarian tumors are categorized as epithelial ovarian cancers (EOC). This sub-group can furthermore also be subdivided into four major groups based on tumor cell morphology:

- serous
- endometrioid
- clear cell
- mucinous (metastases from other malignant carcinomas)

The groups themselves are sub-divided into three further categories to reflect their behavior: being benign, malignant and intermediate (borderline tumor). [1-7] [8]

Another possibility of classification of the ovarian tumors is based on degree of differentiation, also known as staging. Staging is done postoperative based on clinical and pathologic-anatomic findings. The most commonly used grading system is the system of the International Federation of Gynecology and Obstetrics (FIGO). This system is based on the architectural criteria and contains four grades.

Another staging system has been established by the WHO. It contains both architectural and cytological features. The disadvantage of this system is, that it is not based on quantitative criteria and so being rather subjective.

A third staging strategy used, comes from the Gynecology Oncology Group (GOG). In this staging, the most important criterion is the histological type of the tumor. [5, 9, 10]

1.1.3 Diagnosis

Characteristic early symptoms of ovarian carcinomas are very rare and do not really exist. Over 70% of the tumors are detected in progressed stages of III or IV. One reason for this is that the tumors of an early stage can move in the pelvis and neighbored organs like bladder and intestines can be evasive easily.

Most of the tumors (95%) have a diameter about five and ten centimeter at time of diagnosis. Most of the diagnosis of ovarian cancer happens by chance. For other types of cancer, tumor which such size are detected rarely, as normally they would be detected before, based on i.e. clearer early symptoms. When the tumor reaches a certain size in the pelvis, the patients realize some symptoms, like:

- an increase of the girth with feeling of fullness and foreign body sensation (ascites, tumorvolume)
- gaining (ascites) or losing (tumorcachexia) weight
- acute abdomen (rupture, torsion)
- pelvic pain and gastrointestinal symptoms
- irregular female cycle and postmenopausal bleedings (hormonactive tumors)

Benign and malign tumors cannot be differentiated easily in the clinical setting. Every single tumor is seen as malignant at first if functional cysts can be excluded. The nature and its histological characteristics can be defined only postoperative. This happens either by laparoscopy or laparotomy.

The medical history of the patient always plays an important role in the diagnosis of ovarian cancer. There should be detailed questions about the appearance of malignant tumors in the family in the past or other decisive events like exposition of radiation.[8] [11]

1.1.4 Therapy

Therapy of ovarian cancer is staging-dependent. In stage I and II they should be focused on curative therapy, meanwhile in stage III it is important to achieve an initial remission rate of 70% and nothing but palliative therapy is applied in stage IV. Following therapies are used: surgical therapy, chemotherapy and radiotherapy.

1.1.4.1 Surgical therapy

Surgery is the primary therapy of epithelial ovarian carcinoma. The entire disassembly of the tumor tissue is first priority. If the complete removing of the tumor is not possible, the remaining tumor tissue should not be bigger than one centimeter diameter. The prognosis is dependent of the remaining tumor tissue and this is therefore the most important prognosis parameter. The staging is performed intraoperative as well.[8] [11]

1.1.4.2 Chemotherapy

For FIGO staging Ic, II and above after a radical removing of the tumor a systematic chemotherapy is necessary, because capable proliferative tumor cells can still be there. The recurrence rate should be reduced by adjuvant chemotherapy with achieving recovery. This period accounts approximately for six months.

Most of the epithelial ovarian carcinomas have a good response for cytostatics. About 25 years ago, the first effective monotherapy, cyclophosphamid, an alkylating agent, was invented. This was the standard therapy until the end of the 70s. The results were improved by inserting cisplatin that can be also used in polychemotherapies (like CAP: cyclophosphamid, adraimycin, cisplatin). A further step of chemotherapy was the developing of more effective supportive drugs and carboplatin, which has fewer side effects than other platinum-based substances. It could be used as an effective combination with cyclophosphamid.

In FIGO staging III and IV the chemotherapy plays crucial role because a whole removing of the tumor is achieved rarely and as it mentioned above the remained tumor tissue is an important prognosis parameter. The lower the remaining tumor mass, the higher is the probability of a complete response. In case the tumors are bigger than one centimeter, a complete response is expected only for about 25% after primary chemotherapy. Platinum-based combinational therapies are used in general and their effectiveness was approved by several studies.

In 1996 a new therapy, using paclitaxel was established in combination with cisplatin. This combination showed a better response and a longer recurrence free and overall survival than the standard combination of cisplatin and cyclophosphamid. The combination of paclitaxel and carboplatin didn't show any improvement in the outcomes. The advantage of this combination is given by the fewer side effects of carboplatin.

Another possibility to enhance the effectiveness of first-line treatment is a third component besides paclitaxel and platinum-based. Those are the anthracyclines doxorubicin and epirubicin.
Based on current studies the first-line therapy in advanced ovarian carcinomas is the combination of carboplatin and paclitaxel.
In the early stages I and II a surgical removing of the tumor and following platium-based chemotherapy is suggested.
Despite all the progress in first-line treatment of ovarian carcinoma in the last years most of the patients sustain a recurrence. Therefore the second-line therapy is also an important part in treating ovarian cancer. But in most of the cases, the second-line therapy has only palliative effect. This is the reason why fewer side effects and gain of higher quality of life are so important factors.
Ascites and pleural effusion are more frequent in half of the FIGO staged III tumors, here chemotherapy can have a beneficial impact. [8] [11]

1.1.4.3 Radiotherapy

Chemotherapy has replaced the radiotherapy as far as possible. Still, it is used for local restricted tumor nests or recurrences. One of the major problems of radiotherapy is the dosing. It is hard to find a compromise between effectiveness and the protection of surrounded organs, i.e. kidneys and liver. The possibility of intraoperative radiotherapy should be further examined as it is used successfully by colon and pelvic carcinomas.[8] [11]

1.1.5 Follow-up

After first-line treatment over 66% of the patients suffer a recurrence that leads to death. Thus, the prognosis of ovarian cancer is definitely worse than by other gynecological carcinomas. So, one focus of the follow-up is making the right decision about the point of time and the type of therapy for possible incurable patients.
Additional important point of follow-up examination is given by the monitoring of the defecation and miction and functions as well as the girth, which should be performed every three month after finishing first-line therapy. Gynecological examination with transabdominal and vaginal ultra-sound and determination of certain tumor marker are also important procedures of the follow-up. [8] [11]

1.2 Ovarian cancerogenesis and inflammation

In 1863, Rudolf Virchow hypothesized the link between cancer and inflammation based on the observations that (i) some tumors arise at sites of chronic inflammation and (ii) leukocytes were found within neoplastic tissues. After decades Rudolf Virchow's concept being forgotten his visions now became accepted and can be seen as the seventh hallmark of cancer. [12-16].

Epidemiological studies showed that an inflammatory microenvironment is an essential component of all tumors and chronic inflammation predisposes individuals to various types of cancer.[15]

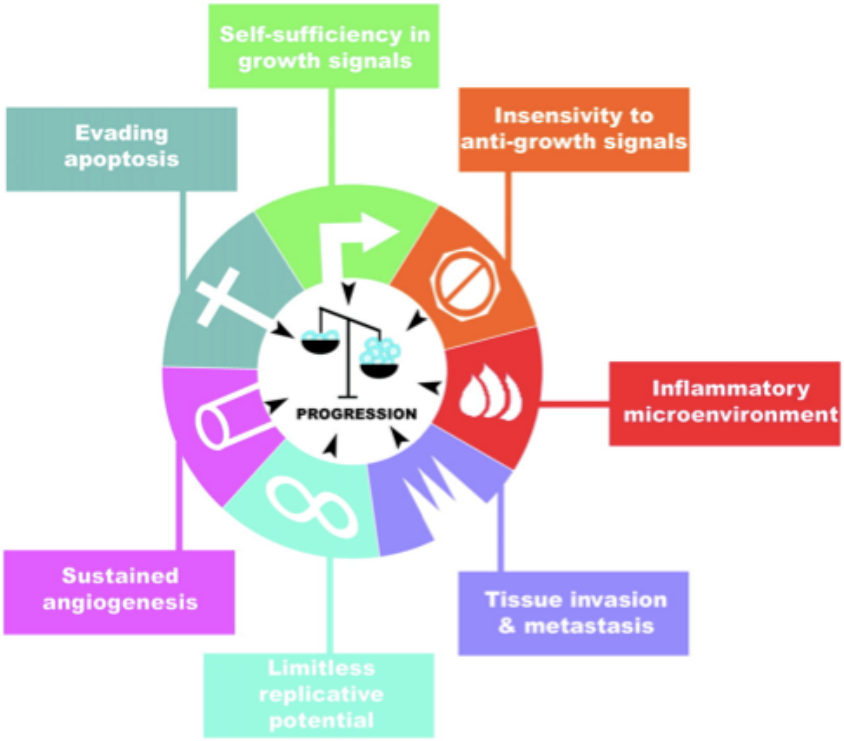

Figure 2: The seven hallmarks of cancer described by Hanahan and Weinberg et all. [12]

To get better understanding on the link between inflammation and canceriogenesis, the terms "chronic inflammation" and "canceriogenesis, including their origins and pathogenesis, will be explained in the following chapters.

1.2.1 Inflammation

Inflammation can generally be considered as a defense action of an organism against damaging *noxa (Latin; harm, damage)*. *Noxa* is described as factors, which can induce tissue injury. Complex inflammatory reactions affect the vascular system, blood cells and parts of the blood plasma. The aim of the inflammatory reaction is the

deletion of the *noxa* and its consequences, as well the recovery of the injured tissue. Therefore, inflammation has additionally an important protective function. In case the tissue injury is very strong, so that a complete healing cannot be achieved, it need to replaced by connective tissue with scar formation. [13, 17, 18] [S. 117-118] [18] [S. 48]

1.2.1.1 Process Of Inflammation

Noxa is a trigger for an inflammatory reaction. There are several examples for a *noxa*-like:

- microbial agents: virus, bacteria, fungus, protozoon
- chemical substances: acids, bases, endogenic substances
- physical reactions: heat, cold, radiation, trauma
- immunreactions

Noxa triggers a tissue injury, whereby which sets inflammation mediators are set free. This comprises a multifactoral network of chemicals triggering cellular reactions that drive the process of inflammation and are involved in healing the affected tissue. Thereby, the activation of leukocytes plays an important role.

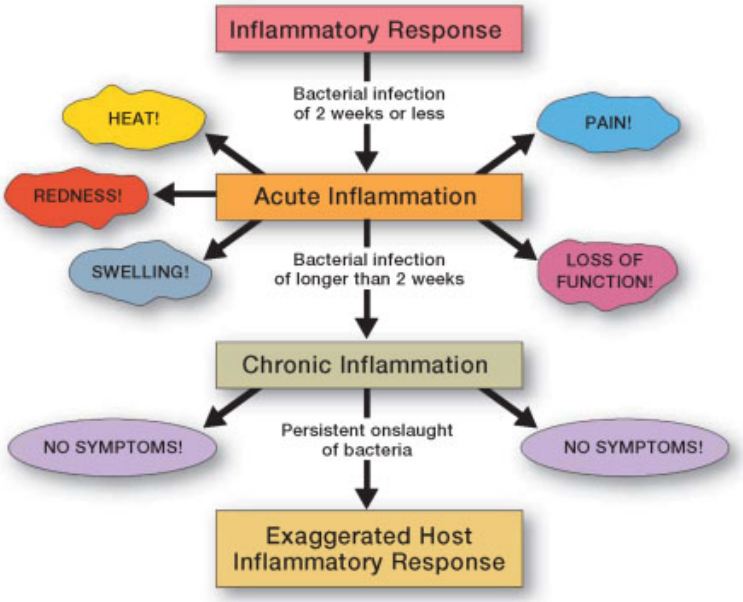

Figure 3: Process of acute and chronic inflammation:
acute inflammation is of short duration, whereas chronic inflammation is a long-lived inflammatory response [67]

1.2.1.2 Types Of Inflammation

There are two major types of inflammation: the acute and the chronic inflammation. The characteristics of the acute inflammation are sudden appearance and a fast and massive progression over a few hours or days.

The well-known four signs of the acute inflammation are:

- rubor: redness through vasodilatation
- tumor: tissue swelling through inflammatory exsudat
- calor: heat through a increased tissue perfusion
- dolor: pain through irritation of the nerve

In contrast, chronic inflammation has a long developmental phase over weeks, months or even years. There is often a subtle beginning with gradually developing symptoms, also called primary chronic inflammation. Another way of development is and arise from an acute inflammation (= secondary chronic inflammation). [17]

1.2.1.3. Chronic Inflammation

The chronic inflammation is characterized by the persistence of the inflammatory stimulus and can last for weeks, months or years. The result is a granulation tissue that consists of cellular infiltrates like macrophages, lymphocytes, capillaries and fibroblasts. The longer persistence of the chronic inflammation, the more massive is the tissue destruction, tissue remodeling and scar formation.

As mentioned above there are two different types of chronic inflammation: the primary and the secondary chronic inflammation. [17]

The primary chronic inflammation is defined to be chronic from the beginning on, known to be triggered through several damaging noxa, i.g. microorganisms (mycoplasma tuberculosis), viral infection like hepatitis type B or autoimmune disease like colitits ulcerosa or morbus crohn. The most presented cell types are macrophages, lymphocytes and plasma cells.

The secondary chronic inflammation arises from an acute inflammation through persistence of the inflammatory stimulus. It can be classified according to the type of noxa and the acute chronic inflammation form:

- chronic purulent inflammation
- abscess
- foreign body associated bacterial purulent inflammation
- persistence of viruses or chemical/physical noxa [17]

1.2.2 Cancerogenesis

By definition, tumor is an abnomral tissue mass, which occurs through progressive increase of endogenously degenerated cells (e.g. transformed cells, tumor cells). The reasons for this degeneration are disregulation of genes that especially are responsible for controlling the processes of proliferation, apoptosis and cell differentiation. Further characteristics of a tumor cell are invasion and distribution of tumor cells in the organism and creation of metastases.

One major difference between a transformed cell and a normal cell is that tumor cells have the ability to proliferate without an external growth stimulus, also called "autonomic tumor progression".

The induction of an additional blood vessel system especially for the tumor and the tumor stroma, tumorangiogenesis, ensures the supply of the tumor. Consequently, every tumor consists of real tumor cells (tumorparenchym) and supporting tissue (tumor stroma). It is important for supporting the tumor and supplying it with blood. [17] [18]

Tumorigenesis is a multistep process with severe genetic alterations that are responsible for the transformation of normal cells into highly malignant derivatives. This multistep process is composed of 4 steps: the first crucial process is the initiation. An initiated cell arises from a normal cell through several mutations in genes responsible for growth regulating or DNA protecting. Next step is promotion, which contains the clonal growth of the initiated cell. The third and last step is progression associated with the selective growth of the tumor clone. Initiation and progression are irreversible processes; in contrast promotion and selective growth are a reversible step.

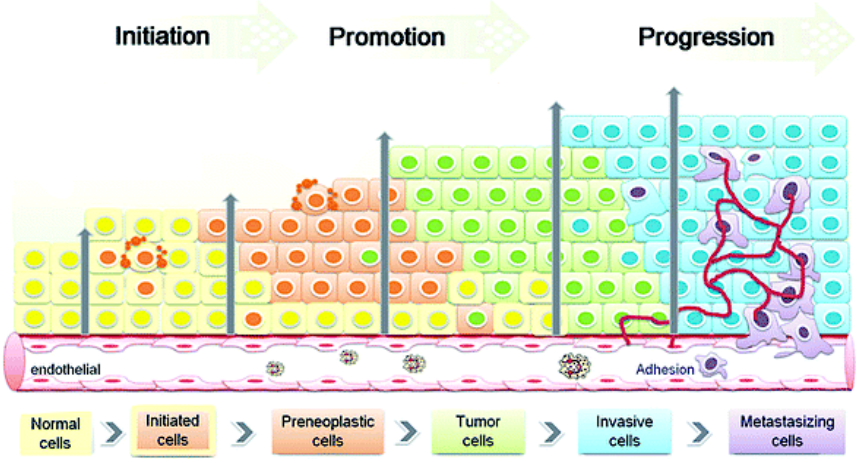

Figure 4: Multistep process of tumorgenesis [68]

Another important role within tumorigenesis plays the oncogenes and tumorsuppressor. Malignant cells can arise only when at least two oncogenes act together in a given cell. Oncogenes are normal cellular genes those expression products regulate the proliferation, mobilization and differentiation of cells. A receptor-ligand-complex is necessary to stimulate cell growth. In malignant tumor cells these become constitutively active which means that the growth signal is activated independent and without any ligand binding on the receptor. Over 200 different types of oncogenes are known to be associated with this phenomenon. Consequently, cancer is a disease of the cellular signaling mechanisms based on genetic damage. In normal cells, complex control mechanisms regulate growth and differentiation to assure an equilibration in the organism. These control circuits are defective in tumor cells and hence, they can proliferate unregulated. [17] [19]

1.2.3 Cancer-related Inflammation

Nowadays, the link between cancer and inflammation is accepted. In 2008 Alberto Mantovani postulated the term "cancer-related inflammation". Of note, it is estimated that 15- 20% of all deaths from cancer worldwide are associated with chronic infection and inflammation.

The following table will give an overview of possible triggers for an increased risk for developing cancer through a chronic inflammation; in general the most common triggers are microbial infections, viral infections, autoimmune disease or inflammatory conditions of unknown origin. [15, 16, 20]

Table 1: Examples for associaton between inflammation and cancer risk [16]

Malignancy	**Inflammatory stimulus/condition**
Bladder	Schistosomiasis
Cervical	Papillomavirus
Ovarian	Pelvic inflammatory disease/talc/tissue remodelling
Gastric	H pylori induced gastritis
Colorectal	Inflammatory bowel disease
Hepatocellular	Hepatitis virus (B and C)
Bronchial	Silicia, asbestos, cigarette smoke

Inflammatory cells and inflammatory mediators are hallmarks of cancer-related inflammation in tumor tissues, tissue remodelling, angiogenesis and tissue repair. The environment of sustained proliferating cells contains inflammatory cells, growths factors, activated stroma and DNA-damage-promoting agents, like peroxynitrite, that can stimulate neoplastic risk and gives the evidence that cell proliferation alone does not cause cancer. These DNA damages result through the generation of reactive oxygen and nitrogen species of leukocytes and other phagocytic cells, which are normal fighters against infection. In general, cell proliferation is increased during the regeneration of injured tissues. After the reparation is completed, proliferation and inflammation are down regulated. In case, cells possess DNA damages or mutations, like point mutations, deletions or rearrangements, the proliferation continues in microenvironment that contains inflammatory cells and growth factors, which promote cell growth. Based on this you can say that tumors are wounds, where wound healing failed. [13, 15, 16]

1.2.3.1 The Transition From Chronic Inflammation To Cancerogenesis

The transition from inflammation to cancer can be described in following chain: it starts with a chronic inflammation caused by two pathways: the extrinsic pathway and the intrinsic pathway. The chronic inflammation induces tumor initiation and promotion followed by the development of cancer.

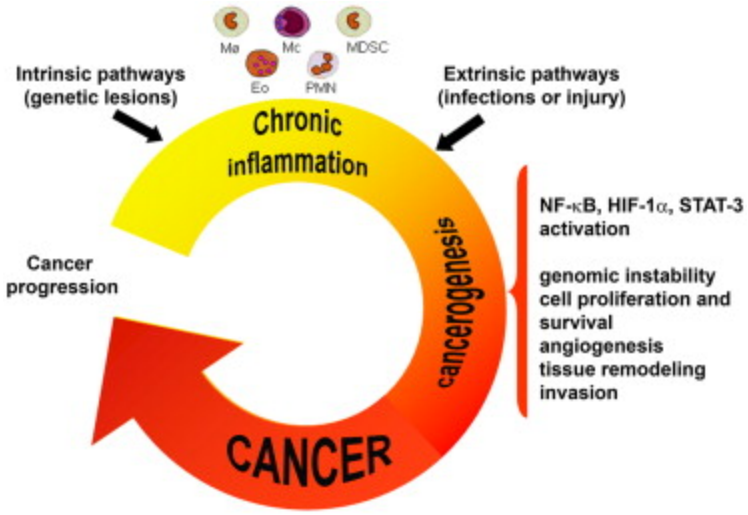

Figure 5: The transition from chronic inflammation to cancer [20]

Two major pathways determine the first step of the transition: the extrinsic pathway and the intrinsic pathway. The extrinsic pathway is characterized by inflammatory or

infectious conditions, which increase cancer risk, like inflammatory bowel disease, especially chronic ulcerative colitis and Crohn's disease. The instrinsic pathway is determined by genetic alterations that cause inflammation and neoplasia. This includes the activation of different types of oncogenes by mutation, chromosomal rearrangement or amplification and inactivation of tumor-suppressor genes. [13, 15]
The transformed cells produce inflammatory mediators and create an inflammatory microenvironment. On the other hand, the extrinsic pathway increases the risk of developing cancer at anatomical sites, like colon, prostate and pancreas, by inflammatory or infectious conditions. The meeting point of these two pathways is the activation of transcription factors in tumor cells, such as the nuclear factor-κB (NF-κB), signal transducer and activator of transcription 3 (STAT3), lipid mediators, nitric oxide (NO) intermediates, and hypoxia-inducible factor 1α (HIF1α). These transcription factors activate again the production of inflammatory mediators, like cytokines, chemokines, and cyclooxygenase 2 (COX2). Inflammatory cells are recruited by these chemokines and cytokines, for example macrophages, eosinophiles, mast cells, neutrophils and myeloid-derived suppressor cells. The inflammatory cells activate again transcription factors in stromal cells and tumor cells, which also produce chemokines, cytokines and COX2 and create a cancer-related inflammatory microenvironment. The resulting cancer-related inflammation has a lot of tumor-promoting effects as described above. [15, 20]

1.2.3.2 Chronic Inflammation And Ovarian Cancer

Several epidemiologic studies have shown that inflammation can be also a fundamental mechanism in the development of ovarian cancer and plays a crucial role in initiation and progression of epithelial ovarian cancer. Besides this, two additional hypotheses describing the potential cause of causing ovarian cancer: the ovulation hypothesis, which links ovarian cancer risk to incessant ovulation and the pituitary gonadotropin hypothesis, which links excessive exposure of OSE to gonadotropin thus triggering increased epithelial cell proliferation and malignant transformation. Epidemiologic and biologic studies have not proved these hypotheses yet. In contrast, more data support the idea that factors causing epithelial inflammation are more crucial in ovarian carcinogenesis. Such factors are for example ovulation, endometriosis and pelvic inflammatory disease. To the contrary, tubal ligation and hysterectomy have apparently protective effects that may decrease

the exposure to local genital tract irritants. Of note, inflammatory mediators produced by activated innate immune cells such as TNF-α, IL-1β and -6 are known to promote EOC genesis, growth and progression. [21-24]
The following figure will give an overview about inflammation as common mechanism underlying ovarian cancer.

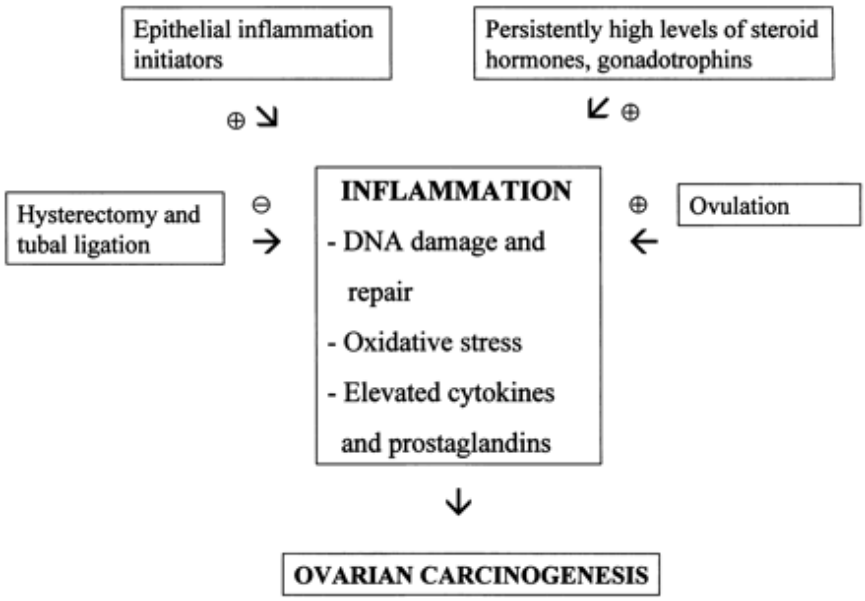

Figure 6: Chronic inflammation and ovarian cancer:
factors such as ovulation and hormones can initiate inflammation which lead to a higher risk of ovarian carcinogenesis [22]

The ovulatory process has a lot in common with the inflammatory response. It is pro-inflammatory in nature and potentially mutagenic. When the pre-ovulatory follicle matures, the proximal ovarian epithelial cells proliferate and then undergo apoptosis to regulate follicular growth. In the meantime, the fibroblastic layers of the tunica albuginea and theca externa are attenuated in preparation for ovulation. The surface of the ovary is wounded by a burst of apical epithelial cells and the underlying follicular layers followed by rapid extrusion of the ovum. These ovulatory processes and the following repair steps are characterized by the present of an interconnected regulatory network of cytokines and chemokines and also matrix-remodeling enzymes, including prostaglandins, bioactive eicosanoids, plasminogen activators, collagenases, interleukines, TNF and various growth factors. Additionally, activated immune cells recruited to the wounded epithelial surface are involved in the activation of the pro-inflammatory network. Thus, the inflammatory stimuli are

furthermore responsible for additional damage to the OSE that is already stressed by the ovulatory rupture of the local epithelial cell layer. In conclusion, the inflammatory responses under physiological conditions can encourage the development of EOC. [23, 25]

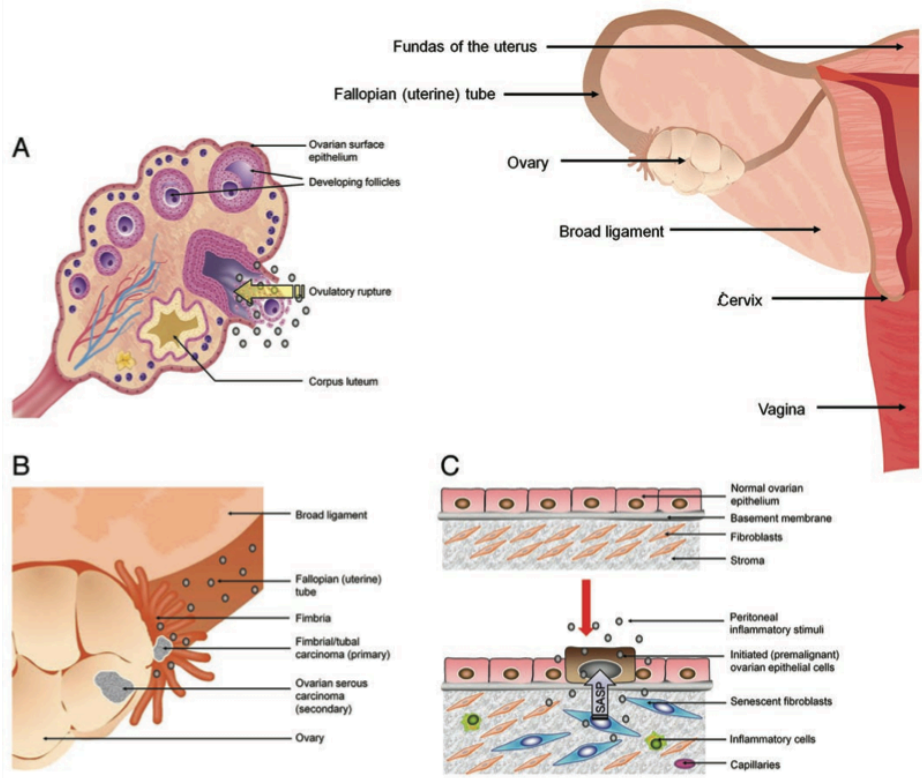

Figure 7: Chronic inflammation and ovarian cancer:
processes contributing to the initiation and/or progression of EOC [23]

1.3 The role of macrophages in ovarian cancer

Chapter 1.2 point out the impact of inflammation in ovarian cancerogenesis. As it is known, in tumor tissues, the innate immune cells are highly represented, especially macrophages. Their functions, classification, tasks, origin and activation will be discussed in detail in the following chapter.

1.3.1 Origin and classification

The innate immune system is responsible for the first line of defense against pathogens. Thereby, the pathogens are recognized unspecifically, whereby to immunological memory as protection against a re-infection is build up. Important effectors of the innate immune system are phagocytes that can be sub-classified into two categories: the polymorphonuclear phagocytes (granulocytes) and the mononuclear phagocytes. The mononuclear phagocytes consist of tissue macrophages, circulating monocytes, the promonocytes and their precursor cells in the bone marrow. [26] [27, 28]

The most immature cell of the mononuclear phagocyte system is the promonocyte. It arises in the bone marrow, where it develops from pluripotent stem cells; it is a multiplicative cell that by cell division gives rise to an undefined number of sub-populations of monocytes. These pass through different multipotent progenitor stages. Finally, monocytes are released into the circulation and enter all tissue compartments of the body. When they find sites, where conditions are advantageously for phagocytosis, these cells differentiate into macrophages and exhibit their main function - under go phagocytosis. The growth and differentiation of macrophages is modulated by lineage-determining cytokines, such as macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF). IL-3, TNF and TNF-receptor-related molecules are in involved in the macrophage determination.

Resident macrophages respond to CSF-1 and have different names and functions according to their tissue residency, e.g. Kupffer cells in the liver, microglial cell in the brain or osteoclast in the bone. They undergo local activation in response to various inflammatory and immune stimuli. The accumulation of tissue macrophages in the tissue results from the enhanced recruitment of monocytes and their precursors from bone-marrow pools. These macrophages can be classified in two different subtypes: classically activated macrophages and alternatively activated macrophages.

The differentiation to inflammatory macrophages, M1 macrophages, is caused under the influence of GM-CSF, interferon-γ (INF-γ) and TNF. They can be found at sites of infections and injury. In contrast, alternatively activated macrophages arise in response to parasitic infections, allergic conditions and during tissue repair through the effects of IL-13 and IL-4. There are also known as M2 macrophages. Of note immature macrophages could also differentiate into dendritic cells. [27-29]

The network of the macrophage differentiation and the control of its development by growth factors are showed below by the two figures.

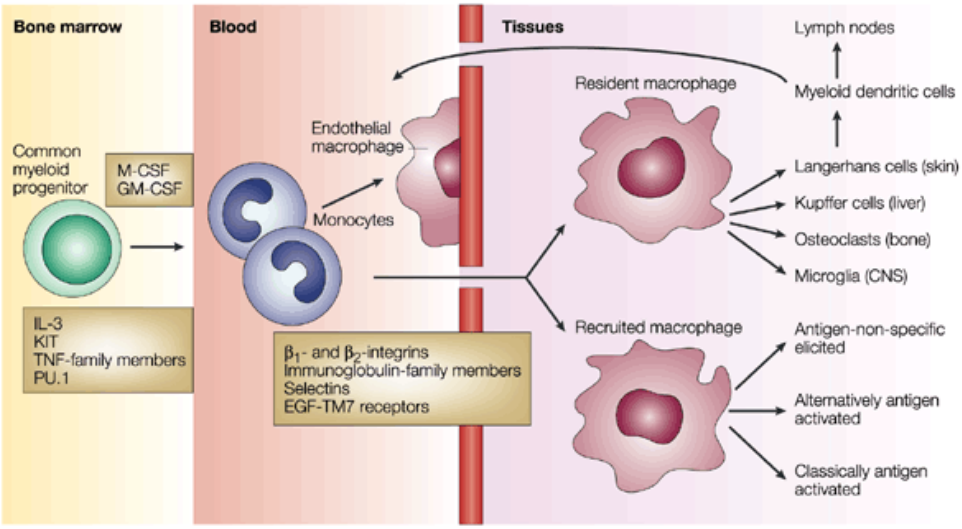

Figure 8: Macrophage differentiation and the control of its development by growth factors [29]

1.3.2 The functions and tasks of macrophages

Macrophages are involved in tissue remodeling, inflammation, immunity and thrombosis. Furthermore, impact of macrophages onto tumor growth and progression was reported; this issue will be discussed in detail in the next chapter.

In innate immunity resident macrophages are responsible for immediate defense against foreign pathogens and coordinate leukocyte infiltration. Macrophages regulate the balance between antigen availability and clearance through phagocytosis of senescent or apoptotic cells, microbes and likely also neoplastic cells. Triggering, instructing and terminating the adaptive immune response are one of the major tasks of macrophages. Thereby, they cooperate with T and B cells as well through cell-to-cell interactions as well as fluid phase-mediated mechanism. This is based on the releasing of cytokines, chemokines, enzymes, arachidonic acid metabolites, and reactive radicals. Activation of macrophages can be pro or anti-

inflammatory thus being involved in tissue cell destruction or tissue regeneration and wound healing. [27, 30, 31]

As above mentioned, there are two different types of macrophages existing with different functions: M1 and M2 macrophages. This overview shows the differences in their tasks.

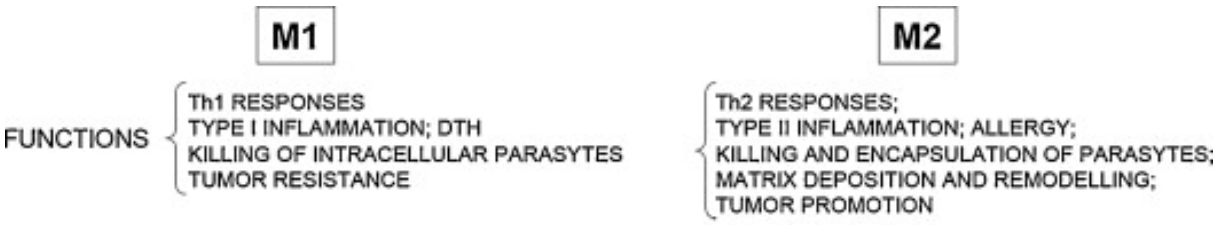

Figure 9: Comparison of M1 and M2 functions [30]

The polarization of the macrophages is based on the production of either IL-12 or IL-10: M1 are producing IL-12 and M2 known to produce IL-10. These interleukins IL-12 and IL-10 support the activation of T helper cells 1 (Th1) and T helper cells 2 (Th2), respectively which in turn play opposite roles in pathological conditions, especially in infection and cancer. The differentiation in to one or the other direction depends on the microenvironment the macrophages are surrounded by. [30]

M1 macrophages, induced by IFN-γ, TNF or microbial products, have the ability to kill microorganisms and, thus, also tumor cells and produce a variety of pro-inflammatory cytokines. In contrast, M2 macrophages differentiated in response to several interleukins (like IL-4, IL-13 and IL-10), glucocorticoids and immunoglobulin complexes, trigger Th2 immunity, scavenge debris, promote angiogenesis and tissue remodeling and repair. [30, 32]

The ability to direct the recruitment, maturation and differentiation of infiltrating monocytes depends on the molecules and microenvironmental conditions, like cytokines and hypoxia which in turn trigger the activation of specific transcriptional programs conducting the cells in M1 or M2 direction. [30, 33]

The process of macrophage polarization and their crucial role in tumor promotion are further described in the next following chapter, paying special attention to the tumor-associated macrophages (TAMs) in ovarian cancer.

1.4. The Role of tumor-associated macrophages in tumor progression

Virchow for the first time postulated in 1863 the presence of leukocytes in human tumors. The majority of the malignant tumors imply plenty of macrophages as the major constituent of the host leukocytic infiltrate. These macrophages are called "tumor-associated macrophages" (TAM) and were shorn to have important impact on tumor progression. [30, 34]

This chapter it will discuss the origin, polarization and function of TAMs giving special attention to their role in tumor progression.

1.4.1 The origin of tumor-associated macrophages

Tumor-associated macrophages represent a mature population of terminally differentiated myeloid-derived cells, that mostly originate from peripheral blood monocytes, recruited into the tumor mass from the circulation, and that proliferate locally at poorly vascularized regions of the tumors. Generally, macrophages belong to the monocyte-macrophage lineage and become activated by cytokines and microbial products. [29, 30, 32, 33, 35-37] TAMs are known to be associated with immune suppression; still their phenotype is distinct from the heterogeneous population of immature myeloid cells exhibiting similar suppressive functions. [27, 38, 39]

Especially chemotactic cytokines, as mentioned above "chemokines", are important for the recruitment of monocytes into tumors, e.g. monocyte chemotactic protein-1 (MCP-1), macrophage colony stimulating factor (M-CSF or CSF-1) and vascular growth factor (VEGF) that are produced by tumor cells.

MCP-1 is a specific chemotactic and activating signal for monocytes and is expressed by tumor cells, endothelial cells, fibroblasts and macrophages in tumors. MCP-1 is a crucial factor of macrophage infiltration into tumors and is strongly expressed in many tumor types including ovarian carcinoma. There is a close association between up-regulation of MCP-1 by tumor cells and TAM accumulation. TAM accumulation is also linked to higher levels of VEGF, TNF-α and IL-8 and thus, MCP-1 and especially VEGF play a major role in the regulation of angiogenesis. Consequently, MCP-1 might be seen as a pro-tumorigenic protein. [34, 40-43]

M-CSF is a monocyte-specific chemoattractant that stimulates the differentiation of monocytes into macrophages and the proliferation of mononuclear phagocytes. It is produced by monocytes and macrophages as well as by several tumor types

including ovarian carcinoma. High levels of expression of M-CSF in ovarian tumors were found to be associated with a poor prognosis and might be used as markers for disease progression.[34, 43-46]

Another chemotactic factor for monocytes is VEGF acting via VEGF receptor. It is a potent pro-angiogenic cytokine produced by macrophages, tumor cells, and T-cells in many types of tumors. The literature reports a correlation between VEGF expression and macrophage content in ovarian tumors. [34, 47-49]

1.4.2 The mechanisms of TAM polarization

Macrophages are known as a heterogeneous population. The plasticity of macrophages is based on continuous exposure to microenvironmental signals. As discussed in the previous chapter macrophages can be classified as "classical", M1 macrophages, or "alternative", M2 macrophages. M1 phenotype is associated with active microbial killing, however M2 phenotype is associated with tissue remodeling and angiogenesis. [31, 33, 38, 50]

Generally, the activation of macrophages is induced by IFN-γ, alone or with microbial products, like LPS, or cytokines, e.g. TNF-α. M1 macrophages are characterized by strong capacity to present antigen, high IL-12 and Il-23 levels and production of toxic intermediates, e.g. NO, or reactive oxygen intermediates (ROI). When monocytes in the tumor tissue are exposed to tumor derived anti-inflammatory molecules like IL-4, IL-10, IL-13, glucocorticoid hormones, or vitamin D3, they trigger a different activation program, driving them in the direction of alternatively activated macrophages or M2 macrophages. [31, 33, 36, 51]

M1 and M2 macrophages can be discriminated by following criteria: they have distinct receptor expression patterns, effector functions and cytokine repertoire. M1 macrophages produce IL-12 and TNF, in contrast M2 macrophages secrete IL-10, IL-1 receptor antagonist (IL-1RA) and type II IL-1 receptor. Hence, M1 macrophages are characterized as potent immune effector cells, which are able to kill microorganism and tumor cells, present antigen, and secrete pro-inflammatory cytokines, thus being anti-tumoral. In contrast, M2 macrophages have poor antigen presenting ability, produce factors suppressing T-cell proliferation and activity, and promote angiogenesis, tissue remodeling and repair. All these properties contribute to the fact that M2 macrophages are pro-tumoral. [31, 33, 52]

It is assumed that those macrophages, which infiltrate tumor tissues, known as TAMs, adopt the properties of a polarized M2 population. TAMs have the same molecular and functional profile as the M2 phenotype and exhibit pro-tumoral functions. They show high constitutive expression of IL-1 and IL-6, and a low secretion of TNF. [31, 34]

The following figure shows functional characteristics of TAMs. They are simultaneously the origin and target for cytokines and chemokines in the tumor microenvironment that regulate tumor growth, progression and invasion. Furthermore, interaction with other components of the stromal compartment is taken place as well as the activation and orchestration of adaptive immunity. [33]

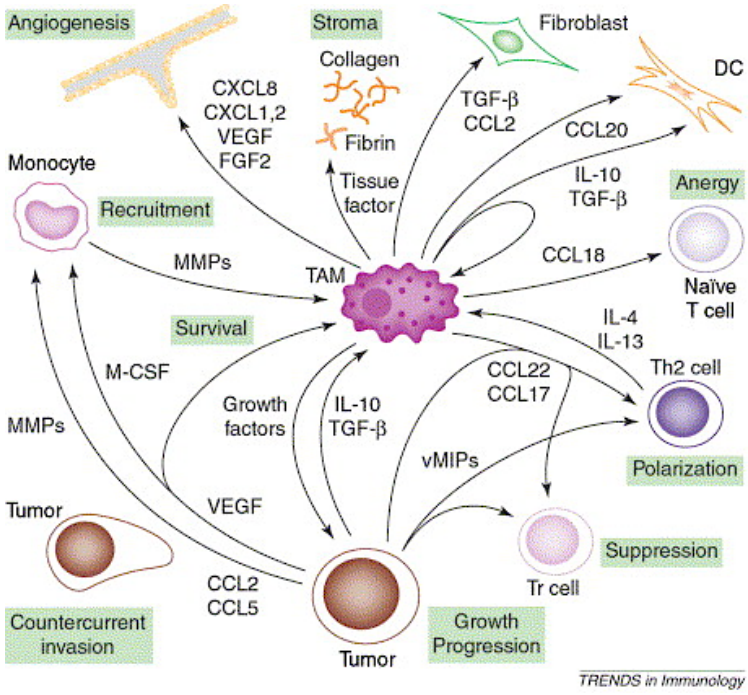

Figure 10: Functional characteristics of TAMs:

They regulate tumor growth, progression and invasion by cytokines and chemokines [33]

Based on these properties, TAMs, beside other factors, are major promoters of tumor progression and metastasis. This knowledge can be used as novel and valuable therapeutic targets in the future. [16, 33, 43]

1.4.3 TAMs and their role and functions in tumor progression

As described above, tumor-associated macrophages have the capacity to exert pro and anti-tumor activity, as phenomeny, which was described by Alberto Mantovani in 1992 as "the macrophage balance". [43] Their pleiotropic functions can influence tumor growth and progression in opposite directions. [15, 27, 33, 53]

This chapter will focus on the pro- und anti-tumoral properties of tumor infiltrating macrophages.

1.4.3.1 The anti-tumor functions of TAMs

Macrophages are not tumoricidal itself. They need to be in an activated state to be anti-tumoral. Activation happens, for example, by "classic" macrophage stimulants as IFN-γ or LPS. When the macrophages are activated, their exhibit cytotoxic functions either directly towards tumor cells or indirectly through secretion of factors that stimulate the anti-tumoral properties of other cell types. [34] Macrophage-mediated tumor cytotoxicity (MTC) and antibody-dependent cellular cytotoxicity (ADCC) are the two major routs of direct cytotoxicity. MTC is associated with the secretion of lytic factors into neoplastic cells thus causing their lysis. This is a slow-going process and is mediated by the secretion of toxic factors as TNF-α, serine proteases, and reactive nitrogen intermediates. Reactive oxygen and nitrogen species are liberated by macrophages, which are activated by T cells or natural killer (NK) cells producing INF-γ. The mechanism of ADCC is similar, important difference is that it is dependent on the presence of antibodies on tumor cells. [34]

The cytotoxicity against tumor cells is orchestrated by several cytokines.

The macrophage migration inhibitory factor (MIF) is one of the important cytokines, which is involved in the anti-tumoral activity of TAMs. Both, macrophages and tumor cells are able to synthesize it. Following properties of MIF are noteworthy as well: it fosters critical macrophages functions, such as phagocytosis, tumor cell killing, and release of TNF-α and IL-1β. [34]

MIF itself is supported by another cytokine: the granulocyte/macrophage-colony stimulating factor (GM-CSF). As mentioned above, this cytokine is important for differentiation, survival, proliferation, migration, and metabolism of macrophages; when released by tumor cells it causes increased cytotoxic activity of TAMs in the tumor. [34]

IL-12 and IL-18 are also participating in the regulatory network. This cytokine belong to the T helper type 1 cell (Th1)-inducing family of cytokines. One of their major functions is the promotion of cytokine production, in particular IFN-γ and IL-2. They are also involved in proliferation and cytotoxic activity of NK and T cells, and drive progenitor cells toward type 1 CD4-positive T helper cells (Th-1). [34]

1.4.3.2 The pro-tumor functions of TAMs

As the functions of TAMs can be seen as a double-edged sword, the pro-tumoral functions of TAMs will be discussed in detail in this chapter.

In general, TAMs have a phenotype and functions of M2 or alternative macrophages. Thus, the pro-tumoral functions of TAMs are associated with tumor growth and progression, invasion and metastasis, angiogenesis, and matrix remodeling. [15, 31, 53]

The figure gives an overview on the role of TAMs in cancer progression.

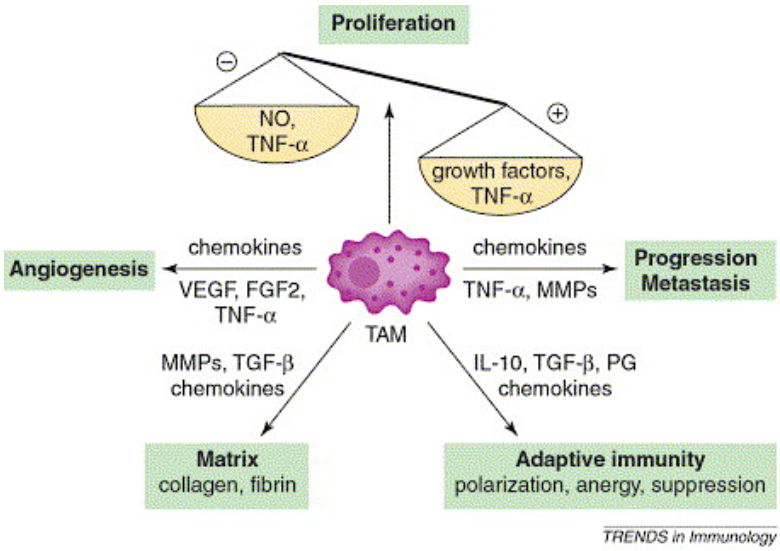

Figure 11: TAMs in cancer progression:

Interaction between various cytokines to tumor progression and metastasis [33]

One of the key features associated with tumor-growth is angiogenesis. Without it, tumors cannot be supplied with essential nutrients and oxygen, or the outflow of waste products is not guaranteed. Creating new blood vessel is in addition needed for metastasizing tumor cells to get out of the tumor and be scattered via bloodstream. In general, angiogenesis is a multiple step mechanism regulated by local signals within the tumor. These local signals are involved in the degradation of

the extracellular matrix around the vessels, the proliferation and migration of capillary endothelial cells, and their determination into functioning capillaries. [34]

The tumor microenvironment drives the recruitment of macrophages followed by conversion to the M2 phenotype. When TAMs get activated, they have the ability to express substances, which promote angiogenesis, such as VEGF, TNFα, and IL-8. Additionally, TAMs liberate also a variety of angiogenesis-modulating enzymes, such as different types of MMP, and COX2. [30, 31, 33, 34, 37]

The proliferation and migration of endothelial cells, matrix remodeling, and formation of stabilized vessel are supported by macrophages by their monocytic precursors. This has the ability to migrate into the sites where they differentiate into macrophages and produce the essential angiogenic molecules on demand in specific locations. [54-56]

TAMs are frequently found in hypoxic regions of tumors and hypoxia is a well-known trigger for angiogenesis. Hypoxia and the cytokines, which are produced in response to this condition, are local attractants for macrophages. Under hypoxic conditions, the transcription factor hypoxia inducible factor-2α (HIF-2α) becomes upregulated and induces VEGF expression. VEGF is one of the key signals in the angiogenic process and both tumor cells and TAMs express it by upregulation of CSF-1. In turn, VEGF is a chemoattractant for TAMs themselves and therefore, the vascularization in tumors is accelerated. [34, 54, 57, 58]

Besides VEGF, TAMs also produce TNFα, which plays a furthermore an important role in angiogenesis. TNFα is responsible for inducing MMP-9, which regulates the destruction of the extracellular matrix and in turn liberates bioactive VEGF from its extracellular-matrix-bound latent form. Matrix metalloproteinases (MMPs) belong to the family of matrix-degrading enzymes and are expressed by tumor cells and stromal fibroblasts. They also participate in angiogenesis by disrupting the tissue architecture to support tumor growth, and breaking down of the basal membranes facilitating metastatic spread. In ovarian cancer it is assumed, that TAMs are a major source of MMP-9 especially and thus, support the metastatic potential of tumor cells acting thereby together with the epithelial and stromal cells of the tumor. [16, 34, 54, 59]

Additional protein produced by TAMs, which promotes angiogenesis, is urokinase-type plasminogen activator (uPA). It is a serine protease activated by CSF-1 receptor signaling TGF-β1 in macrophages and is beside others involved in the degradation of

the extracellular matrix in tumors. The presence of uPA in tumor tissue is correlated with high microvessel density and hence, it can be concluded, that the uPA system is important for the creation of a vascular network in tumors and TAMs are involved in its expression and regulation. [34, 54, 60]
As mentioned above, TAMs are able to produce NO through the induction of iNOS. Is the expression of iNOS increased, an enhanced vascular flow and vasodilatation can been observed. [54]
Endothelin-2 is another vasoactive molecule, which is integrated in inflammation and angiogenesis and is expressed in tumor cells. It is assumed that Endothelin-2 is also chemoattractive to macrophages and recruits them to hypoxic areas. [54]
In summary, TAMs upon entering the tumor can participate in the induction vascularization by synthesizing angiogenic regulators that are responsible for creation of new vessels supporting tumor growth and metastasis.

Through the ability to promote angiogenesis, TAMs are able to affect tumor growth and the survival as well as metastasis of tumor cells. Tumor cells release cytokines and chemokines, thus attracting TAMs into stromal areas surrounding the tumor. The expression of these cytokines such as IL-10, TGF-β and prostaglandine E_2, and chemokines are important in the orientation and differentiation of recruited macrophages and account for the reduction of the anti-tumoral functions of the local immune system. [13, 30, 33, 37, 54, 61]
A typical characteristic of TAMs is the poor antigen presentation ability and consequently, the suppression of T cell activation and proliferation through prostaglandins, IL-10 and TGF-β. This cytokine profile direct the differentiation away from the Th1 direction toward the induction of regulatory T cells (Tregs). Tregs are characterized by an anergic phenotype and suppression of the activity of effector T cells and other inflammatory cells, like monocytes. The anti-tumor activity mediated by Tregs leads to an increased tumor growth especially in ovarian carcinoma tissues. [30, 33, 43]
Generally, macrophages have guarding functions, as there are involved in the organization of immune defenses and coordinate the tissue repair process including epithelial migration, matrix remodeling, and angiogenesis. Thus, tumor cells recruit macrophages and develop a microenvironment, where macrophages decrease immune functions. [13, 30, 33, 37, 54, 61]

In early stages of tumorgenesis, macrophages are organized in dense clusters as part of leukocytic infiltrates. Approximately 50% of the leukocytes in the tumor microenvironment consist of TAMs. They produce proteases that cause the breakdown of the basement membrane and therefore create an access for tumor cells entering the stroma and induce tumor metastasis. The following figure illustrates the infiltrating leukocytes breaking down the basement membrane, escaping into the surrounding stroma and moving out of the tumor mass towards blood vessels. [37, 54]

Tumor cells have the ability to degrade the basement membrane and penetrate the surrounded stroma of healthy tissue. Together with macrophages, they stimulate angiogenesis. The resulting vessels infuse the early tumor and supply it with nutrients and oxygen for tumor growth and create an exit point for metastasizing cells. TAMs produce a variety of chemotactic signals for tumor cells, which promote migration and intravasation into the blood and lymphatic vasculature. These include growth factors that can stimulate the growth and promote the proliferation of tumor cells, like epidermal-growth-factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and transforming growth factor (TGF). Main issue is the paracrine interaction loop involving macrophage-expressed EGF and epithelial cell-expressed CSF (M-CSF). The interaction between them guides the migration of tumor cells and macrophages toward each other. Furthermore, M-CSF promotes macrophage proliferation, survival and tissue recruitment during development, homeostasis, and pathological tissue remodeling such as acute tissue injury.[37, 38, 54, 61]

TAMs are also involved in the process of restructuring of the extracellular matrix (ECM) through the digestion of ECM components. This happens through MMP, especially MMP-7 and -9, plasmin, serine proteases, cathepsin, and urokinase-type plasminogen activator (uPA) derived from TAMs in the tumor microenvironment. The migration on and through ECM is necessary for the cell migration and therefore, TAMs regulate the tumor cell migration/invasion through the ECM remodeling by mechanism including MMP-dependent digestion of the ECM, collagen deposition, and conversion of collagen structure. [30, 38, 54, 61]

In summary, the combined TAM-driven processes are responsible for a more angiogenic, malignant phenotype of the tumor and the microenvironment at sites of initial tumor cell invasion, perivascular sites, stromal regions, and hypoxic/necrotic

areas. As an outlook, TAMs can be seen as a possible target for cytotoxic therapies in these specific areas, because of their presence in in the most aggressive areas of the tumor, as hypoxic or stromal areas where angiogenesis and metastasis are taken place. Another aspect of targeting TAMs by cytotoxic is their phagocytic capacity. This can be used to engulf liposomes carrying cytotoxic drugs and transport them into the tumor, where these drugs would be liberated after cell death. The attraction of TAMs in hypoxic areas can also be used for developing new therapeutic strategies: using TAMs as vectors to bring genes or drugs activated by hypoxia into the specific sites. A delivery system like this has the advantage of reaching avascular tumor areas, which are mostly not affected by most forms of therapy. [37, 54]

2. AIM OF THE STUDY

We aim to perform systematic analysis of macrophage subpopulations within ovarian tissues using large-scale sample analysis of paraffin-embedded sections. The diploma work is a part of an ongoing project in the group of Diana Mechtcheriakova (Molecular Systems Biology and Pahtohysiology), which is entitled "Microscopy-based algorithm for qualitative and quantitative analysis of immune cell sub-population within complex ovarian cancer tissues". The microscopy-based tissue analysis system, TissueFAXS, will be used for automatic scanning of large scale tissue sections followed by and HistoQuest software-based analysis of ovarian carcinoma specimens. Within the study, particular attention will be given to the macrophage/monocyte lineage, stained with the anti-CD68 antibody. Of interest will be the patient specific organization and accumulation pattern as well as the extent of CD68-positive leukocytes found within tumor and stroma tissue. Additionally, HistoQuest-based algorithm will be established to allow quantitative analysis of the stained cell population. To discriminate between the M1 and M2 phenotype, randomly selected group of specimens will be stained for CD163, the marker of the M2 lineage. Overall, qualitative and quantitative analysis will allow to gain insights into the immune landscape and the immune contexture of ovarian carcinoma. As outlook, staining-derived data sets might be used for alignment with clinicopathological parameters and prognostic modeling.

3. METHODS

The examination of 68 ovarian carcinoma specimens regarding the presence of the monocyte/macrophage lineage was based on the established microscopy-based algorithm for qualitative and quantitative analysis of immune cell population within ovarian carcinoma tissue. The study was approved by the Ethics Committee of the Medical University of Vienna (EK-Nr. 1101/2013).

Upon immunohistochemical based staining, large scale-tissue sections were scanned using the microscopy-based TissueFAXS system. Next, using the HistoQuest software regions of interest, also called ROIs, were defined accounting tumor-specific anatomy. In our study we predominantly concentrated on the following ROIs: tumor and tumor stroma. Furthermore attention was given to the following morphological features of the tissue - fibrotic tissue, normal ovarian stroma and adipose tissue. The work is part of a larger project assessing diverse immune cell population in ovarian carcinoma tissue (funded by FWF P22441 to Diana Mechtcheriakova, Priv.Doz. Dr., Department of Pathophysiology and Allergy Research, Medical University of Vienna). The current part of the study focused on the monocyte/macrophage lineage and its patient-specific extent and distribution patterns. Within the analysis, the next critical step was the establishment of profiles specifically designed for the macrophage markers such as CD68 to achieve that positively stained cells are properly recognized and quantified by the HistoQuest software. Finally, the results of the quantitative analysis were exported including parameters such as percentage of positive cells, intensity, density, and area.

3.1 Immunohistochemical Staining

3.1.1 Immunohistochemical staining for CD68

The staining protocol for CD68-detection was established by Diana Mechtcheriakova's group and represents now the standard immunohistochemical staining protocol for this marker in the group.

A collection of formalin fixed paraffin embedded ovarian carcinoma tissues (n=68) were provided by our collaboration partner Prof. Peter Birner from the institute of Pathology.

For paraffin melting, the slides were incubated at 60°C for 25 minutes. Next slides were rehydrated in Xylol and in an Ethanol-series. Thereby, the slides were put three

times for ten minutes in Xylol following by five minutes in each Ethanol dilution (100%, 96%, 70%, 50%, 30%). Afterwards, they were washed three times for five minutes in PBS.

For antigen retrieval the slides were boiled with 100mM EDTA buffer (pH=9) in a pot. First the EDTA buffer was warmed up for five minutes and then the slides were put into the hot puffer and were boiled for 20 minutes without the top. After cooling for 20 minutes at room temperature the slides were washed again twice with PBS for five minutes.

In the next step the tissues were marked with DAKO pen (DAKO, Denmark) to delimit the application area.

The slides were put into PBS-0.2 % Tween for 5 minutes for permabilisation. In the meantime the blocking solution (5% FCS in PBS) was prepared, from which 100µl per slide were applied for 30 minutes.

The first antibody was a mouse anti-human CD68 (Thermo Sc. MS-397-PO) that was diluted in 0.1% BSA (dilution: 1:500). Per slide 80µl were applied and the slides were incubated for 50 minutes in a humidifier chamber at room temperature. After the incubation the slides were washed three times for ten minutes in PBS+0.05% Tween.

Next, 2 drops of secondary antibody, in our system this is the dextran polymer peroxidase envision system (HRP/MOUSE DAKO Envision Kit) were applied and the slides were incubated again for 25 minutes. The slides were washed 3 times in PBS+0.05% Tween for ten minutes followed by visualizing of the antigen using 80µl DAB chromogen per slide for two minutes. Next slides were washed twice for five minutes in bidestillated water and afterwards using 100µl hematoxylin per slide the nuclei staining was performed. Slides were washed for one minute in tap water to stop the salt-reaction slides were rinsed with bidestillated water. At the end the slides were mounted with Fluoromount.

3.1.2 Materials for immunohistochemical staining for CD68

- tissue sections of ovarian carcinoma (n=68)
- primary mouse anti-human CD 68 antibody (Thermo Sc. MS-397-PO, Thermo Scientific, Fremont, USA)
- polymer peroxidase envision system, anti-mouse (Dako Cytomation, Denmark A/S)

- Ultravision LP Detection System (HRP Polymer & DAB Plus Chromogen; Thermo Scientific, Fremont, USA)
- Phosphate Buffered Saline (PBS) pH=7,4
- Tween®20 for electrophorese (Sigma-Aldrich Chemie GmbH, Steinheim, GER)
- Xylol (Merck, Darmstadt, GER)
- Ethanol, absolute (Merck, Darmstadt, GER)
- EDTA (Merck, Darmstadt, GER)
- Dako Pen (Dako Cytomation, Denmark A/S)
- Chem Mate ™ Hematoxylin (Dako Cytomation, Denmark A/S)
- 10% BSA (Bovine Serum Albumin)/PBS (Blocking Puffer)
- Fluoromount (Southern Biotech, USA)
- Eppendorf tubes (Biozym Biotech Trading GmbH)
- drying chamber (Memmert, TV15u)
- slide box (Hellendahl, GER)
- cover glasses 24 x 50 mm Borosilicate Glass, Thickness No. 1 (VWR International)
- steam cooker (Braun, Multigourmet Type 3-216)
- Aqua bidest.
- Vortex Labinico L24
- Microscope Zeiss Imager Z1 (Zeiss, Jena, GER)

3.1.3 Immunohistochemical staining for CD163

The staining protocol for the detection of CD163 in ovarian carcinoma specimens is similar to the protocol of the immunohistochemical staining of CD68 based on the standard protocol established in Diana Mechtcheriakova's group.

The main difference is the usage of a rabbit anti-human CD163 (DB Biotech, CAT: CB 045-T) antibody diluted with 0,1% PBS (dilution: 1:200) and an incubation time of 60 minutes. The volume and incubation time of DAB chromogen for visualizing the antigen are also different: for CD163 staining it amounts 100μl and 10 minutes of incubation.

3.1.4 Materials for immunohistochemical staining for CD163

The same sets of reagent as described in 8.1.2 for CD68. Only those, which differ are listed here:

- primary rabbit anti-human CD163 antibody (CAT: DB045-T, DB Biotech, SK)
- polymer peroxidase envision system, anti-mouse (Dako Cytomation, Denmark A/S)

3.2 Acquisition with the microscopy-based TissueFAXS System

The slides were acquired by microscopy-based instrument, the TissueFAXS (TissueGnostics: www.tissuegnostic.com). It's a combined system of high-tech hardware, microscope and a fast multiple core computer workstation. The system enables to acquire IHC and immunofluorescence (IF).The instrument for cell analysis combines detailed morphologic information offered by microscopy with multi-channel flow cytometry.

For IHC analysis the bright field modus was applied wherein up to eight slides at once can be acquired. First, a preview image of the tissue section is been taken, wherein the regions of interest to be acquired can be selected. The regions are manually drawn by setting single points combined to an object; next the slide is labeled with slide number (e.g. S1) and patient number. The preview is accomplished with the 2.5x objective and the acquisition with the 20x objective. Within the acquisition, single images, called "fields of view" (FOVs), are acquired and will be stitched together to get an entire image. All the parameters for acquisition are established once and then used for all the oncoming slides. Furthermore, the z-interval for focusing has to be defined. Following parameter were used for the acquisition: saturation: 112,2; gamma: 13; TL lamp: 12,2; condenser: 0,9; exposure time: 0,7. For obtaining the right focus, the average of the z-position value of all 8 slides was taken and with this average an interval was calculated of +/- 200µm, where all z-values from the slides to be acquired localize in. For the acquisition of the ovarian tissue the "extended focus" mode was chosen, as the tissue is morphologically very complex. "Extended focus" means, that five images were taken one after another from each FOV at different z-positions and the image with the best focus was chosen by the software. After the acquisition, there is a possibility to correct individual FOVs, or groups of FOVs, that are not in focus by using the "reacquisition" mode. Thereby, the z-value for this FOV is corrected manually at the

microscope. At the end the file with the extension "aqproj" was created and saved, that could be used for further analysis.

3.3 Software-based qualitative and quantitative analysis via HistoQuest

The TissueFAXS-based acquired tissue was analyzed using the "HistoQuest" software (www.tissuegnostic.com). HistoQuest is analysis software from TissueGnostics for evaluation of IHC slides.

The software builds up an analysis file "hiqproj" from the original "aqproj" file, which was acquired at the TissueFAXS. At the beginning, the markers of interest that will be analyzed and the marker, which is set as "Master" have to be defined. In comparison to the analysis strategy established in DM lab or CD45 and CD20, in case of CD68, not the nucleus, but CD68 itself is set as master.

After that, different regions of interests (ROI) and exclusions areas were manually drown based on the complexity of ovarian carcinoma morphology. These regions enabled a differentiated analysis.

Next the ROIs were sub-devided in 3 main groups: tumor_all, stroma_all and phagocytes_all.

"Tumor_all" implicates all kinds of tumor tissue without differentiating intra-tumoral differences in morphology.

The group "stroma_all" is sub-classified in stroma_0.2, stroma_0.5 and stroma. The number defines the distance from the tumor tissue, e.g. "0.2" means 0.2µm away from the tumor border. The "stroma_0.2" describes the intra-tumoral stroma, the "stroma_0.5" the peritumoral stroma and the sub-group "stroma" describes the distant stroma.

The third main group "phagocytes_all" comprises the detected macrophages and is sub-classified into "immune cells (IC)_phagocytes" and "mucosa_monocytes" to subdivide the different stages of the monocyte/macrophage differentiation/activation. "IC_phagocytes" describes dark brown, round shaped phagocytes organized in phagocytic islands, which are mainly located in the tumor tissue. Round, small monocytes, which are found more in the mucosa and stroma, are assigned in the subgroup "mucosa_monocytes".

During the analysis additional group was created: in addition to the groups "tumor_all" and "stroma_all", the group "tumor+stroma_area" was assinged, which

combined both groups. Additional type of analysis used the entire slide as one ROI that was analyzed in total without any morphological sub-discrimination.

The next step in the analysis was the establishment of the profile for the marker CD68. For identification of the nuclei the color blue is used and for recognition of CD68 positive cells the color brown is considered. With the option "view color image" the correct color can be defined and the option "view gray image" allows to assign the right intensity. The profile is based on following parameters for both CD68 and nucleus:

Table 2: HistoQuest-based algorithm: parameters for CD68 detection

Color:	
Red	156
Green	75
Blue	30
Intensity	200
Nuclei Size	30
Discrimination Area	1
Discrimination Gray	1
Automatic Background Threshold	Yes
Threshold Range	[5, 255]
Virtual Channel	No
Post Processing Order	Remove, Merge
Remove Labels	No
Use Merging Rules	No

Table 3: HistoQuest-based algorithm: parameters for detection of nucleus

Color:	
Red	54
Green	119
Blue	137
Intensity	200
Use Ring Mask	Yes
Interior Radius	-0,42μm
Exterior Radius	0 μm
Use Identified Cell Mask	No
Use Nuclei Mask	Yes
Automatic Background Threshold	Yes
Threshold Range	[5, 255]

This established profile was used to recognize the positively stained cells and quantify these cells by the HistoQuest software. Prior to run the analysis, to make the slide comparable to each other the function "illumination correction" was applied. Thereby, the intensity of all slides was normalized.

After the software-based automatic quantitative analysis of all regions of interest, a patient-specific "cut-off" has to be set to correctly determine which cells are positive. With the help of the diagram "nucleus_mean intensity" at the x-axis and the "CD68 - standard deviation (STD) of intensity" at the y-axis, the cut offs were defined. Using the option "view backward data" it could be tested, whether cells located within the "upper right" (UR) indeed correctly represent the positive cell population.

As next step, the data was exported using the "statistic report" tool. For this type of analysis the total stained area defined by the "UR" quadrant as well as the total area of the corresponding ROI or Group was exported. To calculate the ratio in percent of positive area normalized to total area, excel-based calculations were performed. Finally, the obtained staining-derived datasets will be used for follow up analysis including the alignment with clinicopathologial parameters.

3.4 The real-time polymerase chain reaction (RT-PCR)

Real-time PCR-based profiling was performed on a collection of 22 ovarian carcinoma samples provided by our collaboration partner Prof. Peter Birner from the Institute of Pathology, Medical University of Vienna. The analysis included expression profiling CD68 and CD163 isoforms.

3.4.1 The principle of real-time PCR

Real-time PCR is a highly sensitive approach based on the principles of the PCR reaction, whereby a special gene of interest is amplified up to a billion-fold. With this technology even low expressing genes can be precisely and specifically measured. A certain target sequence in a sample, where by the amplification process is monitored in real time by the detection of the accompanying increase in the corresponding fluorescence signal. Thus the number of cycles the signal needs to reach a certain threshold correlates with the amount of original target sequence present in the sample. Furthermore, right after the amplification a melting curve analysis is performed which enables to determine the melting curve that is specific for each analyzed product, and based on the following characteristics such as the product length and nucleotide composition.

One of the major achievements of real-time PCR is the possibility to monitor the progress of DNA amplification in real time. On the one hand this is enabled by special fluorescent dyes, which intercalate into double-stranded DNA (dsDNA), such as SYBR Green. Thereby SYBR Green binds to the minor groove of dsDNA and upon intercalation emitters 1000-fold greater fluorescence than in the unbound form. The gained fluorescent signal is thereby representative to the dsDNA amount present at a certain time point.

Consequently, the greater the amount of dsDNA present in the reaction tube, the greater the amount of dye binding and consequently the fluorescent signal from SYBR green I. In the next step, fluorescent signal is detected and recorded by a special instrument. The instrumentation has to provide the excitation signal with the corresponding wavelength and be able to detect the emission signal. A further important part of the instrument is the thermocycler, where the PCR reaction itself takes place. This consists of as heating block, which has to keep a consistent temperature among all sample wells, because any differences in temperature could cause different PCR amplification efficiencies.

For data analysis, the real-time PCR instrumentation is also linked with a computer hardware for data acquisition and storage. The corresponding analysis software offers the platform for graphical data visualization and interpretation in form of amplification and dissociation curves.

The amplification curve provides information about the kinetics of amplification of the target sequence and the dissociation curve presents the characteristics (melting point) of the final amplified product. [62-64]

3.4.2 Real-time PCR-based expression profiling of CD68 and CD163 isoforms

Expression profiling was performed on 22 ovarian carcinoma samples. Corresponding cDNA was already available in DM group. Next, real-time PCR-based expression profiling was performed. Primers were created by Dr. Martin Svoboda (research group of DM).

The examined isoforms of CD68 and CD163 are listed below.

Table 4: CD68 isoform 1

		Tm
Forward Primer	ACAGGGAATGACTGTCCTCACA	58
Reverse Primer	GCTGGTTGTTCCAGTGCTCTCT	60

Table 5: CD68 isoform 2

		Tm
Forward Primer	AGACAGCCTAGCTGGACTTTGG	59
Reverse Primer	GTTCCAGTGCTCTCTGCCAGTA	58

Table 6: CD163 isoform 1

		Tm
Forward Primer	CCAAATTCAATACCGGGAGATG	59
Reverse Primer	CAGCACTGAAATCAGCTGACTCA	59

Table 7: CD163 isoform 2

		Tm
Forward Primer	ATTCCTCAGGAGGCCATTCTG	59
Reverse Primer	TAGTCCAGGTCTTCATCAAGGTATCTT	59

Real-time PCR-based profiling was performed using 2μl cDNA (10ng/μl) and 8μl master mix in 384 well format. Composition of master mix is listed in table 13.

Table 8: Composition of the master mix

2x SYBR Green	5μl
primer mix (forward/reverse) 5μM	0,5 μl
H_2O	2,5 μl

=8μl + 2μl cDNA = 10μl total.

For profiling the Applied Biosystems machine 7900HT was used. The applied protocol was composed of following steps: 10 minutes at 95°C; 15 seconds at 95°C and 1 minute at 60°C (40 cycles); standard melting curve analysis.
For analysis the software SDS 2.4 (Applied Biosystems) was taken. For normalization average expression level of housekeeping genes (ACTB, TOP1, UBC, and YWHAZ) was used. For relative quantification data was analyzed by the $\Delta\Delta c_t$ method (normalization to housekeeping genes and usage of the reference RNA as calibrator). Calculation of fold differences included the following steps:

1. c_t (gene of interest) - c_t (HKGs) = Δc_t (sample)
   c_t (calibrator) - c_t (HKGs) = Δc_t (calibrator)

2. Δc_t (sample) - Δc_t (calibrator)= $\Delta\Delta c_t$
3. Fold difference: $2^{-\Delta\Delta c_t}$

For real-time PCR-based profiling the following reagents and equipment were used:

- Gibco ultrapure water RNase/DNase free (10977-035)
- POWER SYBR Green PCR Mastermix (Applied Biosystems; USA)
- qPCR Human Reference Total RNA (Clontech Laboratories Inc.; USA)
- MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems; USA)
- MicroAmp Optical Adhesive Film (Applied Biosystems; USA)
- Bioyzm Safe Seal Filter Tips professional (Biozym; GER)
- Real-time instrument: Applied Biosytems 7900HT (USA)

4. RESULTS AND DISCUSSION

The ongoing study within the research group of Diana Mechtcheriakova, entitled "Microscopy-based algorithm for qualitative and quantitative analysis of immune cell sub-populations within complex ovarian cancer tissues", addresses the question, whether the patient-specific immunological imprint can be used as prognostic marker in patients with ovarian carcinoma.

Central to the study is the assessment of the following the main categories, which in sum represent the immunological imprint: (i) the immune landscape, meaning the distribution and organization patterns of various immune populations within the tissue, (ii) the immune contexture, covering the cell type composition, where beside the analysis of the total CD45 positive immune cell population particular attention is given to the B cell lineage and the macrophage/monocyte lineage, and (iii) tumor anatomy representing the morphological complexity of ovarian carcinoma tissue.

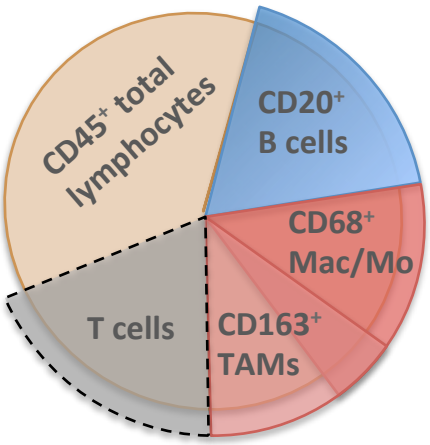

Figure 12: Outline of immune cell sub-population of interest to be assess by qualitative and quantitative analysis:

CD45 is used as general immune cell marker to analyse the entire immune cell population. CD20 is used as marker for B cells. Within the macrophage/monocyte lineage we disseminated between the total population, using CD68 as marker and specifically the M2 population, using CD163. CD3 might be used as marker for T cells. Solid lines represent marker included into the study; dashed line the potential future candidate marker.

Bringing all these parameters together a comprehensive analysis of 68 ovarian carcinoma specimens, which are stained with lineage identity markers can be performed. The following work comprises a part of a large-scale study conducted in DM lab that focused on the qualitative and quantitative assessment of the monocyte/macrophage sub-population in ovarian carcinoma specimens.

4.1 Algorithm for quantitative analysis of macrophage/monocyte lineage within complex ovarian carcinoma tissue

As fist step on the study, an algorithm was established allowing qualitative and quantitative analysis of the macrophage/monocyte lineage in complex ovarian carcinoma tissue. The microscopy-based system, TissueFAXS, was used for automatic acquisition of large-scale tissue section, followed by analysis using the HistoQuest software. In the next sub-chapter the algorithm will be explained step by step in detail.

4.1.1 Immunohistochemical staining of large-scale patient specimens

First, 68 ovarian carcinoma specimens were stained following the IHC protocol, which is described in detail in the Methods section in chapter 8.1.1. Shortly, IHC-based staining of paraffin-ebbed tissue section was performed using the mouse anti-human CD68 antibody to detect the macrophage/monocyte lineage. The composition of patient cohort with different types and stages of ovarian carcinoma used in the study is listed below.

Table 9: The composition of the ovarian carcinoma specimens

	Serous (n=53)	Endometrioid (n=15)
G1	10	5
G2	16	5
G3	26	5
LMP	1	-

Of note, due to the complex tumor anatomy of the investigated tumor type, large-scale tissue section were included into the study, rather than tissue microarrays

(TAMs), which would not allow to perform a comprehensive analysis of immune cell distribution.

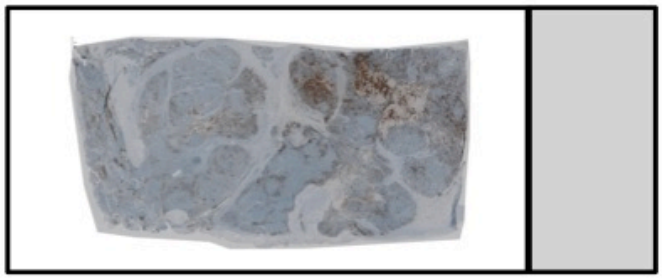

Figure13: An example of a stained large-scale tissue section

4.1.2 Acquisition with microscopy-based TissueFAXS System

Upon immunohistochemical based staining, the large-scale tissue sections were scanned using the microscopy-based TissueFAXS system. The combined system of a microscope, a fast multiple core computer station and a high-tech hardware allowed automatic scanning of the entire slides. The image is thereby built up step by step out of individual Filed of Views (FOVs), which are suing a special algorithm automatically stitched into a large-scale image. A further advantage of the TissueFAXS system is given by an algorithm allowing automatic and fast focusing of the individual FOVs throughout the whole image acquired. At the end of the acquisition process the TissueFAXS-based file containing the information for the follow up analysis is transferred to the evaluation station.

4.1.3 Software-based qualitative and quantitative analysis via HistoQuest

4.1.3.1 Definition of regions of interest based on complexitiy of tissue morphology

The qualitative and quantitative analysis of the scanned large-scale tissue sections occurs with software-based system "HistoQuest".

HistoQuest is immunohistochemistry analysis software from TissueGnostics accounting patient-specific tumor anatomy. Thereby, region of interest, also called ROIs, were defined based on the complexity of ovarian carcinoma tissue morphology and manually drown, enabling a differentiated analysis.

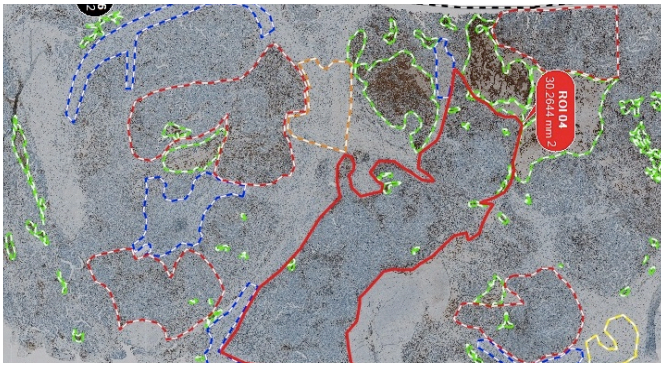

Figure 14: An overview of the defined "regions of interest" (ROIs)

The individual ROIs were thereby pooled into groups of interest. Three main groups - tumor_all, stroma_all and phagocytes_all were defined. The group "tumor_all" included various type of tumor tissue, independent on intra-patient variability in tumor anatomy. The group "stroma_all" was further sub-classified into stroma_0.2, stroma_0.5 and stroma. The number defined the distance from the tumor tissue, e.g. "0.2" represents the area up to 0.2µm away from the tumor border. Thus, "stroma_0.2" describes the intra-tumoral stroma, whereas "stroma_0.5" represents the peritumoral stroma. The sub-group "stroma" comprises the distant stroma. The figure 23 illustrates the tumor morphology defined as tumor versus stroma.

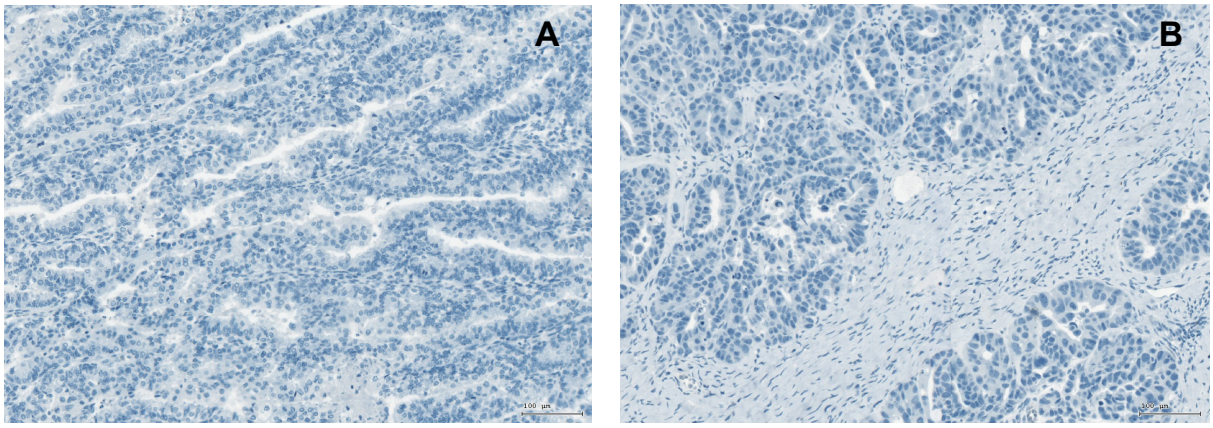

Figure 15: Examples for the two main groups "tumor_all" and "stroma_all"

A: tumor tissue; **B:** stroma tissue; scale-bar: 100µm

The group with the major interest of the following study is named as "phagocytes_all" defining the detected macrophages. To allow detailed analysis of macrophage morphology/activity the group was further sub-divided "immune cells (IC)_phagocytes" and "mucosa_monocytes". These two sub-groups are described in more detail in the chapter 4.2.2.

During the analysis, an alternative strategy was established: the two groups "tumor_all" and "stroma_all" including the corresponding sub-groups were combined to one group called "tumor+stroma_area". Furthermore, the entire slide without predefined ROIs was analyzed as total.

4.1.3.2 Design of HistoQuest profiles for quantitative analysis of CD68 marker

The next critical step was the establishment of profiles specifically designed for the CD68 marker. This profile is used by the HistoQuest software to automatically recognize the positively stained cells and quantify these cells. In contrast to the profiles previously established in DM lab for analysis of CD45 and CD20 molecules in ovarian carcinoma tissue, which recognize the cell as an individual objects based on color and intensity of the nuclear staining followed by recognition of the specific stating, a different strategy was applied in case of CD68. The CD68 profile recognized the area of the positively stained cells, which is the normalized to the total area of the region of interest. The design of an alternative algorithm is based on the fact that due to the localization of CD68 in the endosomal compartment it covers the nuclear staining, thus making it impossible to use the nuclear staining as marker for cell identity. After designing the profile for the CD68 as marker, the profile was loaded and the automatic software-based analysis of positive cells in all defined ROIs was started. Figure 24 illustrates the algorithm allowing proper identification of CD68-positive cells of the macrophage/monocyte lineage.

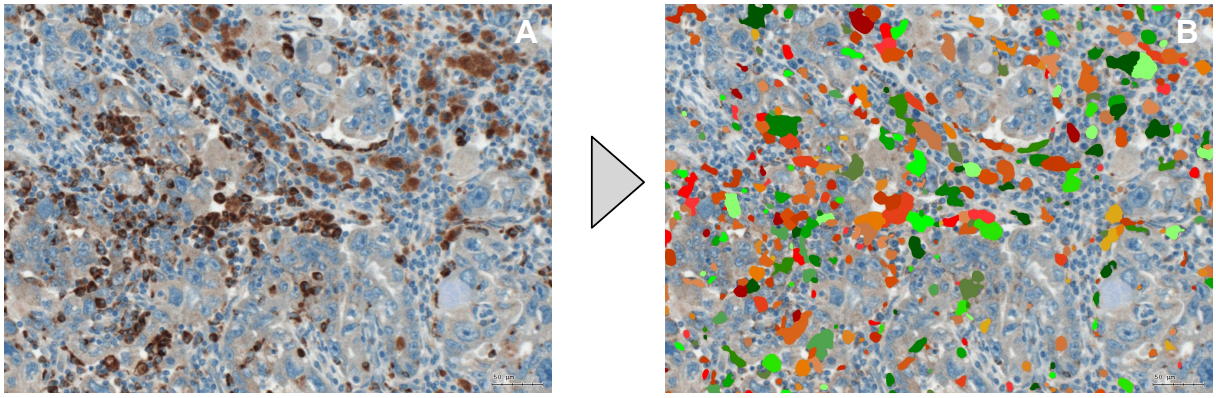

Figure 16: Algorithm for analysis of CD68-positive cells within complex ovarian carcinoma;
A: original; **B:** recognized as positive; **scale-bar:** 50μm

4.1.3.3 Setting of patient-specific cut-offs

As the next step of the quantitative analysis patient-specific cut-off were set. For this purpose the data was visualized by the scattergram Nucleus Mean Intensity versus CD68 STD of intensity. The term "cut-off" describes, which cells are really recognized as positively stained, and thus are allocated within the upper right (UR) quadrant of the scattergram. Depending on the patient-specific background standing intensity and the cut off was set for each patient individually; furthermore for some patients the cut off has to be adjusted separately of the individual group of interest. Using the “view backward data” tool we could ensure, that only the properly recognized cells were taken for follow up calculation. Figure 25 shows an example of a scattergram that was used for setting the cut off with the upper right quadrant highlighted in grey.

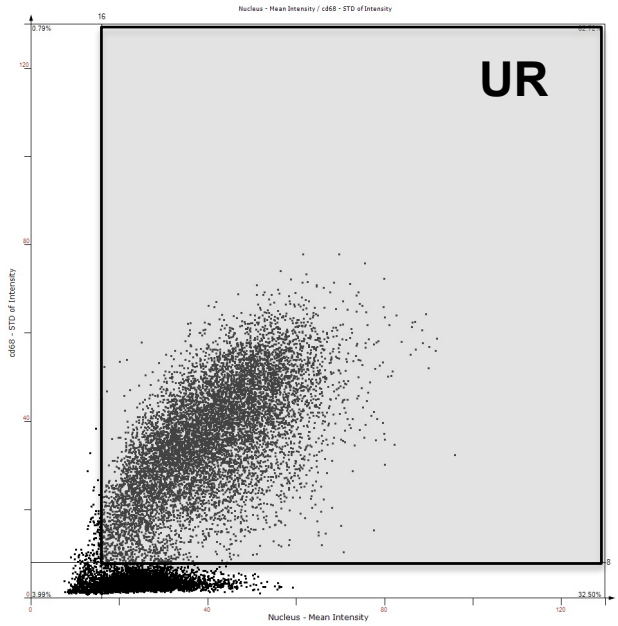

Figure 17: Scattergram with patient-and group-specific "cut off":

The upper right quadrant where cells properly recognized as positive are allocated is highlighted in grey.

4.1.4 Data exporting for statistical analysis and statistical modeling

In the next step, using the "statistic report" tool the data was exported in form of an excel table. Two parameters of interest were exported: the total area of positively stained cells within a group of interest and the total area of this group. Furthermore, to analyze the extend of the "phagocytes_all" and the corresponding sub-groups the total area of the slide was exported as well. All the data was summarized in an excel table, where the follow up calculations were performed. First for the groups,

"tumor_all" and "stroma_all" as well as the corresponding sub-groups the following formula for calculation of the percentage of positively stained area within the total area of the group of interest was applied:

$$\%\ \text{CD68 positive} = \frac{\text{Area positively stained for CD68}}{\text{Area of Group of Interest}}$$

For "phagocytes_all" and the sub-groups "immune cells (IC)_phagocytes" and "mucosa_monocytes" we were interested to calculate the percentage of the area of interest, such as "mucosa monocytes", relative to the total area of the corresponding slide. For this purpose the following formula was applied:

$$\%\ \text{Area of Group of Interest normalized to total slide} = \frac{\text{Area of Group of Interest}}{\text{total area of the slide}}$$

As using such formula we only look on the percentage of the total slide occupied group of interest such as macrophage accumulation in form of phagocytes or mucosa monocytes, in the next step we included in addition the percentage of positive cell into the formula. Thus, the next type of analysis allows to look on the percentage of the total slide occupied by CD68-positive cells within the group of interest.

$$\%\ \text{CD68 positive area within Group of Interest normalized to the total slide} = \frac{\text{Group of Interest area x \%CD68-positive area}}{\text{total area of the slide}}$$

In the next step of analysis the CD68-derived datasets will be imported into SPSS-based table to perfume alignment with clinicopathological parameter.

4.2 Patient-specific extent and organization of CD68 positive macrophages/monocytes in ovarian carcinoma specimens

The qualitative and quantitative analysis of the CD68 positive macrophages/monocytes lineage in ovarian carcinoma specimens demonstrated the presence of a patient-specific extent, distribution and organization pattern of CD68 positive macrophages as well as highlighted differences in morphology attributed to the individual cellular sub-populations.

4.2.1 Patient-specific extent of CD68 positive macrophages/monocytes

As determined by HistoQuest-based quantitative analysis, we observed intra- and inter-patient variability in the extent of CD68-positive leukocytes. To allow sophisticated analysis, quantification was performed separately for the tumor and the stroma tissue. As exemplified in figure 26 and 27, patients could be sub-divided in three main classes, showing low, intermediate and high numbers of CD68-positive macorphages in tumor and stroma, respectively. Software-based analysis over the 68 specimens included into the study revealed the percentage of CD68-positive cells in the tumor ranging from 0.2% to 24.9% with a median of 2.8% and ranging from 0.1 to 14.9 with a median of 1.1 in stroma.

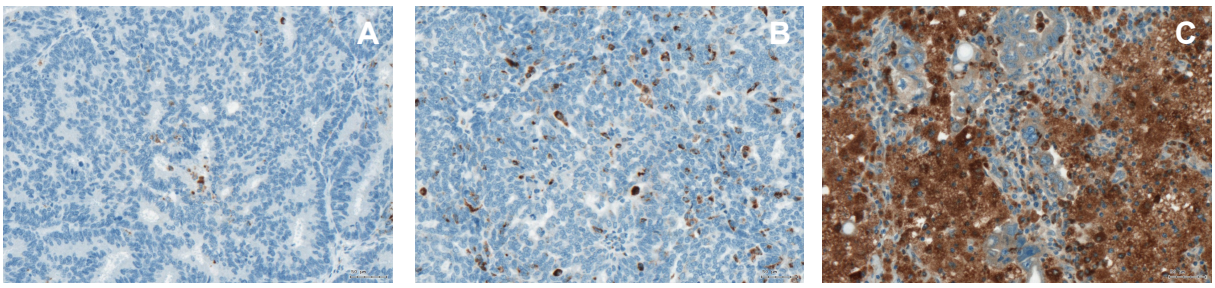

Figure 18: The patient-specific extent of CD68 positive macrophages/monocytes in tumor tissue;

A: low; **B:** intermediate; **C:** high; **scale-bar:** 50μm

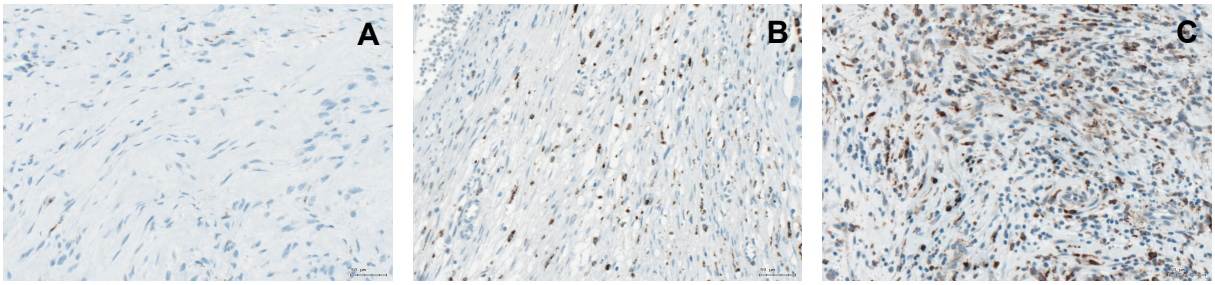

Figure 19: The patient-specific extent of CD68 positive macrophages/monocytes in stroma tissue;

A: low; **B:** intermediate; **C:** high; **scale-bar:** 50μm

4.2.2 Patient-specific organization of CD68 positive macrophages/monocytes

Next finding of the study describes the phenomena of patient-specific organization pattern of the CD68 positive macrophage/monocyte lineage cells. Furthermore, the CD68-positive leukocytes showed differences in the cellular morphology, likely representing diverse sub-populations and/or activation status. Overall, two main sub-classes were observed. First, small round shaped monocytes were detected, with the tendency to accumulate within tumor and/or stroma, thereby strongly associated with the presence of mucosa tissue (figure 28). Those cells likely represent naive monocytes, freshly arrived in the tissue and thus not jet fully differentiated into tissue resident macrophages. Thereby, the mucosa tissue itself was found to show absence (Figure 28A) or presence (figure 28B) of CD68 staining. Those parts were staining was observed might represent an example of presence of the soluble form of CD68, known to be generated by shedding of the CD68 molecule. As described generally for the total CD68-positive population, also in case of the phenomena of mucosa monocytes, those specific structures were detected in a patient-specific manner. Out of the 68 specimens analyzed, 61 were positive and 7 negative for monocytic accumulation. Overall, mucosa monocytes, quantified by the area of accumulation normalized to the total area of the tissue, were found in the range of 0.02% to 33.9% with a median of 0.8%.

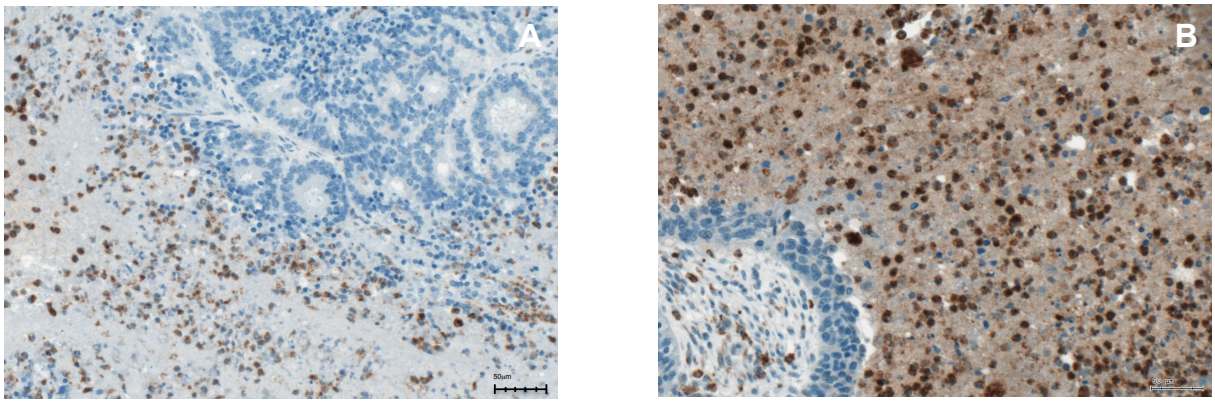

Figure 20: Mucosa monocytes within tumor and stroma parts;

A: mucosa monocytes in tumor; **B:** mucosa monocytes in stroma; **scale-bar:** 50μm

Second type of accumulation was represented by, large phagocytes organized into phagocytic islands. Such islands were found to be building up within tumor, in stroma parts and in fibrotic tissue (figure 29). HistoQuest-based algorithm allowed

quantifying the presence of phagocytic island, as the percentage of area occupied by those islands normalized to the total slide area. Thus, phagocytic accumulation were detected in all specimens analyzed and were observed in the range of 0.01% to 5.7% with a median of 0.2%.

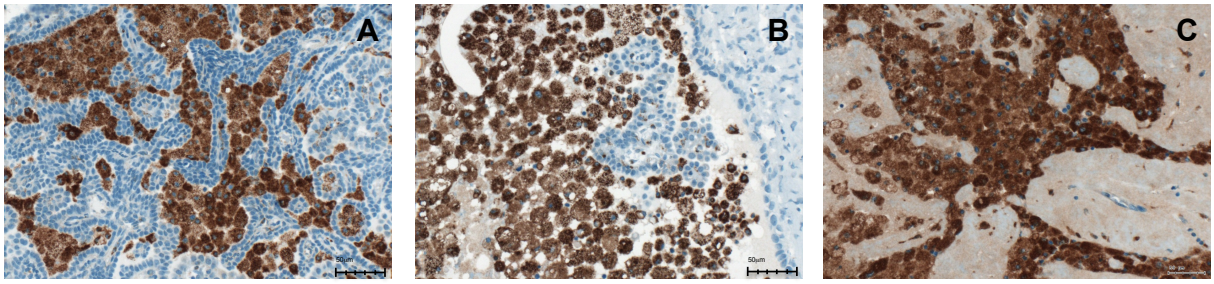

Figure 21: Phagocytes within tumor and stroma parts

A: phagocytes organized in phagocytic islands within tumor parts; **B:** phagocytic island in stroma; **C:** phagocytic islands within fibrotic tissue; **scale-bar:** 50μm

Based on the morphology and localization of those cell, one could hypothesized that those phagocytes are functionally associated with the cellular debris of cellular debris, thus being involved in the process of tumor distraction. Additional phenomena observed within the study describe the accumulation of CD68-positive macrophages within adipose tissue (figure 30) that was detected in some of the specimens analyzed.

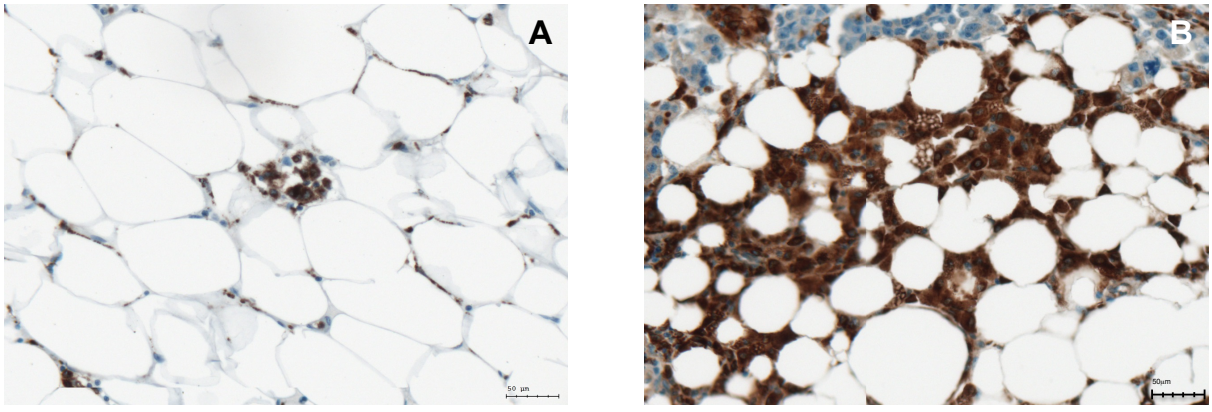

Figure 22: Macrophages wihtin Adipocytes

A and B: Macrophages in adipose tissue; **scale-bar:** 50μm

4.3 Pro- or anti-tumoral functions of CD68 positive macrophages

Next critical question we asked was, whether the CD68-positive monocytes/macrophages detected within complex ovarian carcinoma tissue are of M1 or M2 phenotype, and thus can be associated with pro-or anti-tumoral functions. As the first attempt to answer this question a panel of specimens, being representative for the phenomena described in detail above, was selected and stained with the anti CD163 antibody. In contrast to CD68, which was described to be expressed on the entire macrophage population, CD163 can be used as marker for the M2 phenotype. First class of cells we looked at, were the typical intra-tumoral macrophages, characterized by their elongated, well spread morphology. Here again, we observed patient-specific differences in CD163 staining. Some cells, as shown in figure 31 were found to be CD163-negative, likely representing the M1, pro tumor population. In contrast to that, intra-tumoral macrophages found in another patient (figure 32) showed clear positive staining for CD163, thus likely being attributed to the M2 lineage.

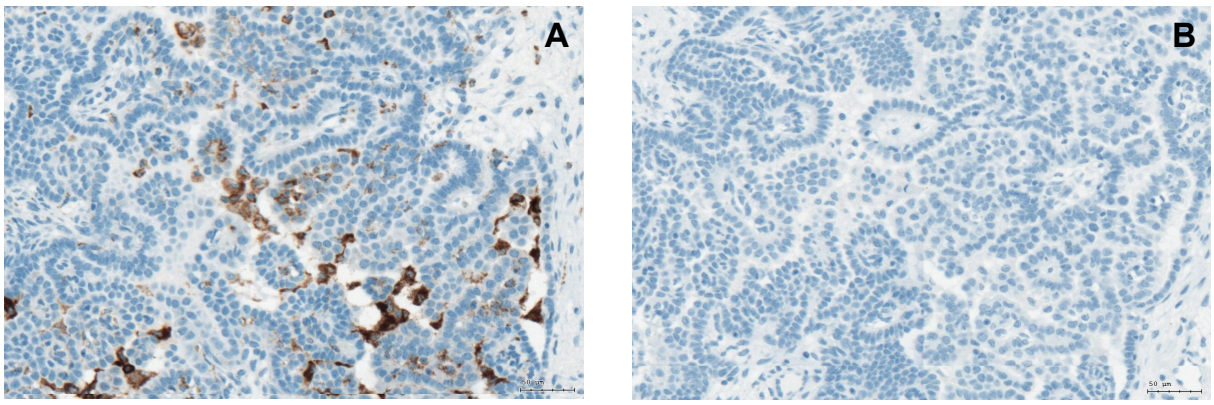

Figure 23: CD163-negative M1 phenotype within tumor tissue

A: CD68-positive macrophages; **B:** CD163-negative macrophages; **scale-bar:** 50μm

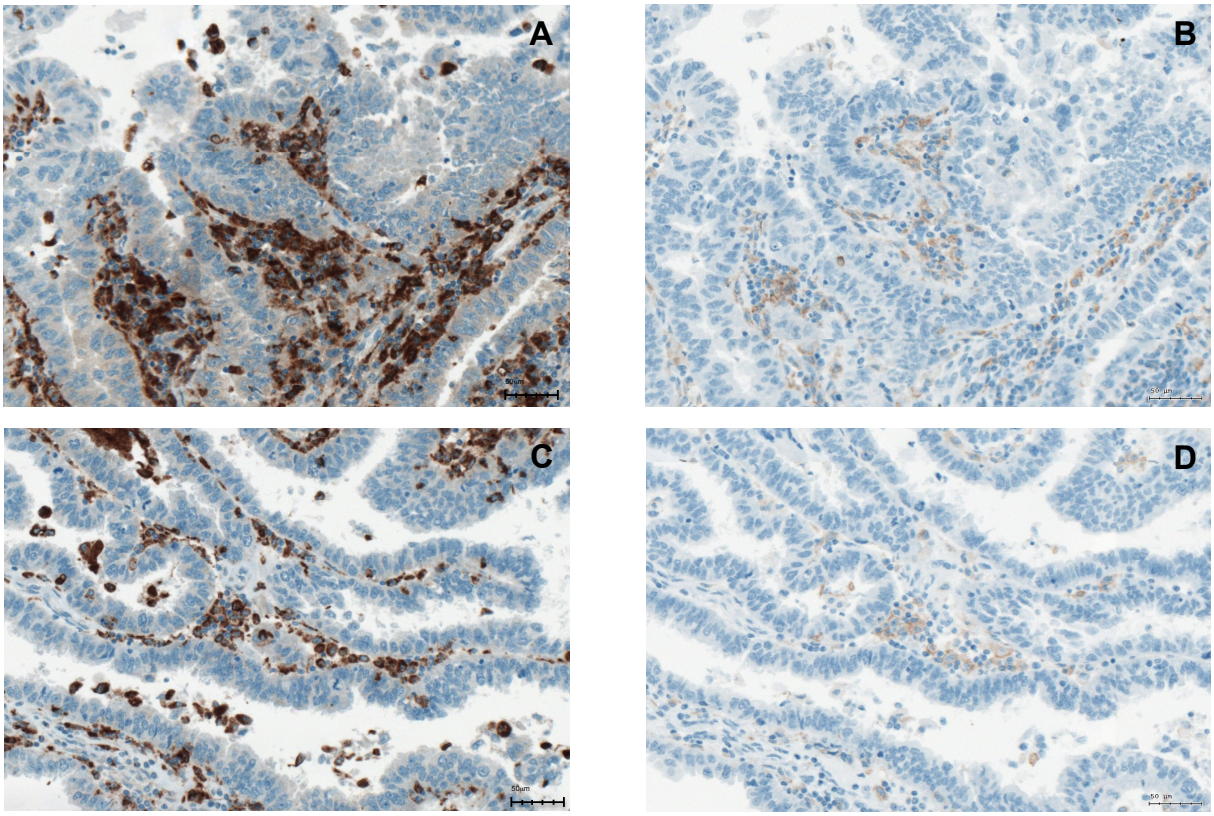

Figure 24: CD163-positive M2 phenotype within tumor tissue

A: CD68-positive macrophages; **B:** CD163-positive macrophages; **C:** CD68-positive macrophages; **D:** CD163-positive macrophages; **scale-bar:** 50μm

Next we looked at the firms main category of cellular organization, namely into monocytes accumulated within mucosa tissue. As shown on figure 33, mucosa monocytes were found to be CD163-negative, thus being suggestive to be part of the M1 population.

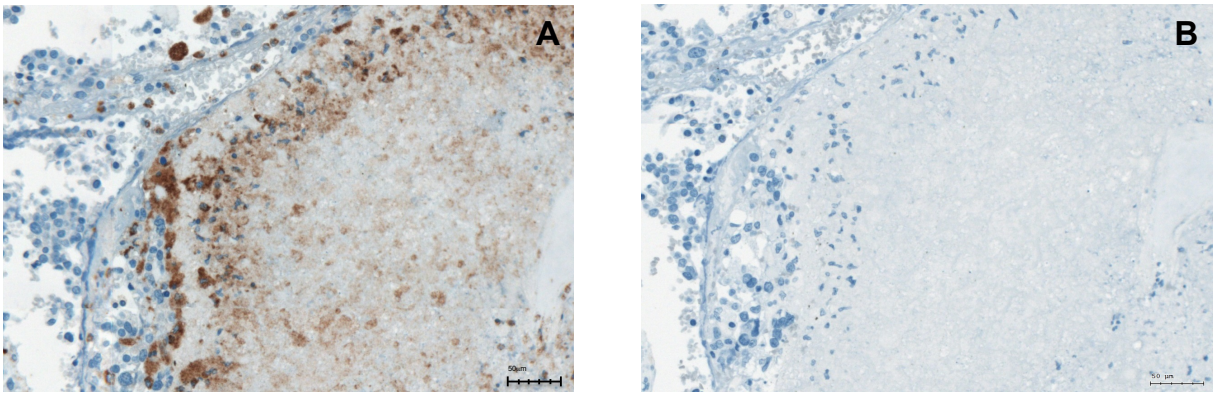

Figure 25: CD163 negative mucosa monocytes

A: CD68-positive mucosa monocytes; **B:** CD163-negative mucosa monocytes

Next category we looked at comprises the large round shaped phagocytes organized in phagocytic islands within tumor and stroma parts. As clearly determined by the anti-CD163 staining, these cells were found to be CD163-negative. In line with our assumption, that those cells represent highly activated phagocytes being involved in clearance processes, absence of CD163 staining further suggested this population to be attributed to the M1 lineage.

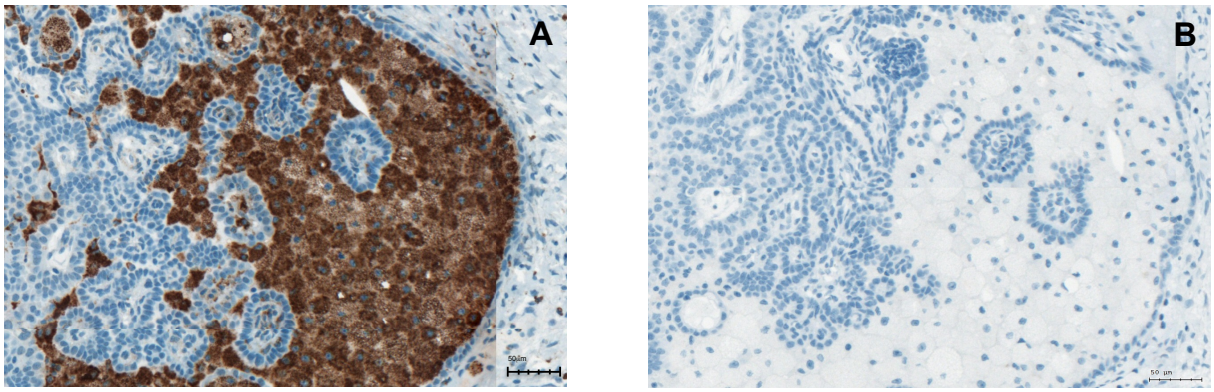

Figure 26: CD163 negative phagocytic islands

A: CD68 positive phagocytes; **B:** CD163 negative phagocytes; **scale-bar:** 50μm

4.4 Expression profiling of CD68 and CD163 isoforms in ovarian carcinoma

A collection of 22 patient RNA samples of ovarian carcinoma specimens was examined for the expression of different types of isoforms of CD68 and CD163 by real-time PCR-based profiling. Table 15 gives and overview on the different types, grades and FIGO stages included into collection of 22 specimens.

Table 10: Characteristics of the 22 samples ovarian carcinoma specimens:

Type of ovarian carcinoma, tumor grade and the corresponding FIGO stage are indicated

Patient ID	Type	Grading	FIGO
7	serous	G1	IIA
18	serous	G1	IC
12	serous	G1	IA
2	serous	G2	IIIb
21	serous	G2	IIIB
22	serous	G2	IC
3	serous	G3	IIIC
5	serous	G3	IV
9	serous	G3	IIIC
10	serous	G3	IIIC
15	serous	G3	IIIc
16	serous	G3	IIIC
17	serous	G3	IIIc
11	endometrioid	G1	IIA
1	endometrioid	G2	IA
13	endometrioid	G2	IA
14	endometrioid/ endocervical	G2	IIIB
6	anaplastic, focalendometrioid	G3	IIIA
4	serous	LMP	IA
20	serous	LMP	IB
8	clear cell	G3	Iib
19	mucinous	G1	IC

As revealed by real-time PCR based expression profiling, within the panel analyzed, the patients show a comparable expression pattern of both isoform of CD68 (figures 35 and 36) and CD163 (figures 37 and 38). The observed results suggest, that there is no isoform of CD68 or CD163 to be predominantly expressed in ovarian carcinoma specimens.

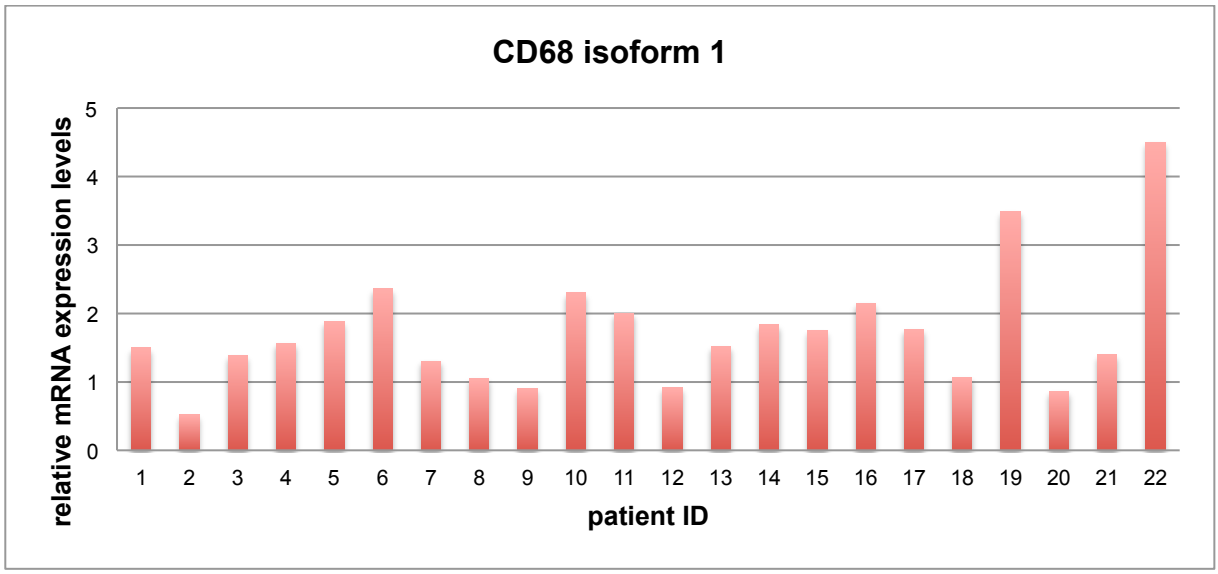

Figure 27: Expression levels of the CD68 isoform 1

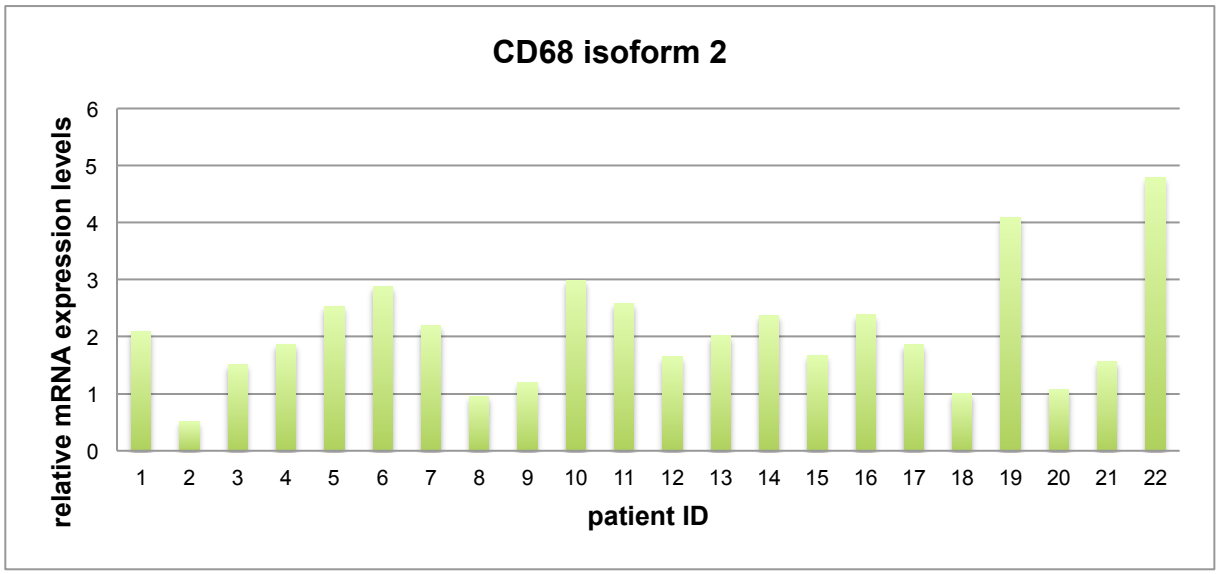

Figure 28: Expression levels of the CD68 isoform 2

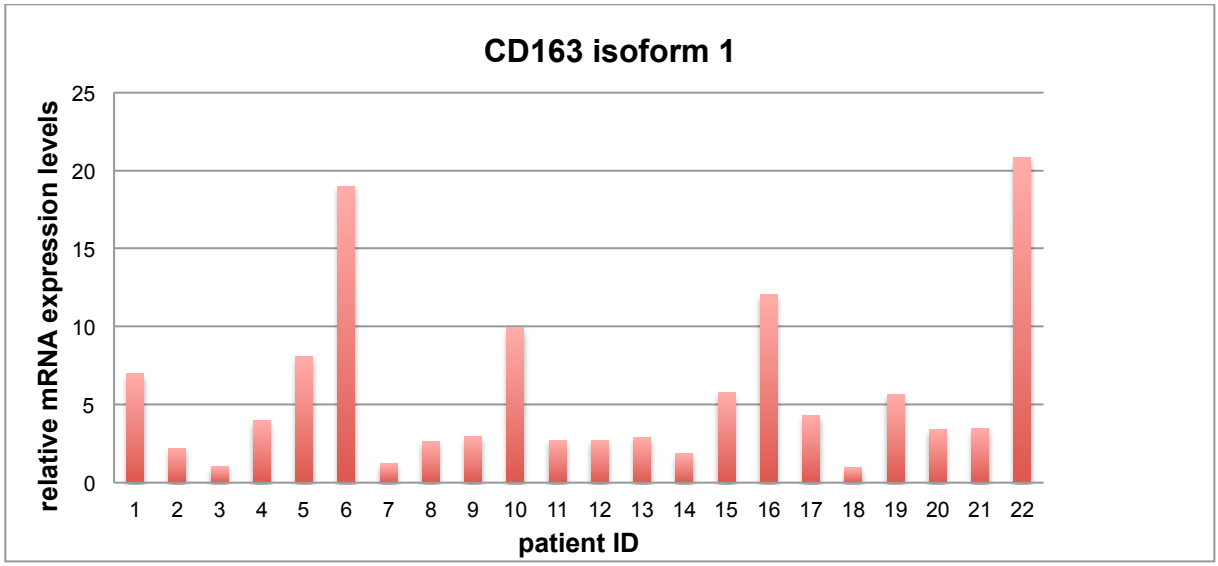

Figure 29: Expression levels of the CD163 isoform 1

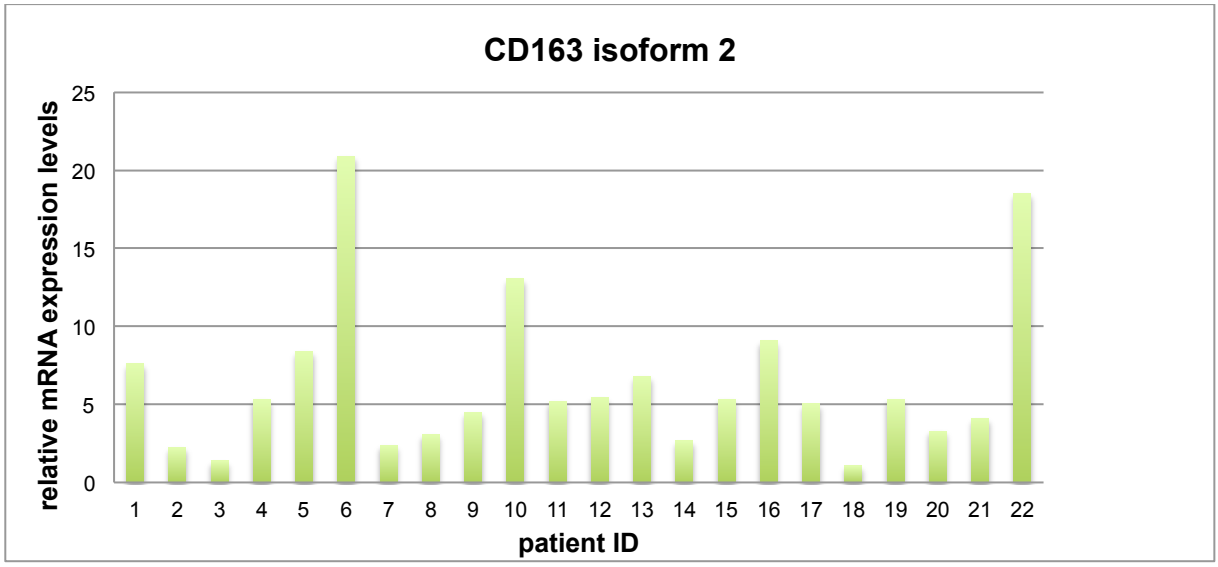

Figure 30: Expression levels of the CD163 isoform 2

5. SUMMARY

Central to the diploma work was the establishment of a microscopy-based algorithm for qualitative and quantitative analysis of the macrophage/monocyte lineage within complex ovarian cancer tissue. A collection of 68 ovarian carcinoma specimens was used for immunohistochemical staining with anti-CD68 antibody followed by scanning of the large-scale tissue sections using the microscopy-based TissueFAXS system. Next, HistoQuest-based algorithm for qualitative and quantitative analysis was established. Shortly, the created algorithm included the following critical steps: (i) manual definition of regions of interest (ROI) within complex ovarian carcinoma tissue which were assigned to groups of interest according to tumor anatomy and the accumulation patterns of CD68-positive leukocytes, (ii) establishment of an algorithm for specific detection of the CD68-positive cell population and automatic quantitative analysis, (iii) setting of patient specific cut offs to properly recognized and quantify the CD68-positive lineage and (iv) export of results. In contrast to the algorithm previously established in DM lab for analysis of CD45-positive and CD20-positive cells, for CD68 as marked a different strategy had to be chosen, given the fact that CD68 localizes in the endosomal/lysosomal compartment and thus covers the nuclear staining. In this case nucleus could not be used as marker for identification of individual cells as object and therefore alternatively, the area of staining was used as parameter for quantification. The analysis of CD68-positive macrophages showed patient-specific distribution and organization pattern as well as extent of CD68-positive cells found in the regions of interest. Regarding the morphology, elongated, well-spread macrophages as well as round-shaped phagocytes organized in phagocytic islands within the tumor and stroma parts were detected. Furthermore, as determined by anti-CD163 staining, the part of the CD68 positive macrophage population was found to be attributed to the pro-tumoral M2 phenotype. Finally, results of quantitative analysis were exported and incorporated into a SPSS data base to be further used for alignment with clinicopathological parameters. Additionally, to explore the knowledge regarding the splice variants of CD68 and CD163, the comparative profiling of individual isoforms was performed by real-time PCR analysis.

6. LIST OF THE FIGURES

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