Exploring Mechanisms behind Beneficial Effects of HIPEC Treatment on Peritoneal Metastasis

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Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

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Zürich, 2024

«Ich habe keine besondere Begabung, sondern bin nur leidenschaftlich neugierig»

Albert Einstein

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I. Abbreviations

APC	antigen presenting cells
CRS	cytoreductive surgery
CRC	colorectal cancer
CC-Score	completeness of cytoreduction score
DFS	disease free survival
EMT	epithelial-mesenchymal transition
HIPEC	hyperthermic intraperitoneal chemotherapy
IFN γ	interferon γ
PCI	peritoneal cancer index
PCT	procalcitonin
PM	peritoneal metastasis
OS	overall survival
OT-I	transgenic mice with an anti-Ova specific T-cell receptor
TCR	T-cell receptor
WBC	white blood cells
WT	wild type

II. Summary

Peritoneal metastasis (PM) arises from different gastrointestinal cancers and ovarian cancer. The most common primary tumor metastasizing to the peritoneum is colorectal cancer (CRC). The treatment of these patients suffering with PM depends on several factors including the extent of the disease in the peritoneal cavity. In case of a limited disease, cytoreductive surgery (CRS) in combination with hyperthermic intraperitoneal chemotherapy (HIPEC) can be indicated. The concept of this treatment approach is to resect the visible tumor mass during CRS and eradicate remnant microscopic tumors via the HIPEC application. One of the two different drug regimens applied for CRC-PM are either the combination of Mitomycin C and Doxorubicin or Oxaliplatin alone. The median overall survival (OS) of patients treated with CRS/HIPEC is roughly 50 months with some long-term survivors, surviving up to 8 years. Why some patients respond better and show long-term survival remains unclear. Clinical studies indirectly suggest a better tumor control probably via the immune system most likely due to the induction of chemotherapeutics-mediated protective immune reactions.

In this thesis, the direct and indirect impact of HIPEC treatment on the immune system to explain induction of tumor-specific immunity were explored. Using patient samples, a systemic inflammatory response after HIPEC and an impaired accuracy of commonly used inflammatory parameters in clinics to diagnose postoperative infectious complications were examined. Furthermore, with the specific analysis of paired (primary tumors and metastatic lesions) PM patient samples, a significant longer disease free survival (DFS) and overall survival (OS) was noticed in the patient group with a higher number of intraepithelial CD8+ T-cells in the PM tumor than with a low number. This was the basis to further investigate HIPEC-mediated effects on CD8+ T-cell infiltration in a murine PM model. The results of these experiments illustrated, that the efficacy of HIPEC was dependent on the function and presence of CD8+ T-cells. Using colorectal cancer cell lines and patient-derived tumor organoids, it was noted that heated chemotherapy (in-vitro HIPEC treatment) treatment induced immunogenic changes via enhanced expression of MHC-class I molecules and cancer testis antigens (CTA). Such immunogenic changes initiated the maturation of monocyte-derived dendritic cells and subsequently the production of intracellular IFN-γ by CD8+ T-cells.

Overall, the work presented in this thesis might help patients suffering with PM by identifying post operation infections at an early stage using additional markers, overall reducing disease and surgery related complications. The work performed using experimental models show that HIPEC treatment seems to enhance immunogenicity of cancer cells making that can activate CD8+ T-cells. This mechanistic finding suggests that

in the future patients with PM might survive better if treated with immunotherapies after HIPEC treatment as immunotherapies are known to provide sustained T-cells activity.

III. Zusammenfassung

Die peritoneale Metastasierung (PM) entsteht durch verschiedene Primärtumore des Gastrointestinal Traktes und auch durch das Ovarialkarzinom der Frau. An unserem Departement behandeln wir am häufigsten PM vom kolorektalen Karzinom. Die Behandlung dieser Patienten hängt von mehreren Faktoren ab, unter anderem vom Ausmass der Erkrankung in der Bauchhöhle. Im Falle einer begrenzten Erkrankung kann eine zytoreduktive Operation (CRS) in Kombination mit einer hyperthermischen intraperitonealen Chemotherapie (HIPEC) indiziert werden. Das Konzept dieses Behandlungsansatzes besteht darin, die gesamte sichtbare Tumormasse während der CRS zu resezieren und die mikroskopischen Tumorreste durch die HIPEC-Anwendung zu beseitigen. Bei PM vom kolorektalen Typ werden zwei verschiedene Chemotherapie Regime eingesetzt. Die eine ist die alte Kombination aus Mitomycin C und Doxorubicin, die andere ist die Verwendung von Oxaliplatin. Aufgrund einer besseren Patientenselektion liegt das mediane Überleben der mit CRS/HIPEC behandelten Patienten bei etwa 50 Monaten. In dieser Kohorte gibt es interessanterweise einige, die ein Überleben von 8 Jahren und mehr aufweisen. Der Grund dafür ist bisher unbekannt. Aus klinischen Studien geht hervor, dass das Immunsystem eine bessere Tumorkontrolle hervorrufen kann, möglicherweise induziert die angewendete Chemotherapie eine protektive Immunreaktion.

Somit haben wir in dieser Arbeit den direkten und indirekten Effekt der HIPEC Behandlung auf das Immunsystem untersucht. Wir haben mit unseren zwei klinischen Arbeiten beschrieben, dass die HIPEC Behandlung eine systemische Entzündungsreaktion hervorrufen kann. Dies bedingte eine verminderte Testsicherheit der routinemässig analysierten Entzündungsparametern während des postoperativen Verlaufes zur Diagnose von infektiösen Komplikationen dieser Patienten. Das Grundlagenforschungsprojekt fokussierte sich auf den Effekt von CD8+ T-Zellen auf die PM Entwicklung und auf die HIPEC Behandlung. Mit der spezifischen Analyse von gepaarten (vom Primärtumor und von der PM Läsion desselben Patienten) PM-Proben von Patienten, die in unserer Abteilung behandelt wurden, konnten wir in der Patientengruppe mit einer höheren Anzahl von CD8+ T-Zellen im PM-Tumor ein signifikant längeres Überleben feststellen. Dies war die Grundlage, um den behandlungsbedingten Effekt von HIPEC auf die CD8+ T-Zell-Infiltration in einem murinen PM-Modell weiter zu untersuchen. Die Ergebnisse dieser Experimente zeigten, dass die Wirkung von HIPEC zu einem guten Teil auf der Funktion und dem Vorhandensein von CD8+ T-Zellen beruht. In einem letzten Schritt untersuchten wir die Mechanismen, welche möglicherweise dahinterstecken. Wir behandelten Krebszellen und Krebs-Organoide, etabliert von Patienten, in HIPEC ähnlichen Bedingungen und detektierten

dabei eine erhöhte MHC-I und CTA Expression. Weiter beobachteten wir in Zell-Co-Kulturexperimenten, dass die HIPEC Behandlung Veränderungen hervorrufen kann, welche zu einer Reifung von Monozyten führt, welche wiederum die IFN-γ Produktion von CD8+ T-Zellen auslöste.

Die Resultate dieser Arbeit sollen helfen postoperative Komplikationen früh zu erkennen, falls notwendig mit der zusätzlichen Bestimmung von anderen Entzündungsparametern um krankheitsbezogene und Chirurgie bezogene Komplikationen zu minimieren. Weiter ging aus den experimentellen Versuchen hervor, dass die HIPEC Behandlung die Immunogenität von Krebszellen erhöht und diese vulnerabler macht für die T-Zell mediierte Erkennung. Die Resultate suggerieren, dass PM Patienten von einer Immuntherapie nach der chirurgischen Behandlung, insbesondere nach HIPEC, profitieren könnten um eine langzeitige Kontrolle des Tumors zu erreichen.

1. Introduction

1.1 Cancer and its incidence

Cancer in general is an accumulation of aberrant cells, which have the capability to divide uncontrollably and have the ability to infiltrate and/or disrupt functions of normal body tissue. Every day, our body produces numerous aberrant cells, which are recognized by the immune system and destroyed. The development of a cancer involves complex mechanisms. An overview of these processes are summarized in the hallmark of cancer (Figure 1).

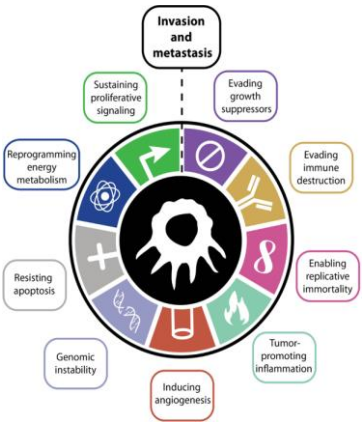

Figure 1: ***the hallmark of cancer illustrates the different processes involved in cancer development***
Figure adapted from: https://www.nature.com/articles/s41388-019-1110-1

Cancer is the 2nd leading cause of death in the world. In 2020, 19.3 million new cases were diagnosed with cancer and almost 10 million cancer related deaths could be counted[1]. Female breast cancer is the most common diagnosed cancer and surpassed lung cancer. The third common cancer is colorectal cancer with around 10% of the new diagnosed cancers worldwide. Lung cancer remained the leading cause for cancer related death. In Germany, it is estimated, that around 51% men, so every second men, and around 43% of women will develop cancer during lifetime[2]. In projection studies, the incidence of primary tumors will change until 2030 compared to 2020. For example, pancreatic cancer will surpass colorectal cancer and rank as the second leading cause for death by 2030[2].

1.2 Cancer and metastasis

Cancer has the capability to spread to other organs via the blood vessels, called hematogenous metastasis or via lymph vessels to form nodal metastasis or directly as it is the case for example for peritoneal metastasis. Cancer cells must undergo certain changes to leave the primary tumor, become motile and invade lymph or blood vessels to form metastasis in a distant organ. Furthermore, how, when and where cancer cells will metastasize, and the mechanisms involved in these processes at the molecular level between the primary tumor and the metastasis site is not yet fully understood. Studies have defined that cancer cells show multiple phenotypic changes, prime target organ for the seeding, initiate formation of new vessels and develop strategies to avoid the recognition by the immune system. Often, the cancer is diagnosed, when distant metastases are formed. Unfortunately, at this stage of the disease, cancer isn`t a local phenomenon anymore, it is considered as systemic disease. In that stage of the disease, cancer is not curable anymore. Therefore, the treatment approach is often systemic chemotherapy, radiotherapy or immunotherapy with the goal of palliation. Colorectal cancer often metastasizes to the liver via the hematogenous route or to the peritoneal

cavity via direct dissemination of the cells. Interestingly, in the setting of metastatic colorectal cancer, liver metastasis can still be a controlled via surgery can be offered as a treatment[3]; whereas, peritoneal metastasis is considered to be systemic and local treatment option in a curative intent can only be offered to selected patients.

1.3 Peritoneal metastasis development from colorectal cancer

The peritoneum is a serous membrane, made of 3-layers, the mesothelium, the basal lamina and the submesothelial stroma. It has several important functions for the abdominal cavity such as the facilitating of the movement of the intraabdominal organs and filtrating the peritoneal fluid[4]. Due to the huge size of the peritoneum, which is almost the size of the human skin, the peritoneal filter capacity can also be used for peritoneal dialysis in case of kidney failure. The peritoneum can be divided in visceral and parietal peritoneum. The visceral is the outer layer of the intraabdominal organs, whereas the parietal peritoneum covers the abdominal wall. Both, the parietal and the visceral peritoneum can harbour metastasis; these metastases are called peritoneal metastasis (PM). Peritoneal metastasis can arise from different gastrointestinal and gynaecological tumors [5,6]. Colorectal cancer, gastric cancer[7] and hepato-pancreaticobiliary[8] cancers are gastrointestinal cancers metastasizing to the peritoneum. Peritoneal tumors are most often metastatic lesions, but in rare cases tumors can also develop in the peritoneum, such as the malignant peritoneal mesothelioma[9].

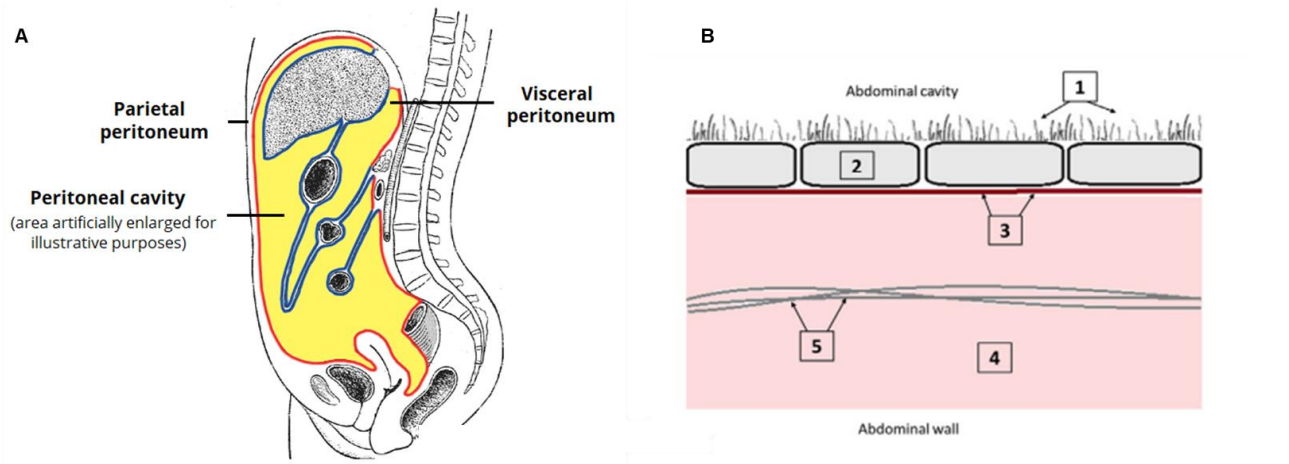

Figure 2*: the anatomy of the peritoneum and the histological structure* *(A) The parietal peritoneum covers the abdominal wall and the diaphragma and the visceral periotneum is the outer layer of the intraperionteal organs such as the liver, stomach, small intestin and spleen. (B) The histological structure of the periotneum is simple with 3 layers. The mesothelial cells (2), the basal lamina (3) and the submesothelial stroma (4,5). The glycocalyx (1) is the exracellular coating towards the abdominal cavity. Figure adapted from: https://teachmeanatomy.info/abdomen/areas/peritoneal-cavity/ (10.10.2022)*

Colorectal cancer is worldwide the 3rd most common cancer in women and men[10] and the 3rd leading cause for cancer related death[11]. In industrialized countries, colon cancer is more frequent than rectal cancer. Risk factors are genetic disorders such as the Lynch Syndrome or familial adenomatosis polyposis (FAP), smoking, lack of exercise, eating red meat, chronic inflammatory bowel diseases as Colitis ulcerosa[12] and medical interventions such as pelvic irradiation[13]. The development of colorectal cancer is a stepwise process involving

several genetic alterations and histological changes. The loss of APC function is common for almost all human colon carcinomatosis and the starting point of cancer development. APC is involved in the so-called wnt-signalling pathway and responsible for the β-catenin degradation. In case of a loss of APC function, β-catenin is not degraded and can initiate cell proliferation. This results in the formation of a polyp.

Around 50% of the patients have in addition an activation in the oncogene K-ras[14], which can then lead to the formation of an intermediate adenoma[15]. Additional genetic changes such as the loss of apoptosis gene p53 can then trigger the development from the adenoma into cancer. At least seven mutations occur during the adenoma-to-carcinoma transformation[16]. Each step of cancer development takes around 10 years and pre-cancerous lesions such as low-grade or high-grade adenomas can be detected during a colonoscopy. That`s why the colonoscopy is a recommended screening method for colorectal cancer from the age of 50 years without any inherited risk factors. This stepwise process of colorectal cancer formation is visualized in Figure 3.

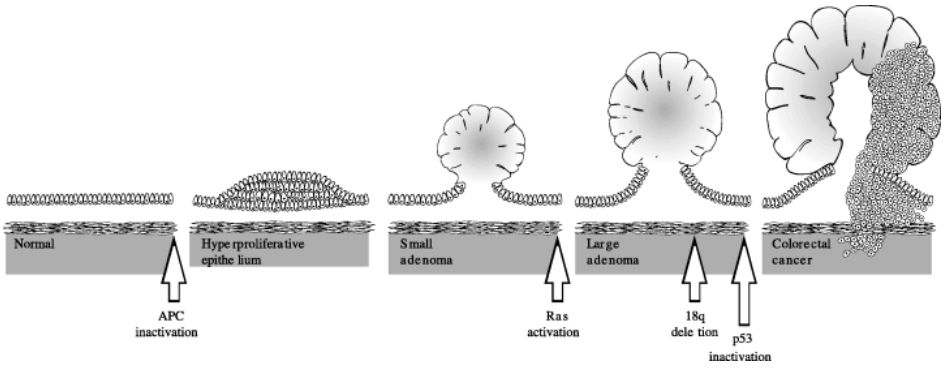

Figure 3: ***the stepwise progression of colorectal cancer:*** *the starting point is the loss of the APC function, which results in a hyperproliferative epithelium of the colon or retum. With additional mutation for example in the Ras oncogene, a small adenoma develops to a large adenoma. The loss of apoptosis gene p53 can then trigger the formation of cancer, which becomes invasive. Figure adapted from: https://epomedicine.com/medical-students/adenoma-carcinoma-sequence-in-colorectal-cancer-mnemonic/*

About 90 – 95% of colorectal cancers are adenocarcinomas, arising from the epithelial part of the colon wall[10]. Mucinous and signet-ring adenocarcinomas are subtypes and associated with a worse prognosis[17]. Non-epithelial tumors are for example gastrointestinal stromal tumors (GIST) or leiomyosarkomas.

The signs and symptoms of colorectal cancer can be very different or even absent. In that scenario, a screening colonoscopy will lead to the diagnosis. The classical symptoms of colon cancer are anaemia, bloody stool and/or abdominal pain. Depending on the size of the tumor, colon cancer can become apparent due to a malignant obstruction with or without bowel perforation. As soon as the diagnosis is made, the cancer disease needs to be classified according to the TNM-stage (8th version of the American Joint Committee on Cancer (AJCC)) as summarized in the Table 1[18,19].

T	
Tx	The primary tumor cannot be evaluated.
T0	No evidence of primary tumor
Tis	Carcinoma *in situ*: involvement of the *lamina propia* without extension to the muscularis of the muscosa.
T1	Invasion of the submucosa (through the muscular tissue of the mucosa but without involvement of the *muscularis propia*)
T2	Invasion of the *muscularis propia*
T3	Invasion through the *muscularis propia* twoards peridolorectal tissues
T4	Invasion of the visceral peritoneum or invasion or adherence to organs and adjacent structures.
T4a	Invasion through the visceral peritoneum (including thick perforation of the intestine and invasion by contiguity through areas of inflammation to the surface of the visceral peritoneum.)
T4b	Direct invasion or adherence to organs and adjacent structures.
N	
Nx	The regional lymph nodes cannot be evaluated
N0	No involvement of the regional lymph nodes
N1	Metastasis in 1–3 regional lymph nodes (tumoral involvement $\geq$ 0.2 mm) or the presence of tumoral deposits but all the lymph nodes identified are negative
N1a	Metastasis in one lymph node
N1b	Metastasis in 2 or 3 lymph nodes
N1c	Lymph nodes without tumoral involvement but with the presence of tumoral deposits in the subserous, mesenterium or non peritonealized pericolic tissues or in perirectal/mesorectal tissue.
N2	Metastasis in 4 or more regional lymph nodes
N2a	Metastasis in 4–6 regional lymph nodes
N2b	Metastasis in 7 or more regional lymph nodes
M	
M0	No distant metastasis found in complementary studies
M1	Metastasis in 1 or more localizations or distant organs or peritoneal metastases
M1a	Metastasis in 1 localization or organ without peritoneal disease
M1b	Metastasis in 2 or more localizations or organs without peritoneal disease
M1c	Metastasis on the peritoneal surface, alone or in association with dissemination in other localizations or organs

Table 1: ***the current TNM classification from colon cancer:*** *T stands for the invasion depth of the primary tumor into the colon wall, N for the nodal involvment and M for distant metastasis. Table adapted from: https://www.researchgate.net/figure/American-Joint-Committee-on-Cancer-AJCC-staging-system-for-colorectal-cancer-29_tbl1_236636422*

This classification is important, because the stage of the cancer disease has an influence on the outcome and on the treatment. The five year relative survival of colon cancer patients remains relatively poor with 64.7% and is dramatically low in a metastasized situation which is below 20%[11]. These numbers illustrate the huge difference in prognosis depending on the presence of distant metastasis. In colorectal cancer, the metastatic routes are hematogenous to the liver and the lungs or directly to the peritoneal cavity. Lymphatic metastases are found in draining lymph nodes next to the tumor and summarized in the N-stage of the TNM classification. The presence of lymphatic metastasis has a better prognosis, than distant metastasis. 21% of the colorectal cancer patients present with a metastatic disease at the time-point of diagnosis [20,21]. The most common localisation for metastasis is the liver. Rectal cancer patients present more often lung metastasis or liver and lung metastastis[20]. The involvement of distant organs is summarized in the M-stage of the TNM classification. If a metastasis is present, this is a stage IV (UICC) cancer, another tumor classification, and often considered as palliative scenario with distinct exceptions regarding liver metastasis, as previously described.

Even though, the most common localisation for metastasis is the liver, the peritoneum is often involved organ for distant metastasis through yet unclear mechanism. Approximately 25% of patients with CRC have or develop peritoneal metastasis. Similar to a hematogenous metastasis, cancer cells must undergo certain changes to become invasive and motile. Colorectal cancer cells lose their epithelial phenotype and gain a mesenchymal-like phenotype, which is called the Epithelial to Mesenchymal transition (EMT)[22]. In contrast to hematogenous metastasis, the cancer cells forming peritoneal metastasis will not need to enter the vessels.

Peritoneal metastasis can arise directly from single cells of the primary tumour or from so-called tumour spheroids with inverted polarity (TSIPs)[23]. The initial process of exfoliation is initiated by the downregulation of several adhesion molecules, like E-cadherine, selectins, CD44 and various leukocyte-associated antigens[24]. This is part of the EMT process and allows cancer cells to become motile. The intraabdominal spread follows the physiologic route of the peritoneal fluid flow. Most cancer cells seed therefore on the omentum, in the pelvis and the subdiaphragmatic space to form peritoneal metastasis[25]. The attachment to the mesothelial cells is mediated by adhesion molecules as ICAM-1, PECAM-1 and VCAM-1[24]. The further invasion into the submesothelial layer is also promoted by mesothelial cells. Interestingly, colorectal metastasis recapitulates the morphology and differentiation of their primary tumor, the so-called Mesenchymal to Epithelial transition (MET).

The occurrence of PM can either be synchronous in 5-10% of the patients or metachronous (> 6 months after diagnosis of the primary tumor[26]). The clinical presentation of patient with PM can vary from an emergency due to a bowel perforation or become apparent due to the formation of ascites. Further, the diagnosis can be made during a laparoscopy as an accidental finding or during a staging laparoscopy or as a finding during the staging process of a colorectal cancer. As soon as the diagnosis is made, patients are discussed at an interdisciplinary tumor board to decide for the best treatment strategy.

1.4 Treatment options for peritoneal metastasis from colorectal cancer

Patients with PM from CRC have limited treatment options [27]. The treatment option ranges from systemic chemotherapy application to two local approaches: CRS/HIPEC and PIPAC. The majority of the patients are treated with systemic chemotherapy. However, selected patients may qualify for a radical local treatment options that includes cytoreductive surgery (CRS) together with hyperthermic intraperitoneal chemotherapy (HIPEC). CRS/HIPEC is indicated in a curative treatment approach. Whereas pressurized intraperitoneal aerosol chemotherapy (PIPAC) is another form of intraperitoneal chemotherapy application without surgical tumor resection and therefore always in a palliative intention. This treatment can be repeated for several times and also be indicated for advanced peritoneal cancer disease.

A lot of clinical research was performed to characterize different criteria's, which are for example summarized in the BIOSCOPE score to identify patients, who will profit from CRS/HIPEC[28]. The BIOSCOPE score consists of four different categories such as the peritoneal cancer index (PCI), the nodal status (N-status), the differentiation status of the tumor (G-status) and the RAS/Raf mutation status. The higher this score, the worst

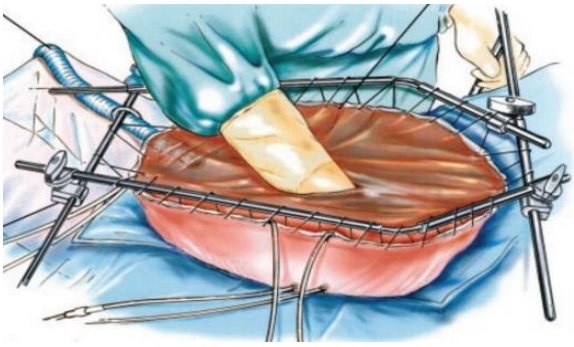

Figure 4: ***HIPEC application in an open coliseum technique:*** *the abdominal wall of the patients forms the coliseum and is filled with heated chemotherapy. Figure adapted from: https://theoncologist-onlinelibrary-wiley com.ezproxy.uzh.ch/doi/pdfdirect/10.1634/theoncologist.2008-0275*

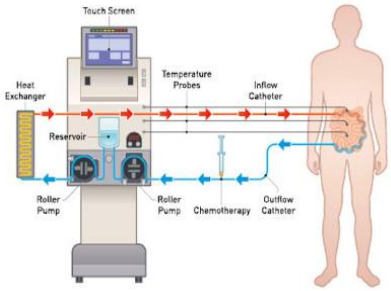

Figure 5: ***the pump system involved in HIPEC treatment.*** *The heated chemotherapy is under constant flow and a heat exchanger controls the temperature of the fluid and heats it up. Figur adapted from: https://www.foxchase.org/blog/2015-01-23-a-new-procedure-called-hipec*

the prognosis for the patients. The PCI is an important criterion and reflects the extent of the cancer disease intraperitoneal.

During CRS procedure, the macroscopic tumor mass is resected. After this extensive surgical procedure, hyperthermic intraperitoneal chemotherapy (HIPEC) is applied as shown in Figure 4. HIPEC is a local process where heated chemotherapies are circulated in an open abdomen setting using perfusion pumps, as illustrated in Figure 5. The concept behind the HIPEC treatment is to eradicate remnant microscopic tumor cells or clusters. The idea of heating up the chemotherapy is to increase the tissue penetration of the chemotherapeutic agent and to potentiate the cytotoxic effect of chemotherapeutics[29].

For PM from CRC two different regimens are often used:

1. MitomycinC/Doxorubicin for 90 minutes at 42°C

2. Oxaliplatin for 30 minutes at 43°C

The CRS/HIPEC approach provides significant survival benefit for these patients with up to 50 months mOS[30]. On the opposite of the survival benefit is the risk for complications from this very invasive procedure. This factor needs to be balanced, because the occurrence of a major complication is associated with an impaired OS[31]. The most severe complications after CRS/HIPEC are sepsis and infection[32]. Therefore, it is crucial to recognize infectious complications early during the postoperative course of these patients. Surgical patients are monitored during the postoperative course via clinical examinations and regular blood tests of inflammatory markers such as C-reactive protein (CRP) and white blood cells (WBC). Elevations of these markers often serve as early sign for an infectious complication. We detected two important findings studying postoperative blood samples of patients after CRS/HIPEC:

1. The application of a prolonged HIPEC protocol (Mitomycin C/Doxorubicin) leads to an increase of CRP between 5 - 8 days postoperatively without any underlying infectious complication[33]. This observation suggests

that the HIPEC procedure seems to induce systemic changes in patients, even though it is considered as local treatment.

2. WBC`s don`t increase after the use of a prolonged HIPEC regimen in case of an infectious complication and CRP is unspecific in the diagnosis of an infectious complication [34].

This illustrates a clear limitation of these markers after CRS/HIPEC. The knowledge of these physiological changes, especially of CRP, is very important to consider by every surgeon performing this procedure.

The overall limitation of CRS/HIPEC is the recurrence of the cancer disease. Work from our lab performed by Breuer et al. described for the first time the importance of the recurrence localization, where most cases recur in the peritoneal cavity, resulting in dramatically impaired survival compared to the recurrence in the liver or lung[30]. The recurrence in the peritoneum could be due to limited efficacy of the HIPEC treatment. However, in the Zurich cohort of CRS/HIPEC treated patients, few patients showed an unexpected long-term survival of up to 7 – 8 years. Why some PM patients show long-term survival after CRS/HIPEC remains unclear. However, it is tempting to assume that systemic inflammatory changes can induce protective immunity. One experimental study claimed the induction of a protective immune response by HIPEC[35] and certain chemotherapies (Oxaliplatin) used in HIPEC are known to be immunogenic[36,37].

Another local treatment option for PM from different primary tumors is PIPAC therapy. In contrast to the HIPEC treatment, PIPAC is usually applied several times via small abdominal incisions as performed for a laparoscopy. And PIPAC can also be indicated in advanced situations, in which a resection is technically not possible anymore. The goal is to achieve a tumor load reduction or a tumor growth control with the local application of chemotherapy. The performance of PIPAC treatment is still within clinical trials, because the outcome of the treatment needs still to be investigated[38,39].

1.5 The immune system in cancer

The immune cells can be classified as part of the innate (neutrophils, macrophages and dendritic cells) and the adaptive (Lymphocytes such as T, B and NK cells) immune system. The innate immune system recognizes pathogens in a non-specific manner. Wheras the adaptive immune system is highly specific to a pathogen. In the context of tumors, many studies have shown that CD8+ T-cells are important adaptive immune cells that control tumor development. Basically, CD8+ T-cells, which are called naïve CD8+ T-cells before interacting with an antigen, need 3 signals to get activated and become an effector CD8+ T-cell: **first**: T-cell Receptor (TCR) - antigen interaction, **second:** costimulation, including checkpoint – inhibition/activation, **third:** differentiation via cytokines. In reality, this is a complex process between different cells of the immune system. The antigen presentation is done by antigen presenting cells (APC). To interact with specific T-cells, antigens

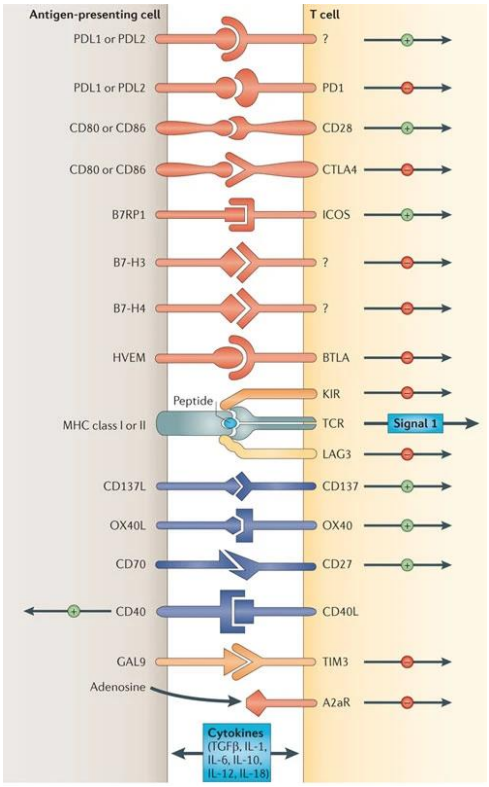

Figure 6: ***checkpoint signals of T-cell activation:*** *these are just a few examples of checkpoint molecules between and APC and the T-cell. Signal 1 with MHC-class I molecule, antigen and TCR interaction is as well shown as the third signal (cytokines). Figure adapted from: https://www-nature-com.ezproxy.uzh.ch/articles/nrc3239/figures/1*

(proteins) need to be processed into smaller pepetides and bound to MHC-I molecule of an APC. Various cells can act as APC`s, typically dendritic cells (DC`s) present antigens and belong to the innate immune system. Once, the antigen is presented, the CD8+ T-cell with the specific TCR to that antigen can bind it and get in contact with for example the cancer cell. This is the first signal of acitvation. Costimulatory molecules get activated or will prevent the activation of the CD8+ T-cell. This part is also known as checkpoint acitivation or inhibition[40] and is in physiologic condition important to prevent the overstimulation of the specific immune response. In the context of cancer, these costimulatory molecules, such as PD-L1, can be expressed by the cancer cells or the antigen presenting celsl that can interact with PD-1 on the CD8+ T-cell to block its activation. Another checkpoint inhibition occurs between CD80/CD86 and CTLA-4. The clinical relevance of the CD8+ T-cell activation control in particular became apparent with the first study of testing Ipilimumab (monoclonal antibody against CTLA-4) in patients with metastazied melanoma. Patients treated with Ipilimumab alone or in combination with gp 100, a well studied cancer vaccine inducing limited antitumor activity, were compared to the administration of gp 100 alone. The OS was 10.0 months in the Ipilimumab groups compared to 6.4 months in the gp 100 group alone[41]. This is a surprising result in such an end-stage metastatic cancer disease and demonstrates the impact of tumor control by CD8+ T-cells.

1.6 Immune interactions within the tumor microenvironment

Tumor control by the immune system isn`t a new phenomenon. Already in 1909Paul Ehrlich described that the immune system could control tumor development. Later, Brunet and Thomas made an unproven claim that lymphocytes can eliminate transformed cancerous cells. Robert Schreiber coined the term the 3 E`s. The 3 E concept of the immunoediting process stands for Elimination, Equilibrium and Escape as shown in Figure 7.

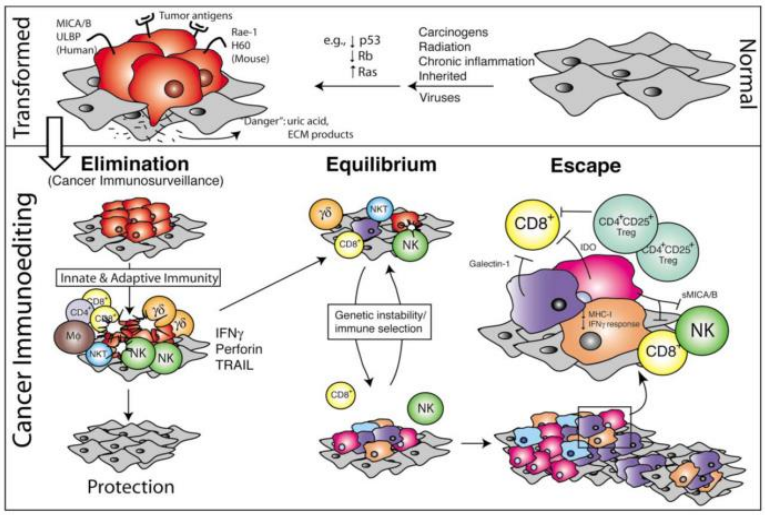

Figure 7: ***the concept of 3 E with elimination of cancer cells, equilibrium and escape.*** *Figure adapted from: https://www.healio.com/hematology-oncology/learn-immuno-oncology/cancer-and-the-immune-system-history-and-theory/immuno-oncology-theories-immunoediting-and-immune-surveillance*

During the Elimination phase, cancer cells or aberrant cells are recognized by the immune system and are eliminated. The switch from the Elimination phase to the Equilibrium allows cancer cells to survive under the control of the immune system. The tumor can remain in this dormant state for many years. However, through this constant pressure from the immune system, cancer cells gain strategies to escape the immune control and become clinically apparent cancer. Such strategies include the decrease of MHC-I molecule expression, enhanced production of collagen IV to create a mechanical barrier towards the immune system. Thus, it is important to use strategies to reprogram the immune system and convert the clinical apparent phase (Escape) to the Equilibrium or better to the Elimination phase. Another mechanism to avoid the recognition by the immune system is the enhanced expression of checkpoint molecules such as PD-L1 on the surface of cancer cells or APC`s.

In general, the effect of immunotherapy depends on the immunogenicity of the cancer and the number of CD8+ T-cells infiltrating the tumor. The malignant melanoma is for example known to be a very immunogenic tumor, due to frequently occurring neoantigens[42]. Neoantigens arise from tumor – specific mutations and can be recognized by CD8+ T-cells[43]. The formation of neoantigens varies between different primary tumors and is graphically illustrated in Figure 8.

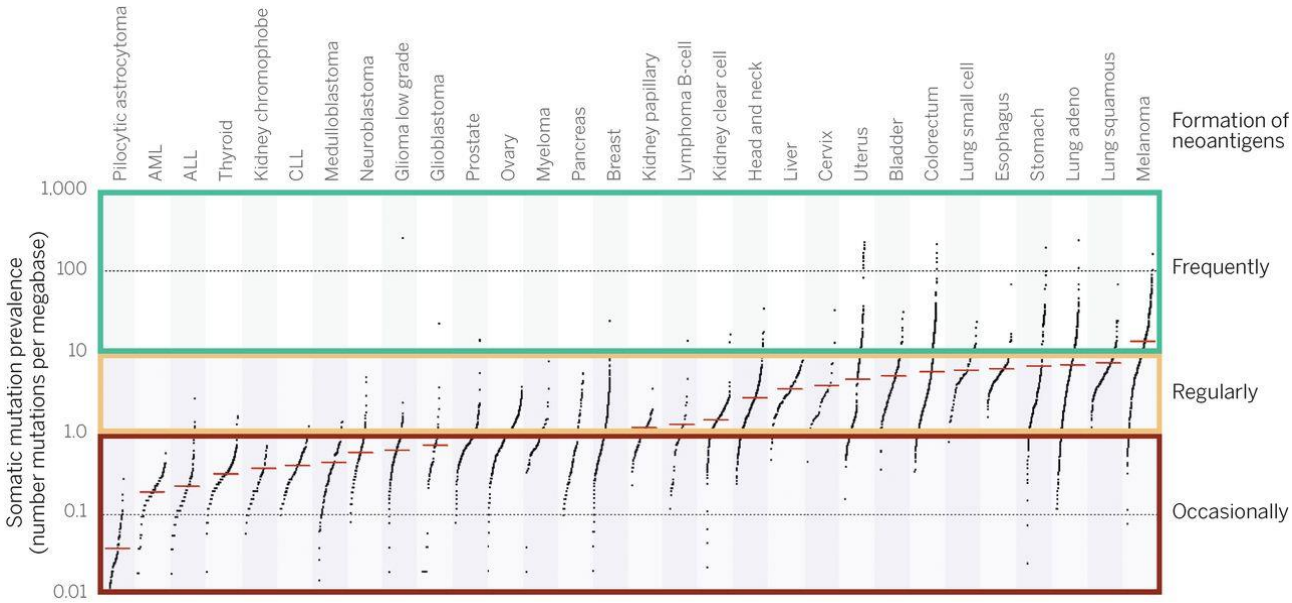

Figure 8: ***the formation of neoantigens correlates with the somatic mutation prevalence and these differs between the primary tumors.*** *Figure adapted from: https://www-science-org.ezproxy.uzh.ch/doi/full/10.1126/science.aaa4971*

In contrast to melanoma, colorectal cancer was long-time considered to be a poor immunogenic cancer. Especially, with the description of an important molecular phenotype of CRC, namely the defect in mismatch repair proteins, it became evident, that microsatellite instable (MSI) colorectal cancers are different from the microsatellite stable (MSS) tumors with regard to prognosis, neoantigen formation and tumor infiltrating lymphocytes. Approximately, 15% of CRC harbour a sporadic or hereditary defect in the mismatch repair proteins This can either be caused by a gene silencing of MLH 1 in sporadic MSI CRC, or by a sporadic germline mutation of MLH 1 and 2 in the Lynch Syndrome (hereditary). The expression of more neoantigens is associated with a higher degree of tumor infiltrating lymphocytes leading to a better immunological recognition of cancer cells, resulting in an improved CRC-specific survival[44,45]. The variety of morphological and molecular differences in colorectal cancer is huge and led to the definition of the consensus molecular subtypes of colorectal cancer. This classification summarizes 4 different categories, which are associated with different prognosis due to different molecular patterns. Roughly 13% of CRC cannot be categorized with the CMS classification score. The score is illustrated in the Figure 9 and shows the impact on survival.

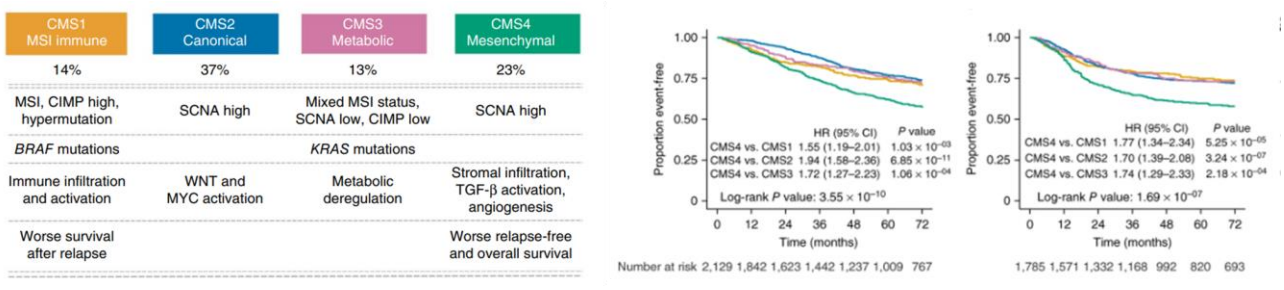

Figure 9: ***the consensus molecular subtypes of CRC****: the mesenchymal type was associated with the worst overall survival (left Kaplan-Meier plot) and the worst relapse free survival. Figures adapted from: https://www-nature-com.ezproxy.uzh.ch/articles/nm.3967/figures/5, https://www-nature-com.ezproxy.uzh.ch/articles/nm.3967/figures/4*

The role of CD8+ T-cells in metastatic CRC is poorly investigated. It has been shown that patients with a higher CD8+/CD3+ ratio in liver metastasis from CRC have a better DFS and OS[46]. The role of immune cells in peritoneal metastasis is not yet clear. Seebauer et al. claimed a functional reorganization of the tumor microenvironment of peritoneal metastasis with an increased number of cytotoxic natural killer (NK) cells[47]. In contrast, Halama et al. assessed the number of NK cells in primary colon cancer and liver metastasis. They compared the results to normal mucosa and normal liver tissue and detected a significantly reduced number of infiltrated cells in the tumor tissue. They also reported significantly more T-cells in the tumor tissue. So, that they concluded an impaired NK cell migration into CRC tumor, whereas the T-cell migration is not affected[48]. Nevertheless, the prognostic impact of CD8+ T-cells in PM lesions from CRC is not yet clear.

The analysis of 43 PM samples from gastric cancer showed lower numbers of CD8+ T-cells, NK cells and myeloid DC`s in advanced (G2 and G3) and histologically aggressive stages (signet ring vs non-signet ring)[49]. These findings can be interpreted as immunosuppressive tumor microenvironment to facilitate tumor progression. Another common primary tumor metastasizing to the peritoneum is ovarian cancer. The peritoneal tumor dissemination from serous ovarian cancer was proposed to categorize into miliary and non-miliary spread[50]. The non-miliary form was associated with a longer survival and more CD8+ T-cells with a higher expression of PD-1, indicating an activated specific immune response[51].

1.7 Chemotherapeutic drugs and immunity

Chemotherapeutic drugs used in the HIPEC setting are either the combination of Mitomycin C/Doxorubicin or Oxaliplatin. Mitomycin C is an intercalating cytostatic and thus binds the two DNA strands covalently, making replication and transcription impossible leading to apoptosis. Doxorubicin belongs to the chemotherapeutic class of anthracyclines. The mechanism, how Doxorubicin acts is not fully understood. It is also an intercalating molecule, which blocks DNA and RNA synthesis. Furthermore, Doxorubicin forms a ternary complex with Topoisomerase IIβ and thus prevents the repair of double-strand DNA breaks, which leads to apoptosis. And Oxaliplatin is an alkylating agent. It binds mostly to Guanin- and Cytosin- units in the DNA[52]. This results in a cross-linking of the DNA and prevents DNA replication and transcription. Oxaliplatin induces therefore also apoptosis. Most chemotherapeutic drugs mediate their effect via apoptosis, which was considered to be a non-inflammatory process or in case of apoptosis induction in immune cells, an immunosuppressive effect. However, certain clinically relevant chemotherapeutic drugs, such as Doxorubicin, Oxaliplatin, Epirubicin, Cyclophosphamide[53] are known to induce immunogenic cell death (ICD).

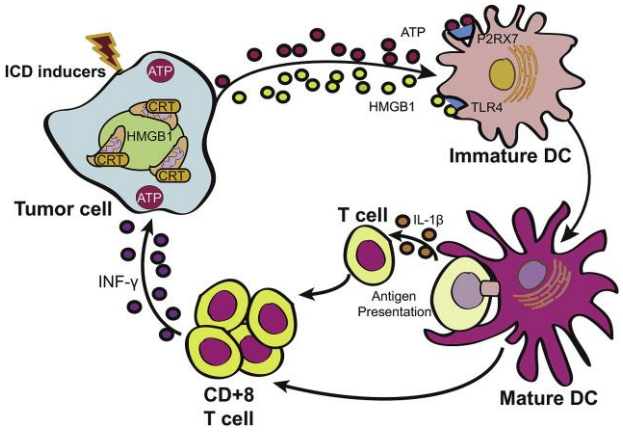

Figure 10: ***immunogenic cell death can be induced by certain chemotherapies.***
Figure adapted from: *https://ars-els-cdn-com.ezproxy.uzh.ch/content/image/1-s2.0-S030438351830555X-gr1_lrg.jpg*

Dying cancer cells release antigenic molecules such as Calreticulin (CRT), HMGB-1 and ATP. These molecules activate Toll-like receptor 4 (TLR-4) on DC`s. Which leads to antigen uptake by DC`s and DC maturation. Chemotherapeutic agents can not only induce immunity via ICD. For example, Oxaliplatin in combination with Cyclophosphamide (Oxa-Cyc) can act as immune sensitizer to checkpoint blockade therapy. This immunogenic combination resulted first in the delay of tumor progression in a KP (**K**RAS and T**P**53 mutated) lung tumor model. This effect was based on significantly more CD8+ T-cells in the tumor. Interestingly, the combination of chemotherapy Oxa-Cyc with checkpoint blockade resulted in a dramatic reduction of the lung tumor mass[54]. One finding, that HIPEC could have an influence on immunity was described by Zunino B. et al. They demonstrated the impact of HIPEC inducing a specific immune reaction via the exposure of heat shock protein 90 (Hsp 90)[35].

2. Aims of the thesis

The aim of this thesis was to characterize inflammatory responses after HIPEC using patient samples. Furthermore, experiments were used to understand the protective systemic and local influence of HIPEC treatment. To do so, the following aims were pursued:

Aim 1: To describe HIPEC mediated changes on inflammatory markers and the consequently influence on the accuracy to diagnose postoperative infectious complications.

Aim 2: To characterize the influence of CD8+ T-cells on PM development in human patients.

Aim 3: To elaborate HIPEC-mediated effects on immune cells and on the immunogenicity of cancer cells and patient derived tumor organoids.

3. Results

The goal of our first publication was to elaborate the dynamics of CRP during the postoperative course from 140 patients undergoing CRS/HIPEC. We detected a significant increase of CRP between postoperative day 5 and day 8 without any underlying infectious complication, after the use of a prolonged HIPEC protocol (for 90 minutes). This phenomenon suggests a systemic inflammatory response, which could be confirmed by measuring another inflammatory marker such as pancreatic stone protein (PSP). Interestingly, the load of bacterial DNA in the peripheral blood of patients after the use of a prolonged HIPEC protocol was significantly higher. This could be due to bacterial translocation from the gastrointestinal tract provoked by the longer application of heated chemotherapy.

3.1 Systemic inflammatory response after hyperthermic intraperitoneal chemotherapy (HIPEC): The perfusion protocol matters!

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European Journal of Surgical Oncology xxx (xxxx) xxx

Contents lists available at ScienceDirect

European Journal of Surgical Oncology

journal homepage: www.ejso.com

Systemic inflammatory response after hyperthermic intraperitoneal chemotherapy (HIPEC): The perfusion protocol matters!

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ARTICLE INFO

Article history:
Received 24 February 2019
Received in revised form
17 March 2019
Accepted 26 March 2019
Available online xxx

Keywords:
Cytoreductive surgery (CRS)
Hyperthermic intraperitoneal chemotherapy (HIPEC)
Peritoneal metastasis

ABSTRACT

Background: CRS/HIPEC gained acceptance as a treatment for selected patients with peritoneal metastasis. However, the pathophysiology behind HIPEC is poorly understood, and a variety of regimens are currently in use. In this study, we describe for the first-time changes in the postoperative systemic inflammatory reaction, highly different among HIPEC treatment protocols.
Methods: HIPEC was performed with three protocols, different with regard to perfusion times and drugs: (mitomycinC/doxorubicin, 90min), (cisplatin, 90min) (oxaliplatin, 30min). Serial blood samples were assessed for C-reactive protein (CRP), white blood cells (WBC), pancreatic stone protein (PSP) and bacterial component (16s rDNA). The study was approved by the local ethics committee and registered at clinicaltirals.gov (NCT02741167).
Results: Overall, 140 patients from two European centers were included. In patients without postoperative complications, a secondary peak of inflammatory parameters, CRP ($p = 0.015$) and PSP ($p = 0.004$) was observed after HIPEC for 90 min with mitomycinC/doxorubicin or cisplatin but not after 30 min oxaliplatin. In patients after 90 min HIPEC, postoperative serum bacterial 16srDNA level were 2.1 times higher (95% CI 0.646–3.032, $p = 0.015$) compared to 30 min oxaliplatin.
Discussion: In conclusion, we identified a secondary inflammatory reaction after 90 min HIPEC, either with mitomycinC/doxorubicin or cisplatin, not observed after short course HIPEC with oxaliplatin. This protocol dependent physiology of acute phase proteins should be known in the clinical management of patients after HIPEC.

Introduction

Peritoneal metastasis (PM) occurs from many gastrointestinal tumors, e.g. colorectal cancer or appendix cancer, and has an inferior prognosis than metastasis to the liver or lungs [1]. Cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC), together with multimodal systemic treatment, has become a valuable option for selected patients [2–4] translating into considerable survival benefits [5,6]. There is good *in-vitro* data about cytotoxic effects of HIPEC on cultured colorectal cancer cells [7], and data in humans show favorable pharmacokinetic effects for HIPEC. In contrast, specific effects of HIPEC on patient physiology and the postoperative course are poorly explored. In addition, existing HIPEC protocols differ with regard to drugs, temperatures and treatment duration. For example, in patients with colorectal PM, distinct HIPEC protocols are currently in use. One protocol, initially developed by Sugarbaker et al., is mitomycinC-based, and used for 90 min at 42 °C, currently preferred by many US centers [8], while a majority of European centers use a shorter protocol for 30 min at 43 °C which is oxaliplatin-based, and was originally published by French groups [9]. So far, no difference regarding overall survival between the two protocols has been shown in retrospective studies [10], and the choice for a specific protocol is center dependent. Currently, the main argument for the majority of surgeons preferring the French

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https://doi.org/10.1016/j.ejso.2019.03.036
0748-7983/

Please cite this article as: Roth L et al., Systemic inflammatory response after hyperthermic intraperitoneal chemotherapy (HIPEC): The perfusion protocol matters!, European Journal of Surgical Oncology, https://doi.org/10.1016/j.ejso.2019.03.036

Abbreviations

CRS	Cytoreductive surgery
HIPEC	Hyperthermic intraperitoneal chemotherapy
PCI	Peritoneal cancer index
CC-Score	Completeness of cytoreduction score
CRC	Colorectal carcinoma
CRP	C-reactive protein
PSP	Pancreatic stone protein
WBC	White blood cells

protocol is the shorter perfusion time compared to the US protocol. Many other protocols exist, for example with cisplatin, which is frequently applied during 90 min at 42 °C and used for PM from ovarian cancer or peritoneal mesothelioma. Cisplatin is an alkylating agent like oxaliplatin, while the perfusion time and the applied temperature is similar to the mitomycin C protocol.

CRS/HIPEC induces complex physiological changes in patients, particularly during the operation and in the early postoperative phase [11]. During HIPEC, absorption of chemotherapeutic agents may systemically affect WBC counts [12–14]. In addition, local heat exposure and chemotherapy can induce direct toxic damage to abdominal organs with so far unknown effects on a patient's physiology [15]. This knowledge about HIPEC is currently not available but may help to improve efficacy without increasing risks of HIPEC in the near future. Here, we aimed to assess the systemic inflammatory response in patients after CRS/HIPEC without postoperative complications.

Material and methods

Patients & ethics

Patients from two centers (Zurich, Switzerland, and Vienna, Austria) treated with CRS/HIPEC for malignant gastrointestinal tumors between 2009 and 2017 were included in this study. Patient data were collected retrospectively (n = 42) between 2009 and 2015, and within a prospective protocol (n = 98) between 2015 and 2017. The study protocol was approved by the ethical committee and registered at clinicaltirals.gov (NCT02741167).

Treatment

Patients were discussed at interdisciplinary tumor boards after exclusion of extra-abdominal tumor manifestations by 18FDG-PET/CT or thoracic-abdominal CT. Patients received standard of care pre- and postoperative chemotherapy according to tumor entity and international guidelines. Anesthesia was conducted with propofol and volatile anesthetics combined with thoracic epidural anesthesia as described previously [11]. CRS was performed according to international standards and defined as radical (CC-Score 0) if no macroscopic residual tumor was visible [16]. HIPEC for appendix and colorectal tumors was performed using peritoneal dialysis solution for mitomycinC (30 mg/m^2 body surface area, BSA according to the Mosteller formula) in combination with doxorubicin (15 mg/m^2 BSA) for 42 °C for 90 min, or oxaliplatin (300-400 mgm^2 BSA) as single agent at 43 °C for 30 min. The type of protocol used for appendix or colon cancer was changed in both centers in 2016 from the mitomycinC/doxorubicin protocol to the oxaliplatin protocol, which was then consistently used for these primary tumors. Patients with mesothelioma or ovarian cancer were treated with cisplatin-based HIPEC (75 mg/m^2 BSA) for 90 min at 42 °C.

Clinical parameters

Patients after CRS/HIPEC were visited daily by the operating surgeon according to standard clinical routine. In case of clinical symptoms or signs of infection, blood, urine and central catheters tips (jugular or subclavian) were taken for cultures. Imaging studies, usually an abdominal CT, were performed if CRP levels increased >30% after postoperative day 4. Complications were graded according to the Clavien-Dindo classification [17], while infectious complications were defined according to the Center for Disease Control and Prevention (CDC) definitions [18].

Serum probes

C-reactive protein (CRP), white blood cell (WBC) counts, pancreatic stone protein (PSP) were measured in blood samples by the clinical laboratory service on a daily routine basis prior to surgery and for the 14 consecutive postoperative days [19]. PSP was measured from frozen serum samples with enzyme-linked immunosorbent assays (ELISA) as previously reported [20]. DNA was extracted from fresh frozen serum samples using the DNeasy Blood and Tissue Kit (Qiagen). Quantitative polymerase chain reaction (qPCR) with TaqMan (Pa04230899_s1) was performed to assess bacterial components (16srDNA) [21].

Statistical analysis

Continuous variables were compared with the student t-test, the Mann–Whitney U or the Wilcoxon test, where appropriate. Fischer's Exact tests was used to compare differences among proportions derived from categorical data. Normally distributed data are shown as mean ± SD, non-normal variables as median and interquartile range (IQR). Missing values in the dataset were excluded. All p values were two-sided and considered statistically significant if $p \leq 0.05$. Statistical analysis was performed using SPSS version 25 and GraphPad Prism version 7.0.

Results

Demographic data

Overall, n = 140 patients (n = 91 from Zurich, n = 49 from Vienna) were included in the analysis. Patient characteristics are summarized in Table 1. Patients after HIPEC with oxaliplatin (n = 44) were compared to patients after HIPEC with mitomycinC/doxorubicin (n = 53) or cisplatin-based protocol (n = 43). The mitomycinC/doxorubicin protocol and the oxaliplatin protocol were applied to similar types of primary tumors. Cisplatin-based HIPEC was primarily performed for mesothelioma or ovarian cancer. Detailed patient characteristics and differences between the treatment groups are summarized in Supplementary Table 1.

Prolonged HIPEC with mitomycinC/doxorubicin or cisplatin induces an unspecific secondary C-reactive protein peak

We expected to find a similar level of postoperative inflammation among protocols in patients without postoperative complications, However, in patients after CRS/HIPEC without any complication, we identified an unspecific secondary increase of CRP after HIPEC with mitomycinC/doxorubicin or cisplatin at 42 °C for 90 min. This CRP increase was significant for mitomycinC/doxorubicin (p = 0.015), and cisplatin (p = 0.026) (Fig. 1A and B). This effect was not observed in patients undergoing 30 min HIPEC with oxaliplatin at 43 °C, where CRP levels gradually declined and

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Table 1
Patient characteristics.

	All patients (n = 140)
Age	56 (47–64)
Sex (male/female)	86 (61.4%) 54 (38.6%)
Primary tumor	
Colorectal	54 (38.6%)
Appendix tumors	35 (25%)
Mesothelioma	10 (7.1%)
Others	41 (29.3%)
PCI	6 (3–14)
Operation time (min)	360 (291.25–449.5)
Anastomosis (number)	1 (0–1)
ICU stay (days)	1 (1–2.75)
Hospital stay (days)	15 (12–20.75)
Complications	
none	85 (60.7%)
Major complications (Clavien-Dindo ≥ IIIb)	9 (6.4%)
Mortality (Clavien-Dindo Grade V)	1 (0.7%)

Patient characteristics are shown for the entire cohort and reported as median ± IQR.

returned to almost normal reference values within the first 14 postoperative days (Fig. 1C).

Pancreatic stone protein confirms the presence of an inflammatory trigger

Pancreatic stone protein (PSP) is an acute phase protein produced in the pancreas after a septic stimulus [22]. Serum levels of pancreatic stone protein (PSP) dramatically increased in patients after HIPEC with the mitomycinC/doxorubicin protocol (p = 0.004) and cisplatin-based HIPEC (p = 0.031) between day 2 and 6, in contrast to patients after HIPEC with the oxaliplatin protocol, where no increase was observed (Fig. 2A–C). The secondary rise of CRP (generated in the liver) and PSP (produced in the pancreas upon inflammation) suggest a triggering source from the gastrointestinal tract.

Bacterial components identified as a potential triggering source of inflammation

To explore the trigger of this secondary inflammatory reaction, we analyzed fresh frozen serum samples for the presence of bacterial components. Bacterial 16srDNA levels from patients with septic complications served as positive controls. The relative amount of bacterial DNA (16s rDNA) was 2.1 times higher (95% CI 0.646–3.032, p = 0.015) in patients after HIPEC with the mitomycinC/doxorubicin protocol compared to the oxaliplatin protocol (Fig. 3). In line with this finding, the relative bacterial components were 2.5 times higher (95% CI 0.567–4.85, p = 0.015) after the treatment with the cisplatin-based protocol compared to the oxaliplatin protocol, suggesting prolonged perfusion times as the triggering factor.

White blood cells (WBC) increases after platin-based HIPEC

Median WBC counts remained within a normal range of 5–10 G/l among all three HIPEC regimens. However, a secondary WBC increase could be observed between day 4 and 7 after platin-based HIPEC (Fig. 4B and C) treatment in patients without any complications. WBC did not change after mitomycinC/doxorubicin HIPEC treatment, even though these patient population showed a secondary CRP increase.

Discussion

Our study identifies novel findings related to the pathophysiology of acute phase proteins in patients after HIPEC. For the first time, we describe a secondary inflammatory reaction, associated to the presence of bacterial components in the systemic circulation after CRS/HIPEC. The data provides insight into the human pathophysiology after HIPEC which is necessary to understand the physiology and manage the postoperative course. In the future, it will help to direct future modifications of the HIPEC procedure.

Our finding of an unspecific secondary inflammation phase after HIPEC was unexpected. First, we thought about an influence of major surgery or the postoperative management, but a comparison with open colorectal and open gastroesophageal surgery (data not shown) highlighted an impact of HIPEC itself. To our surprise this secondary inflammatory peak was also not observed after HIPEC with oxaliplatin. Since these patients shared surgical characteristics of patients after HIPEC with mitomycin/doxorubicin, we could exclude a potential role of the cytoreduction part or the perioperative management (e.g. parenteral nutrition, epidural anesthesia, intraabdominal drainage, central vein lines) as an additional source of inflammation. In our study, patients after 90 min HIPEC with mitomycinC/doxorubicin or cisplatin showed a secondary CRP peak, simultaneously with a marked elevation of PSP, and the

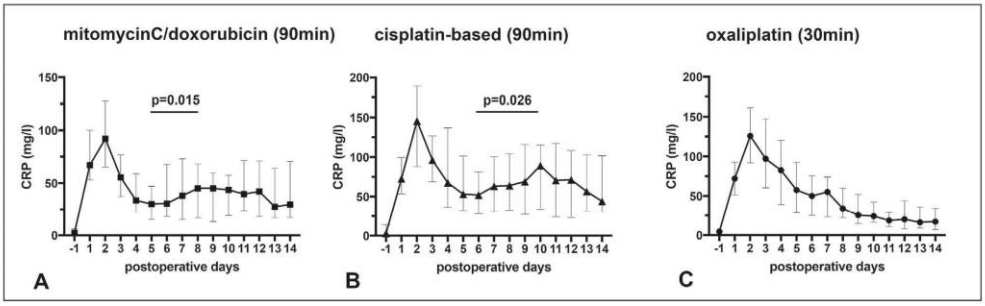

Fig. 1. Kinetics of CRP during an uncomplicated course
Fig. 1A: Kinetics of C-reactive protein (CRP) levels in patients without postoperative complications (n = 107) showing a significant secondary CRP increase after HIPEC with mitomycinC/doxorubicin (P = 0.015, n = 34) between day 5 and day 8. Fig. 1B: CRP in patients without complications (n = 38) after HIPEC with cisplatin showing a secondary CRP increase (P = 0.026) between day 6 and 10. Fig. 1C: Patients treated with oxaliplatin (n = 35) present a linear decline of the CRP. Data are shown as median and IQR.

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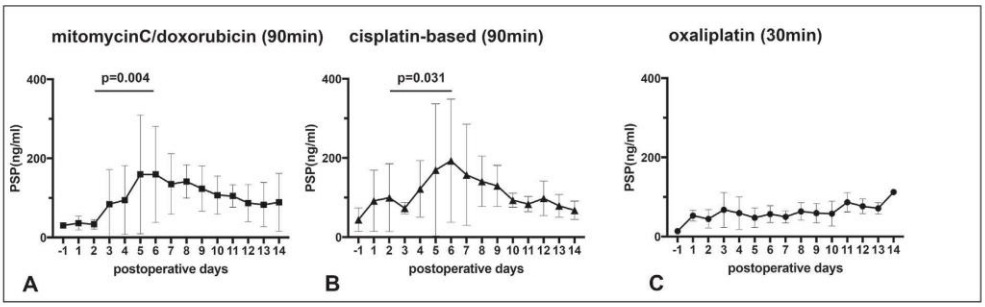

Fig. 2. PSP confirms an ongoing inflammatory process. Fig. 2A: Postoperative pancreatic stone protein (PSP) in patients without postoperative complications after HIPEC with mitomycinC/doxorubicin (n = 34). There is a significant PSP increase in patients between day 2 and 6 (P = 0.004). Fig. 2B: Patients after cisplatin – based HIPEC show a a significant PSP increase (P = 0.031) between day 2 and 6. Fig. 1C: No increase of PSP can be observed within the first postoperative week after HIPEC with oxaliplatin. Data are shown as median and IQR.

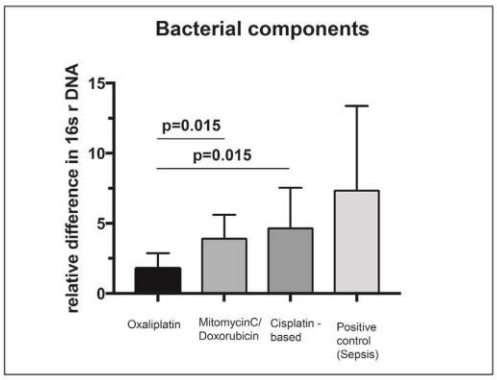

Fig. 3. Bacterial components associated to the inflammatory response.
Relative changes of bacterial 16srDNA during the postoperative course in patients after uncomplicated CRS/HIPEC. Patients after HIPEC with mitomycinC/doxorubicin (P = 0.015) or cisplatin (P = 0.015) have a significantly higher load of bacterial ribosomal DNA (16s rDNA) in their circulation compared to patients after HIPEC with oxaliplatin. Septic patients served as a positive control group. Data are shown as median and IQR.

presence of bacterial ribosomal DNA in the patient serum. Although difficult to prove in the human setting, this finding is suggestive of intestinal bacterial translocation. Support for this hypothesis comes from rat experiments, where increased bacterial components were identified in mesenterial lymph nodes on the third postoperative day after HIPEC [23]. Whether translocation occurred because of direct damage to the intestinal mucosa or due to decreased host defense remains so far unclear. To address the question whether different drugs or longer perfusion times are responsible, we compared a cisplatin-based protocol with the mitomycinC/doxorubicin and oxaliplatin protocol. Cisplatin is a compound similar to oxaliplatin but is usually performed at conditions similar to the mitomycinC/doxorubicin protocol (42 °C, 90 min). Finally, after prolonged perfusion with cisplatin, we also observed a secondary inflammatory peak, indicating that prolonged exposure time to HIPEC might be the critical factor. This observation is similar to a report from Spain, where this observation was not reported but the data and figures indicate the same kinetics for CRP after a paclitaxel-based HIPEC protocol for 60 min at 42 °C in absence of postoperative complications [24].

A justified question is the clinical relevance of our finding. Indeed, we did not observe a clinically relevant overall difference regarding postoperative complications between the different protocols. However, the ratio of deep organ space (CDC definition) infections was higher after HIPEC with mitomycinC/doxorucicin

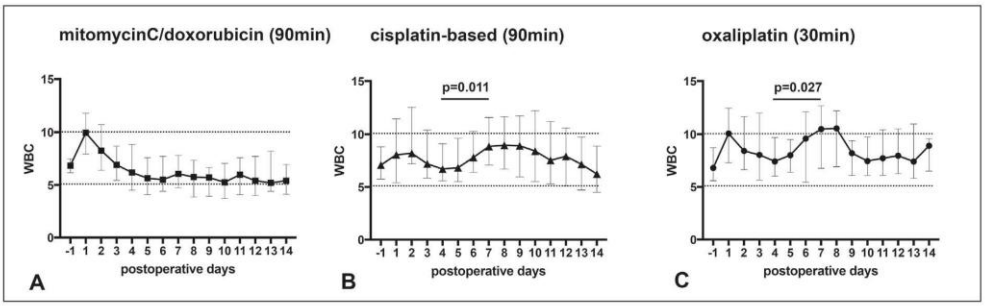

Fig. 4. Kinetics of WBC during an uncomplicated course Fig. 4A: Kinetics of white blood cells (WBC) levels in patients without infectious complications after CRS/HIPEC (n = 107). After an initial increase, WBC decrease and remain in normal range after mitomcycinC/doxorubicin HIPEC (n = 34). Fig. 4B and C: There is a significant secondary WBC increase in patients after platin-based HIPEC (n = 38, cisplatin-based, p = 0.011, n = 35, oxaliplatin-based, p = 0.027) between day 4 and day 7. Data are shown as median and IQR.

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compared to oxaliplatin (30% vs 11%, p = 0.028), and two patients in this protocol group had postoperative peritonitis without an underlying digestive fistula, and observation also made by others [25]. This may indicate that, although a rare event, bacterial translocation may contribute to adverse postoperative outcomes. Another question is whether HIPEC with oxaliplatin is still relevant after the results of PRODIGE7, since most centers may have changed to other protocols. Indeed, the goal of the present study is not to show a benefit of one over another protocol. Our data just highlights that protocol parameters, particularly temperature and duration, may significantly impact on the pathophysiology of our patients.

There is, however, another clinical value of these findings. When we initialized the study, we did not know about a potential increase in acute phase proteins in absence of complications after prolonged perfusion. Consequently, our patients regularly underwent a complex postoperative work-up including CT scans, and sometimes surgical reexploration, without revealing any infectious focus. This finding urged us to investigate the pathophysiology behind the secondary increase and we started measuring markers such as PSP and 16sDNA. Our findings finally highlight the complexity of HIPEC and underline the need for a dedicated and specialized team, not only for the procedure itself but also in the postoperative management and the interpretation of the clinical status of a patient after CRS/HIPEC. Based on our data, we learned that, while a secondary inflammatory reaction can occur after prolonged (90 min) HIPEC protocols, it was almost never observed after a short, 30 min protocol.

CRS/HIPEC treatment improves survival of patients with peritoneal metastasis. However, a majority of patients still has recurrent disease within the first two years [26], and future improvements of HIPEC are therefore needed. Today, parameters of HIPEC including drugs, perfusion time and temperatures are determined empirically, resulting in a huge variation, and major difficulty to compare and identify the role of a specific component. In addition, data from animal experiments may not be transferable to the human situation. A better understanding of molecular mechanisms, and the human pathophysiology of HIPEC is therefore required to intensify and improve existing protocols without increasing perioperative complications. Based on our findings we suggest that expanding perfusion times of HIPEC should include a monitoring of acute phase proteins, and probably also parameters to exclude increased intestinal translocation.

Another open question is the long-term impact of systemic inflammation after CRS/HIPEC. The role of postoperative inflammation and infection on patient survival is controversially discussed in the literature, and there is data showing a negative impact on patient outcomes, while other studies show no impact on survival [27,28]. The relevance of our present finding, inflammation in patients without complications, regarding patient survival is yet unclear, and would open another door to a molecular understanding of HIPEC.

An interesting finding in our study is the kinetics of WBC during the postoperative course of patients without a complication. Although, WBC values remained within normal range, we observed changes after platin-based HIPEC. Expecting a secondary increase after prolonged HIPEC treatment, we only detected an increase in patients after cisplatin-based HIPEC, which suggests a myelodepressive effect of mitomycinC/doxorubicin [29]. WBC also increased after oxaliplatin-based HIPEC, even though CRP values decreased in a linear manner almost to normal, indicating that a minimal bacterial translocation probably also occurs after oxaliplatin-based HIPEC, indicated also by the small quantity of bacterial components in the systemic circulation. Regarding the diagnostic workup of patients after CRS/HIPEC we would like to highlight a study published in 2016 which assessed the role of procalcitonin in the early postoperative phase after CRS/HIPEC. This study concluded that procalcitonin may improve the diagnosis of postoperative infection, but needs careful interpretation within the clinical context, similar to CRP and white-cell counts [30].

We would like to acknowledge the limitations of our study. The cohort of patients is heterogeneous regarding the type of primary tumors. However, treatment associated surgical factors are comparable among the three groups which will limit this bias. Finally, we acknowledge that the observed association of bacterial components in the bloodstream to systemic inflammation is not a mechanistic proof of intestinal translocation. This hypothesis is difficult to investigate and proof in humans, and animal experiments may finally be required. Overall, in the majority of patients, this effect did not result in a more complicated course. However, it indicates a critical mechanistic step in the pathophysiology of HIPEC and may explain the rare finding of peritonitis without a cause, sometimes observed in patients after HIPEC [25].

In conclusion, we identified novel aspects in physiologic changes after CRS/HIPEC, a secondary inflammatory reaction in patients after 90 min perfusion with HIPEC, associated to bacterial components in the systemic circulation. These protocol-specific effects after HIPEC should be known to physicians dedicated to the treatment of peritoneal surface malignancies for a better understanding of a patient's physiology. In future, they may help to direct the next evolution of technical refinements in perfusion protocols.

Conflicts of interest

The authors declared no conflict of interest. No third-party financial funds or materials were accepted or necessary for execution of this research project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejso.2019.03.036.

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The consequence of the first study with the increase of CRP without any infectious complications lead to the second study, in which we assessed the accuracy of CRP, WBC and procalcitonin (PCT). In that study, we included 248 patients with PM from different primary tumors, also ovarian cancer. The specificity of CRP in diagnosis of infectious complications after CRS/HIPEC is low, especially after the application of a prolonged protocol. Therefore, in case of a CRP elevation during the postoperative course, we recommend to assess also Procalcitonin (PCT). If PCT increases as well, an infectious complication needs is very likely and needs to be diagnosed or the patient closely monitored. If it decreases or is not elevated, an infectious complication is very unlikely.

3.2 Serum procalcitonin improves diagnosis of infectious complications after CRS/HIPEC

Roth *et al. World Journal of Surgical Oncology* (2023) 21:5
https://doi.org/10.1186/s12957-022-02884-9

World Journal of Surgical Oncology

RESEARCH Open Access

Serum procalcitonin improves diagnosis of infectious complications after CRS/HIPEC

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Abstract

Background Cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) improve the survival of selected patients with peritoneal metastasis. A major cause of treatment-related morbidity after CRS/HIPEC is infection and sepsis. HIPEC alters the diagnostic sensitivity and specificity of blood and serum markers and therefore has an impact on early diagnosis of postoperative complications. This study aimed to assess the sensitivity and specificity of blood and serum markers after CRS/HIPEC.

Methods Patients from two centers, operated between 2009 and 2017, were enrolled in this study. Perioperative blood samples were analyzed for white blood cells (WBC), C-reactive protein (CRP), and procalcitonin (PCT); postoperative complications were graded according to Clavien-Dindo and infectious complications according to CDC criteria.

Results Overall, *n*=248 patients were included with peritoneal metastasis from different primary tumors treated by CRS/HIPEC. Depending on the applied HIPEC protocol, patients presented a suppressed WBC response to infection. In addition, a secondary and unspecific CRP elevation in absence of an underlining infection, and pronounced after prolonged perfusion for more than 60 min. PCT was identified as a highly specific — although less sensitive — marker to diagnose infectious complications after CRS/HIPEC.

Discussion/conclusion Sensitivity and specificity of WBC counts and CRP values to diagnose postoperative infection are limited in the context of HIPEC. PCT is helpful to specify suspected infection. Overall, diagnosis of postoperative complications remains a clinical diagnosis, requiring surgical expertise and experience.

Synopsis

HIPEC treatment after CRS influences the accuracy of common inflammatory parameters to diagnose a postoperative infectious complication. The additional determination of procalcitonin increases the specificity in the diagnosis.

Introduction

Cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) have become an accepted component of multimodal therapy of peritoneal metastasis. While CRS refers to a systematic and radical resection of visible peritoneal implants, HIPEC is an innovative strategy to control microscopic disease by an intraoperative, heated chemo-perfusion. Over the last years, the concept of CRS/HIPEC changed the landscape of treatment for peritoneal metastasis and demonstrated impressive survival rates, e.g., for colorectal [1], gastric [2], or ovarian [3] metastasis. Despite all advances

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made in the treatment of peritoneal metastasis, HIPEC is still performed in various ways, and several parameters remain poorly defined, varying among centers. For example, this includes the treatment duration, the degree of hyperthermia, or the substances or combinations used, which are likely to change in the future. Today, many HIPEC protocols use combinations of mitomycin C/doxorubicin, oxaliplatin or cisplatin, and use a temperature range between 41 and 43°C for 30 to 90 min.

CRS/HIPEC, a radical and potentially curative treatment modality, is associated with the risk of treatment-related morbidity and mortality. By far the major contribution relates to the surgical procedure. However, HIPEC may add to the overall morbidity, and have some specific morbidity. For example, mitomycin C is known to have a negative impact on WBC counts in up to 39% of patients [4, 5], oxaliplatin may be associated with hemorrhagic complications [6], and cisplatin can induce severe nephrotoxicity [6, 7]. A recent study from the USA compared treatment-associated morbidity of CRS/HIPEC with other major surgery, e.g., liver resection, Whipple's procedure or esophagectomy, and identified an overall lower morbidity and a low mortality rate of 1.1% [8]. After CRS/HIPEC, the major cause for treatment-related death is sepsis and infection [9]. Early recognition of complications has been recently defined as a major factor to reduce failure-to-rescue after CRS/HIPEC [10]. Therefore, a reliable diagnosis of infectious complications after CRS/HIPEC is crucial. Although the clinical picture of patients remains the fundament of surgical diagnosis of postoperative complications, blood parameters may be helpful to screen or specify.

We reported in a previous report, that HIPEC can provoke a systemic inflammatory response [11]. This is very likely to have an impact on sensitivity and specificity of laboratory values, e.g., WBC counts, C-reactive protein, or procalcitonin. In the present study, we assessed the role of standard blood parameters (WBC counts, C-reactive protein, procalcitonin) to diagnose postoperative infectious complications after CRS/HIPEC.

Material and methods

Patients and ethics

The study includes patients from two centers (University Hospital Zurich, Switzerland, and Hanusch Krankenhaus, Vienna, Austria) operated between 2009 and 2017. The study protocol was approved by the ethical committee (KEK-ZH-Nr.2017-01656) and registered at clinicaltrials.gov (NCT02741167).

Surgery and perioperative management

All patients were discussed prior any treatment in a multi-disciplinary tumor board. Extra-abdominal tumor was excluded by ^{18}FDG-PET/CT or contrast-enhanced thoraco-abdominal CT. Patients received standard of care pre- and postoperative chemotherapy according to their tumor entity and international guidelines. Anesthesia was conducted with propofol and volatile anesthetics combined with thoracic epidural anesthesia as described previously [12]. CRS was performed according to international standards, and defined as radical (CC-score 0) if no macroscopic residual tumor was visible, except for pseudomyxoma, where a CC-1 score (<0.25cm remnant macroscopic tumor) was accepted [13]. For appendix and colorectal tumors, peritoneal dialysis solution with mitomycinC (30mg/m^2 body surface area, BSA according to the Mosteller formula) in combination with doxorubicin (15mg/m^2 BSA) was applied at 42°C for 90 min, or oxaliplatin (300-400mg/m^2 BSA) as a single agent at 43°C for 30 min. Patients with mesothelioma or ovarian cancer were treated with a cisplatin-based regimen (75mg/m^2 BSA) at 42°C for 90 min. In 2016, the type of protocol used for the appendix and colon cancer changed in both centers from mitomycinC/doxorubicin to oxaliplatin, which was then consistently used for these tumors until the end of the study. Patients received pre/intraoperative antibiotic prophylaxis (cefuroxime 1.5g, metronidazole 500mg) which was not continued to the postoperative phase.

Serum probes

C-reactive protein (CRP), white blood cell (WBC) counts, and procalcitonin (PCT) were measured in blood samples by the clinical laboratory service on a daily routine basis prior to open surgery or CRS/HIPEC and for the 14 consecutive postoperative days or until the date of discharge. A positive event for WBC counts or CRP and PCT levels was defined if the value at day 8 was higher or equal compared to the value at day 5. In addition, only WBC counts above the normal range (>10G/l) were considered as a positive event.

Definition and diagnosis of postoperative infection

For the grading of complications, the Clavien-Dindo classification was used [14]. Definition of infectious complications was done according to the Center for Disease Control and Prevention (CDC) definitions [15]. Patients after CRS/HIPEC were visited and examined daily. In case of clinical symptoms or signs of infection, urine and central catheter tips were sent for cultures. Imaging studies, usually an abdominal CT, were performed if CRP levels increased >30% after postoperative day 4.

Statistical analysis

Continuous variables were compared with the Student *t*-test, the Mann–Whitney *U*, or the Wilcoxon test, where

appropriate. Fischer's exact test was used to compare differences among proportions derived from categorical data. Normally distributed data are shown as mean +/-SD, non-normal variables as the median and interquartile range (IQR). Missing values in the dataset were excluded. All p values were two-sided and considered statistically significant if $p \leq 0.05$. Statistical analysis was performed using SPSS version 25 and GraphPad Prism version 8.0. Sensitivity and specificity of each diagnostic parameter were determined by the kinetics between postoperative day 5 and day 8 and the number of patients with an infectious versus non-infectious complication.

Results

Overall, n= 248 patients after CRS/HIPEC were included in this analysis. Overall, 41% (n=145) of patients had any complication, in 10% (n=25) of patients major morbidity (≥Clavien Grade 3b) was observed, and one patient died (Table 1). For HIPEC, three protocols (mitomycin C, oxaliplatin and cisplatin) were used. Patients differed in terms of primary tumors and median operation time, but not the PCI (Table 2). With the primary goal to test the diagnostic accuracy of serum parameters, we first assessed if WBC counts and serum CRP are able to diagnose postoperative infectious complications.

Table 1 Patient characteristics

	CRS/HIPEC
Number of patients	248
Age	54 (46–63)
Gender (male/female)	141(57%)/107(43%)
Preoperative systemic chemotherapy	106 (43%)
Anastomosis (number)	1 (0–2)
Complications (Clavien Dindo)	
none	145 (59%)
Grade I	11 (4%)
Grade II	42 (17%)
Grade IIIa	22 (9%)
Grade IIIb	21 (8%)
Grade IVa	4 (2%)
Grade IVb	0
Grade V	3 (1%)
Infectious complications	61 (25%)
Superficial	16/61 (26%)
Deep	2/61 (3%)
Organ space	43/61 (71%)

Patient characteristics for patients after CRS/HIPEC. Categorial data are presented as absolute numbers with percentage and nominal data as median and IQR

Low specificity of CRP and low sensitivity of WBC counts after CRS/HIPEC

In general, CRS/HIPEC is associated with a low specificity of CRP to diagnose an infectious complication during the postoperative course. The reason for this is the secondary peak of CRP between days 5 and 8, also present in absence of any infection (Fig. 1A). Generally, the CRP levels after HIPEC remained elevated during the observation time of two weeks. In contrast to CRP, WBC counts remained within a normal range, even in presence of postoperative infections (Fig. 2A), which results in a very low sensitivity of 36.4% (Fig. 3C).

CRP levels are unspecific after 90-min platin-based protocols

After the observation that HIPEC can elevate postoperative CRP levels in absence of infection and suppress WBC counts in response to infection, we next explored whether these effects depend on the HIPEC protocol. In this study, HIPEC was performed with oxaliplatin (n=48), mitomycinC/doxorubicin (n=123), or cisplatin (n=77). Upon infection, CRP levels increased after any protocol as expected (Fig. 1A). In contrast, in patients without infection, patients after a 90-min protocol with mitomycinC or cisplatin, the above-mentioned secondary CRP peak was observed between postoperative day 5 and day 8 (Fig. 1C). Consequently, CRP levels demonstrated a poor specificity (37–40%) to diagnose postoperative infection in these two protocols (Fig. 3B). As a consequence, infection was suspected and over-diagnosed in 16% (13/84) of patients after HIPEC with mitomycinC, who underwent an abdominal CT scan without a diagnosis of complications

White blood cell counts to diagnose infection after HIPEC

In contrast, WBC counts have a moderate sensitivity to diagnose infection (Fig. 3C). This effect is more pronounced after HIPEC with mitomycinC or cisplatin (Fig. 2B), where WBC kinetics show no response to infection. This is different after oxaliplatin-based HIPEC, where WBC counts are able to react to infection, resulting in a higher sensitivity (Fig. 3D) of this marker. Overall, WBC counts seem to have only a moderate utility to diagnose infection after the CRS/HIPEC.

Serum procalcitonin (PCT) improves specificity to diagnose infectious complications

Given the low specificity of CRP to diagnose postoperative infection after CRS/HIPEC, we assessed the diagnostic value of PCT in this setting. PCT values reacted similarly to infection, regardless of the perfusion protocol (Fig. 4A), and did not show a nonspecific reaction as seen

Table 2 Comparison of patient characteristics between HIPEC protocols

	Mitomycin C	Oxaliplatin	Cisplatin	*p*-value
Number of patients	123	48	77	
HIPEC				
Perfusion time (min)	90	30	90	
Temperature (°C)	42	43	42	
Primary tumor				0.000
Colorectal	44 (36%)	25 (52%)	7 (9%)	
High-grade appendix	32 (26%)	19 (40%)	2 (3%)	
Low-grade appendix	38 (31%)	0		
Mesothelioma	0	0	14 (18%)	
Others	9 (7%)	4 (8%)	54 (70%)	
PCI	10 (4–21)	8 (3–17)	9 (4–20)	0.17
Operation time (min)	540 (445–685)	361 (284–479)	405 (281–546)	0.000
Splenectomy	26 (21.2%)	9 (18.8%)	18 (23.4%)	0.50
ICU stay	1 (1–2)	1 (1–4)	2 (1–5)	0.000
Hospital stay	18 (13–25)	17 (15–31)	16 (12–20)	0.035
Infectious complications	39 (32%)	10 (21%)	12 (16%)	0.028
Superficial	7/39 (18%)	4/10 (40%)	1/12 (8%)	0.53
Deep incisional	1/39 (3%)	1/10 (10%)	1/12 (8%)	1.00
Organ/space	31/39 (79%)	5/10 (50%)	10/12 (84%)	0.000
Intestinal leak	7/31 (26%)	0		
Urinary infection	5/31 (16%)	0	1/10 (10%)	
Positive blood culture	8/31(26%)	1/5 (20%)	2 (20%)	
Pneumonia	3/31 (9%)	1/5 (20%)	4 (40%)	
Intraabdominal abscess	4/31 (13%)	2/5 (40%)	2 (20%)	
Infected pancreatic fistula	1/31 (2%)	1/5 (20%)	1 (10%)	
Bacterial peritonitis	2/31 (6%)	0	0	
Other [a]	1/31 (2%)	0	0	

Patients after CRS/HIPEC were assessed according the HIPEC protocol, which differ with regard to the drugs used for HIPEC and the perfusion time. Particularly the early kinetic of inflammatory markers (Figs. 1, 2, 3 and 4) should be read with the information that the groups differ the primary tumor and operation times. [a]Other infectious complications include cholangitis and colpitis. Categorial data are presented as absolute numbers with percentage and nominal data as median and IQR

for CRP (Fig. 4B). Despite a low sensitivity, PCT demonstrated a high specificity of >85% to diagnose infection for all protocols (Fig. 4C). Assessment of PCT in addition to CRP can be helpful to distinguish between infectious complications and a non-specific CRP increase, particularly for protocols with prolonged perfusion times (Fig. 4D).

Discussion

This study highlights the specific role of HIPEC on the pathophysiology of postoperative serum inflammatory parameters. We observed that certain HIPEC protocols can suppress the WBC response to infection and may cause secondary and unspecific CRP elevations without underlining infection. This has a major impact on the sensitivity of WBC counts or the specificity of CRP values. We observed that this effect depends on the specific HIPEC protocol and seems more pronounced after prolonged perfusion for 60 min or more. To overcome this diagnostic limitation, we assessed the role of PCT, which was identified as a highly specific — although less sensitive — marker to diagnose infection. These findings may help to discriminate and diagnose infectious complications in the setting of CRS/HIPEC.

Due to the complexity of the procedure including HIPEC which induces additional tissue damage and inflammation, diagnosis of postoperative infection can be challenging. Knowledge about the potential suppression of the WBC reaction in response to infection after HPEC with mitomycinC and cisplatin is an important detail which should be known to any surgical oncologist in charge of these patients. Myelosuppression is a well-known hematologic side effect of doxorubicin, cisplatin, and mitomycinC [16–18]. Although is well known in the field, that HIPEC is overall well tolerated with acceptable myelosuppression rates compared to the systemic use of chemotherapeutic agents [18, 19], special care should be taken to this attenuated

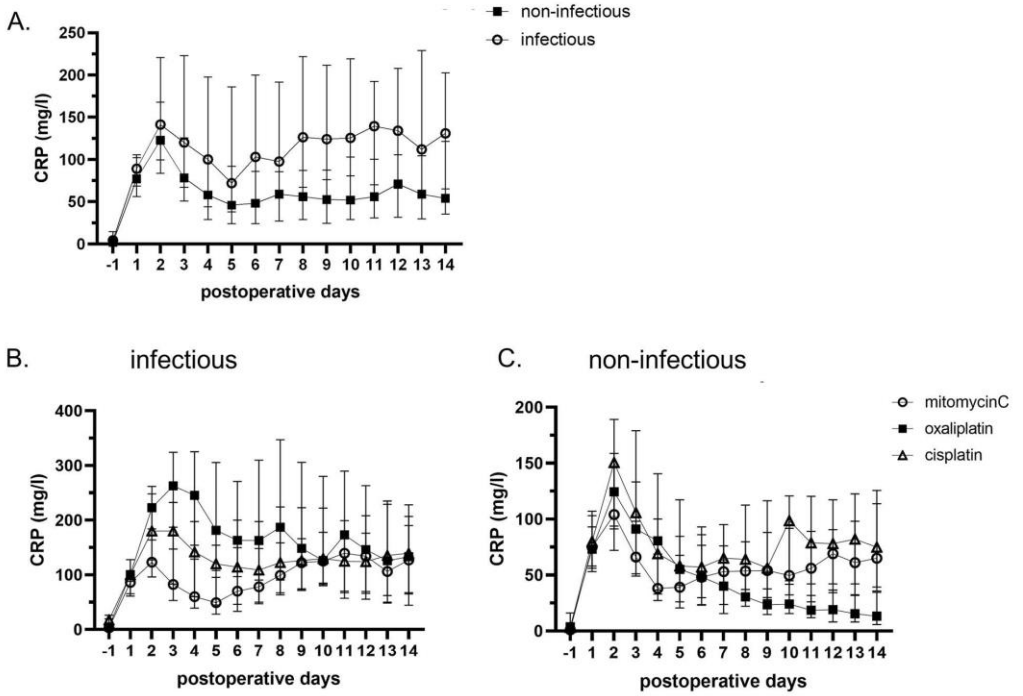


Fig. 1 HIPEC treatment influences postoperative CRP levels. CRP levels after CRS/HIPEC do not return to normal, even without any infectious complication (**A**). With any infectious complication, CRP increases after HIPEC performed with any of the three protocols (**B**). In absence of infectious complications, CRP increases after mitomycinC and cisplatin based HIPEC (**C**), while returning to normal values after HIPEC performed with oxaliplatin (**C**). The graphs illustrates the postoperative CRP values, plotted as median and IQR

myelosuppressive effect which is not a clinical problem per se but may affect the diagnostic utility of WBC counts. This puts HIPEC treatment in line with other clinical situations, e.g., immunosuppression, old age, transplant patients, where the immune system is not able to react properly, and WBC counts or other serum parameters require critical evaluation.

While myelosuppression can be explained by the systemic effect of locoregional chemotherapy, the underlining mechanism of the secondary inflammation wave and CRP peak remains unclear. However, the clinical consequence is relevant. In the present study, 16% of patients after HIPEC with mitomycinC/doxorubicin underwent a CT scan due to increased CRP levels without diagnosing any postoperative infection. We speculated in a recent study, that prolonged perfusion protocols may trigger a systemic inflammatory response by translocation of intestinal bacterial components [11]. The pathophysiologic mechanism behind this remains, however, still elusive. We observed in this study that patients treated with a 90-min protocol, who also shows depressed WBC and unspecific late CRP elevations, had more organ space infections compared to the short protocol with oxaliplatin. We do interpret this result with the highest care, due to the heterogeneity of groups which could explain this observed difference.

To improve diagnostic accuracy, PCT was introduced earlier for postoperative infection [20]. We share the opinion of these authors that the diagnostic value of serum parameters in the first postoperative days is limited and is highly triggered by the amount and type of surgery. In this critical phase, the experience of the surgeon and particularly the clinical picture of the patient is more relevant, and serum parameters are of limited use to predict complications. However, towards the end of the first postoperative week, when the first peak of surgery-related inflammation flattens, these markers may help to improve patient management. PCT is produced

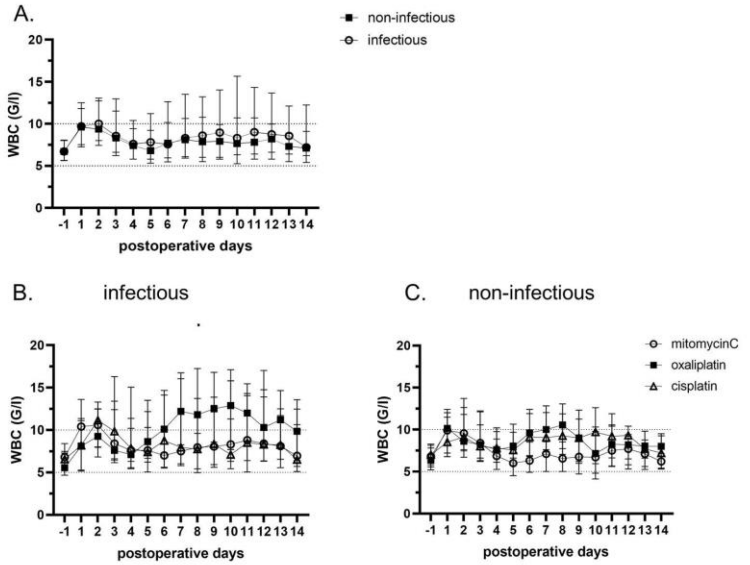


Fig. 2 HIPEC treatment suppresses WBC counts. Postoperative WBC counts remain within normal ranges, even in presence of infectious complication (**A**). WBC`s remain reactive to infection only after oxaliplatin based HIPEC (**B**). Without any infectious complications, the WBC`s counts remain within the normal ranges of 5–10 G/l illustrated for each HIPEC protocol (**C**)

A.

CRP	CRS/HIPEC
SENS	66.1%
SPEC	49.3%
PPV	33.9%
NPV	78.7%

B.

CRP	mitomycinC	oxaliplatin	cisplatin
SENS	80%	44.4%	75%
SPEC	37.3%	75%	39.9%
PPV	40.0%	36.4%	15.0%
NPV	78.1%	80.8%	91.7%

C.

WBC	CRS/HIPEC
SENS	36.4%
SPEC	74%
PPV	33.9%
NPV	76%

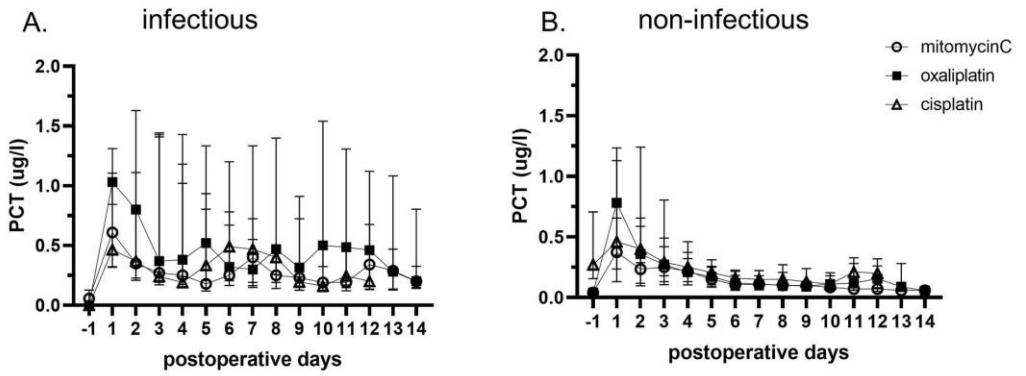
D.

WBC	mitomycinC	oxaliplatin	cisplatin
SENS	25.7%	55.6%	33.3%
SPEC	91.9%	54.8%	64.1%
PPV	60.0%	26.3%	12.5%
NPV	72.3%	81.0%	86.2%

Fig. 3 Sensitivity and Specificity of CRP and WBC counts after HIPEC. The specifitiy of CRP after CRS/HIPEC is only 49.9% (**A**). Specificity is reduced after mitomycinC and cisplatin based HIPEC to 37.3% and 39.9% respecitivly (**B**). WBC counts demonstrate a low sensitivity of 36.4% in general (**C**), pronounced after prolonged protocols with mitomycin C or cisplatin (**D**). SENS, sensitivity; SPEC, specificity; PPV, positive predictive value; NPV, negative predictive value

by the C cells of the thyroidal gland and some other cell types upon bacterial infection and is stimulated by bacterial endotoxins and lipopolysaccharides, and indirectly by inflammatory markers, such as tumor necrosis factor-alpha, interleukin-6, and interleukin, and has a high specificity in the diagnosis of bacterial infections and sepsis [21]. In this study, the high specificity of PCT to diagnose infectious complications could be confirmed and was independent from the applied HIPEC protocol. Despite its low sensitivity, the specificity of PCT, which remains unchanged by the perfusion protocol, is an important tool that may be helpful to discriminate between inflammation and infection in the sometimes challenging management of patients after CRS/HIPEC.

We would like to acknowledge the limitations of our study. Overall, the patient cohort includes different



C.

PCT	mitomycinC	oxaliplatin	cisplatin
SENS	26.7%	60%	33.3%
SPEC	92.9%	86.2%	84.6%
PPV	80.0%	50%	33.3%
NPV	54.2%	90.5%	84.6%

D.

CRP/ PCT	mitomycinC	oxaliplatin	cisplatin
SENS	80%	60%	100%
SPEC	92.3%	85%	88.9%
PPV	80%	33.3%	14.3%
NPV	52.2%	89.5%	100%

Fig. 4 Serum procalcitonin (PCT) improves specificity to diagnose infectious complications. PCT is highly specific to detect postoperative infection after HIPEC, independent of the protocol. SENS, sensitivity, SPEC, specificity; PPV, positive predictive value; NPV, negative predictive value

primary tumors and therefore the amount of surgery or CRS is not entirely comparable. Some differences in the early postoperative kinetics of the assessed parameters could also be related to this. For example, patients with pseudomyxoma were treated with mitomycinC, which translates into a longer operation time compared to the other protocols. However, the aim of the study, to look at the kinetics of blood and serum parameters, and to assess their diagnostic sensitivity and specificity, in the presence or absence of infection should not be influenced by this heterogeneity. The difference among groups with regard to ICU stay, hospital stay, and infectious complications should not influence the analysis of diagnostic parameters. While we assessed the most commonly used markers, it would be certainly interesting to assess the diagnostic potential of other inflammatory markers such as IL-6, IL-1, or TNF-a to get a deeper insight of the impact of HIPEC on a patient's physiology.

In conclusion, we analyzed kinetics and the diagnostic value of CRP, WBC, and PCT after uncomplicated and complicated CRS/HIPEC. We identified a major impact on CRP levels and WBC counts, depending on the type of HIPEC protocol. In addition, we propose the use of PCT as a marker for infection which demonstrated to be independent from the treatment and offers a good specificity despite a still low sensitivity. Together our data highlight the complexity of HIPEC treatment which goes beyond technical excellence in the operating room but requires a dedicated holistic care of the surgical oncologist.

Abbreviations

CRS	Cytoreductive surgery
HIPEC	Hyperthermic intraperitoneal chemotherapy
PCI	Peritoneal cancer index
CC-score	Completeness of cytoreduction score
CRC	Colorectal cancer
CRP	C-reactive protein
PCT	Procalcitonin
WBC	White blood cells

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12957-022-02884-9.

Additional file 1: Figure S1. The postoperative CRP course is PCI independent. The CRP course for three different PCI groups is plotted in Fig. 1A and illustrates the secondary increase or stable CRP level after CRS/HIPEC. As shown in Suppl. Figure 1B. – D., the HIPEC protocol mainly influences the course of the CRP in all three PCI groups and the main findings remain consistent. Whereas mitomycinC and cisplatin are associated with a CRP increase, after oxaliplatin HIPEC, the CRP decreases almost to normal.

Authors' contributions

D.E. and K.L. established and initiated the study. D.E., L.R., F.L., F.K., and S.R enrolled the patients in the study and collected blood. D.E. and L.R. analyzed the blood samples. L.R. and K.L. wrote the main manuscript text. L.R. and L.R. prepared Figs. 1 2, 3, and 4 including suppl figure 1 and applied statistical

tests. All authors reviewed the manuscript. The author(s) read and approved the final manuscript.

Funding
The project is supported by a research grant from the Swiss National Science Foundation (310030_185029) to K.L. The funding covered the salary of a PhD student.

Availability of data and materials
Human data is stored on a server at the University Hospital of Zurich. All measured serum samples of the patients are stored at −80°C at the University Hospital of Zurich.

Declarations

Ethics approval and consent to participate
The study protocol was approved by the ethical committee (KEK-ZH-Nr.2017-01656) and registered at clinicaltrials.gov (NCT02741167).

Competing interests
There are no conflicts of interests of any author in this manuscript regarding finances or in a personal nature.

Received: 19 November 2022 Accepted: 29 December 2022
Published online: 12 January 2023

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Publisher's Note

Based on previous findings and the observation of long-term survivors after CRS/HIPEC, I intended to investigate the particular effect of CD8+ T-cells on PM development and on the HIPEC treatment efficacy using experimental models. The manuscript is currently revision at *Nature Communications*.

3.3 CD8+ T-cells restrict the development of peritoneal metastasis and support the efficacy of hyperthermic intraperitoneal chemotherapy (HIPEC)

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Abstract

Background: Multimodal therapy for peritoneal metastasis (PM) including cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) provides long-term survival in highly selected patients. Mechanisms behind HIPEC are unknown, and may include induction of adaptive immunity. We therefore analyzed human PM samples and explored the impact of HIPEC in experimental models.

Methods: Paired human samples from colorectal primaries and associated PM (n=19) were examined for CD8+T-cells and correlated with disease free (DFS) and overall survival (OS). CD8+T cell response after HIPEC was assessed using an in-vivo PM mouse model, tumor cell lines and patient-derived tumor organoids.

Results: Human patients with high intraepithelial CD8+T cell counts showed longer DFS and OS. In the mouse model, HIPEC controlled growth of PM and increased numbers of functional CD8+T cells.. In-vitro (cell lines and human organoids) heated chemotherapy induced immunogenic changes, reflected by significantly higher levels of MHC-class I molecules and expression of cancer testis antigens cyclin A1 and SSX-4. Using in-vitro co-culture assays, cancer cells after heated chemotherapy primed dendritic cells, which subsequently activated CD8+ T cells to produce significantly higher amounts of IFNγ.

Conclusions: Our data concludes that presence of CD8+T-cells within PM lesions correlates with prolonged survival of human patients. With the help of in-vivo and in-vitro experiments, we show that heated chemotherapies induce immunogenic changes on cancer cells leading to protective CD8+T-cells mediated immunity, which seems to contribute to improved survival rates observed after HIPEC.

Introduction

Metastatic colorectal cancer (CRC) is a major cause of cancer death [30,55,56]. Although less frequent than hematogenous metastasis, peritoneal metastasis (PM) occurs in up to 15% of CRC patients[7]. Systemic therapy remains the treatment of choice for patients with any metastatic CRC, however there is evidence that hematogenous metastasis is better controlled than PM [30,56]. The reason for this is not known but might be attributable to different molecular subtypes among metastatic sites [57] or the specific microenvironment in the peritoneal cavity [47]. In patients with CRC PM only a highly selected subset of patients qualify for radical resection [58]. In those highly selected patients, so-called cytoreductive surgery where all metastatic lesions are resected, followed by local application of hyperthermic intraperitoneal chemotherapy (HIPEC), can be performed. Although clinical trials remain unclear about the role, duration, and composition of HIPEC [59,60], the benefit of surgery as a part of multimodal treatment of PM is highly evident. In many cohorts, median survival rates significantly increased up to 50 months for a disease which was considered terminal until not so long ago [30,59].

The current concept of CRS/HIPEC follows a two-step process, where in the first step the macroscopic tumor lesions are removed through CRS and in the second step, heated chemotherapy is applied locally to ensure destruction of remnant microscopic cancer lesions. Drugs for HIPEC are selected based on their cytotoxic ability to kill tumor cells, usually in a combination with mild hyperthermia (41-43°) for 30-90 minutes to increase the cytotoxic effect [61]. In patients with CRC PM, a variety of protocols evolved historically and include drugs such as mitomycin C, doxorubicin or oxaliplatin [62-64]. Since some of these drugs can induce immunogenic effects[36,37,54], we assumed long-term survival after CRS/HIPEC for CRC PM may result from induction of protective immunity. In the present study, we first analyzed the accumulation of CD8+ T cells in paired human samples from primary tumors and PM and their impact on disease free (DFS) and overall survival (OS). In a next step we turned our focus on in-vitro and in-vivo assays to investigate the influence of heated chemotherapy (mimicking HIPEC) on CRC cell lines and patient-derived tumor organoids. Using in-vitro assays, we discerned that heated chemotherapy induced immunogenic changes on cancer cells that activated DCs and subsequently primed CD8+ T cells. Using a PM mouse model, we finally assessed the accumulation of functional CD8+ T cells within PM lesions after HIPEC and could show that CD8+ T cells are essential to control PM lesions.

Materials and Methods

Cancer cell-lines

Human CRC cells HCT-8 and HT-29, a gift from Prof. M. Scharl's Laboratory (University Hospital Zurich), were used for in-vitro studies. HCT-8 cells were cultured in RPMI 1640 medium and HT-29 cells in Dulbecco`s modified Eagles medium (DMEM) (both from Gibco, Life Technologies, Zug, Switzerland), respectively. The medium was supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, Zug, Switzerland) and penicillin/streptomycin (100U/ml). In-vivo studies were performed with syngeneic mycoplasma negative (tested with PCR Mycoplasma Test Kit, PromoCell, Heidelberg, Germany) murine colorectal cancer MC-38-OVA cells obtained from Prof. M. van den Broek, University of Zurich, Switzerland. MC-38-OVA cells were also cultured in complete DMEM media.

Patient-derived tumor organoids

Tissue samples or ascites were collected during CRS following cantonal ethics number: 2019-01031 at the University Hospital Zurich. Organoids were prepared at the laboratory of Prof. Chantal Pauli at the Department of Pathology and Molecular Pathology, University Hospital Zurich. The organoids were expanded by splitting every 3-4 weeks. Organoids were cultured in Matrigel in suspension plates (6-well TC plates from Sarstedt, Nümbrecht, Germany) with WRN media (provided by Chantal Pauli`s Laboratory, exact details are listed in Supplementary Table 1). The cell-cell connection and cell-Matrigel connection was detached with Triple-LE (Gibco, Life Technologies, Zug, Switzerland). After a few washing steps, the cells were dissolved in a

Supplementary Table 1

GHM for pancreas and colon (tumor and normal tissue)

Reagent Name	Location	*Amount Conc*	*Volume Solvent*	*Solvent*	*Stock Conc*	*Aliquot*	*Aliquot Storage*	*Final Conc in Media*	*Ordering*
DMEM advanced	4°C					500ml			Invitrogen 12491-023
N-Acetylcysteine	4°C	5g	61.278mL	DW	500mM	1250uL	-20°C	1.25mM	Sigma-Aldrich A9165
Human Recombinant EGF	-20°C	500ug	25mL	PBS/BSA** (.1% BSA)	20ug/mL	1250uL	-20°C	50ng/mL	Sigma-Aldrich E9644
Human Recombinant FGF-10	-20°C	500ug	5mL	PBS/BSA** (.1% BSA)	100ug/mL	100uL	-20°C	20ng/mL	Peprotech 100-26
Recombinant Human FGF-basic	-20°C	50µg	10mL	PBS/BSA** (.1% BSA)	5ug/mL	100uL	-20°C	1ng/mL	Peprotech 100-18B
Y-27632 (Rocki)	-20°C	50mg	1.56mL	DMSO	100mM	50uL	-20°C	10uM	Selleckchem S1049
A-83-01	-20°C	5mg	2.37mL	DMSO	5mM	50uL	-20°C	500nM	Sigma-Aldrich SML0788
SB202190	-20°C	25mg	1.51mL	DMSO	50mM	100uL	-20°C	10uM	Selleckchem S1077
Nicotinamide	RT	6.11g	50mL	DW	1M	5mL	-20°C	10mM	Sigma-Aldrich N0636
PGE2	-20°C	10 mg	5.674 mL	DMSO	5mM	100uL	-20°C	1uM	R&D Systems 2296
Noggin (conditioned media)	-20°C					50mL	-20°C	10ng/mL	
R-Spondin (conditioned media)	-20°C					25mL	-20°C	10ng/mL	
B27 Supplement	-20°C	50x				10mL	-20°C	1x	Invitrogen 17504001
A/A	-20°C	100x				5mL	-20°C	1x	Invitrogen 15240062
Glutamax	4°C	100x				5ml		1x	Invitrogen 35050038
[Leu15]-Gastrin I Human	-20°C	500ug	0.474 mL	DW	500 uM	10 uL (dilute stocksol. 1:10 with DW)	-20°C	1nM	Sigma-Aldrich G9145

**PBS/BSA: 0.01 g in 10 mL PBS

WRN/Matrigel ratio of 1/1 and distributed on a new dish. The Matrigel was ordered at Corning (Lot number: 9238003).

Mice

C57BL/6 mice (8-10 weeks old) were purchased from Envigo (Horst, Netherlands). All mouse experiments and treatments were performed in accordance with the Swiss Federal Animal Regulations and approved by the Veterinary Office of Zurich (no. 165/2017 and 022/2021). OT-I transgenic mice were purchased from Jackson laboratories.

In vitro experiments

Human cancer cells (0.5×10^6) were seeded into 6-well culture plates (TPP, Switzerland) containing 1ml of the corresponding media. After 24 hours cells were treated either with control (a carrier solution used for the chemotherapy) or with the chemotherapy at 37°C or at 43°C. Chemotherapies - either Oxaliplatin 300mg/l or the combination of MitomycinC/Doxorubicin 10mg/l - were used for respective experiments. After 30 minutes of the treatment, the medium was removed, the cells were once washed with phosphate-buffered saline (PBS; Gibco, Life Technologies, Zug, Switzerland) then fresh corresponding medium was added to the wells. The cells were then incubated for additional 72 hours.

For qPCR, cells were lysed with TRIzol (15596026, Thermo Fisher Scientific) and consequently lysate was stored at -80°C until the RNA extraction was performed. For western blotting, cells were lysed by adding 400ul of 5ml RIPA buffer + 1 Roche Protease Inhibitor tablet + 50ul PMSF (200nM). For FACS analysis, cells were detached with 0.05% Trypsin-EDTA and transferred as a cell-suspension to FACS tubes for further processing.

For co-culture experiments, peripheral blood was collected in an EDTA-containing vial. Peripheral blood mononuclear cells (PBMC) were isolated with a Ficoll gradient (Ficoll Histopaque-1077: Sigma-Aldrich, Schaffhausen Switzerland). Monocytes were isolated with the magnetic cell separation (MACS) technology as per manufacturer`s instructions. The purity >95% of the monocyte fraction was determined with FACS. $3 \times 10^5$ monocytes were added to each well of a 12- well culture plate. To generate monocyte-derived dendritic cells (Mo-DC`s), monocytes were cultured for 7 days with DC medium supplemented with Cytokine A (Dendritic Cell Generation Medium, PromoCell, Schaffhausen, Switzerland). Every second day, the medium was exchanged. HCT-8 cells were seeded in 6-well plates on day 6 after monocyte purification and treated as described above. Mo-DC`s were added to treated tumor cells (ratio Mo-DC`s/tumor cells 1:5). 24 h after co-culture, Mo-CD`s were collected for FACS analysis. The subsequent effect of Mo-DC maturation was further assessed on CD8+ T-cells. This experimental set-up was identical. At day 8 after monocyte purification, CD8+ T-cells were purified from the same healthy volunteer with MACS technology (CD8+ MicroBeads: Miltenyi

Biotec). 1×10^5 CD8+ T-cells were cultured in 96 round bottom tissue culture plates (TPP, Sigma-Aldrich, Schaffhausen Switzerland) and Mo-DC`s exposed to different treated HCT-8 tumor cells were added to the CD8+ T-cells. The positive control condition for cytokine induction was PMA and Ionomycin treated CD8+ T-cells. In last 3 hours of the culture, Brefeldin A (BioLegend) was added to block the vesicular transport to measure intracellular IFN-γ production by CD8+ T-cells.

For the murine co-culture experiments, splenocytes from wt (wildtype) C57BL/6 mice and from OT-I transgenic on C57BL/6 background were used to set-up the co-cultures. Spleens were harvested from the mice and meshed though a 70µm filter (Corning cell strainer, Sigma-Aldrich, Schaffhausen Switzerland) to create a single cell suspension. Red blood cells were lysed with 1ml RBC Lysis Buffer (RBC Lysis Buffer, BioLegend). 1 day before the harvest, 1×10^5 murine tumor cells (MC-38, MC-38-Ova) were seeded into 24 well tissue culture plates and treated 24h after in different conditions. Directly after the treatment of the tumor cells, 2.5×10^5 splenocytes suspended in DMEM supplemented with IL-2 100U/ml were added to the tumor cells. 6h before the collection of the splenocytes, Brefeldin A was added to the cultures. The supernatant of each well, consisting the splenocytes, was collected 48h after co-culture set-up and processed for FACS analysis.

In vivo experiments

Mice were intraperitoneally injected with 0.5×10^6 MC-38-Ovalbumin+ (MC-38-Ova) murine colon carcinoma cells. Macroscopic peritoneal tumor formation occurred mostly by day 7 or 8. The anesthetized mice underwent a median laparotomy to assess PM lesions. PM-lesions bearing mice were randomly assigned to different treatment groups (heated M/D, M/D, heated PBS, PBS). The treatments were performed in an open abdomen coliseum technique. Temperature during treatment was constantly measured with a thermometer. The abdomen was rinsed with saline solution after the treatment. The abdomen was closed with a two layered continuous suture technique with ethilon 4.0 (Ethicon). Six days after the surgery, the mice were sacrificed and the tumor load was assessed with the peritoneal cancer index (PCI)[65]. Peritoneal tumors were harvested for FACS analysis and histology.

Immune cells depletion

CD-4+ and CD8+-T-cell depletion in mice was achieved by the intraperitoneal injection of 100ug CD4 or CD8a depletion antibody (BioXCell, clone GK 1.5 for CD4+ T-cells and clone YTS 169.4 was used for CD8+ T-cells) 1 day prior to tumor cells injection and 1 day prior to the treatment. The macrophages were depleted using anti-CSFR1 antibody (BioXCell, clone: 5A1; 150µg/mice) injected 1 day prior to tumor cells injection and every 3rd day until the end of the experiment.

Patient samples

This study includes patients with visible peritoneal metastasis (PCI>0) from colorectal origin. All patients underwent the CRS/HIPEC procedure at the University Hospital of Zurich. Patients with synchronous metastatic disease were operated for the primary tumor and the PM lesion at the same time at the University Hospital Zurich. Patients with a metachronous disease were operated before the CRS/HIPEC procedure at the University Hospital Zurich or at another Hospital in Switzerland. Patients with a MSI or BRAF mutated primary tumor were not included. All patients gave an informed consent for the further analysis of their samples. The study was approved by the ethical committee (cantonal ethics number: 2019-01031). From each patient, paired samples were selected from the primary tumor and from PM lesion. The most important criteria was the size of vital microscopic tumor area on an H&E stain.

Immunohistochemistry

Tissue samples were collected in 4% buffered formaldehyde and paraffin-embedded. Mouse tumor tissue blocks were sliced into 4 µm and Haematoxylin and eosin (H&E) at our department according to a standard protocol. Additionally, murine tumor samples were immunohistochemically stained for CD8a+ (abcam ab 209775, Dilution 1:500), Granzyme B (abcam ab 2555598, Dilution 1:1000) and Macrophages F4/80 (abcam ab 100790, Dilution 1:100) with the autostainer Link 48 from Dako. Tumor blocks were subsequently sectioned at 4 µm and stained at the Department of Pathology and Molecular Pathology, University Hospital Zurich, Switzerland. Haematoxylin and eosin (H&E) stains were performed according to standard protocol. Immunohistochemistry was performed with double-stain for CD8 (Dako/Agilent M710301, Dilution 1:40, pre-treatment with EDTA buffer (pH8.4), at 100°C for 32min, OptiView Kit Ventana) and with pan cytokeratin antibody (panCK, Dako/Agilent M351501, Dilution 1:100, processed with no further pre-treatment, UltraView Red Kit Ventana) using a Ventana Benchmark Ultra platform with Haematoxylin counterstaining. Primary tumor and the corresponding PM lesion were stained accordingly and scanned using a 3D Histech Pannoramic 250 Flash III Scanner (3DHISTECH Ltd., Budapest, Hungary) at 40x and a resolution of 0.24µm/pixel.

Digital pathology

CD8-panCK double stained slides were scanned and the tumor area was annotated. Artificial intelligence (AI)-based histomorphological tissue and CD8+ T-cell classification was performed using deep neural net algorithm (DNN) to quantify tissue area and to count CD8+ T-cells within the corresponding area in HALO (Indica Labs, Albuquerque, NM, USA). DNN classification was used to

segmental annotated tumor areas into the following compartments and to quantify the tissue area in mm^2 [66]: Background (white space and tissue folds, excluded from subsequent analysis), Necrosis, Epithelium (intraepithelial area), Stroma. Cell nuclei in each compartment were segmented, and CD8+ T-cells were

identified based on Ultra View Red Chromogen signal. Density of CD8+ T-cells cells in the stromal and intraepithelial compartment was calculated as cells / mm^2 of tissue and analysed with clinicopathological variables and outcome.

RT-PCR

RNA was extracted from treated and untreated cancer cells using TRIzol Reagent (Invitrogen, Basel, Switzerland). RNA from tumor organoid was extracted with RNA columns (Qiagen, Hombrechtikon, Switzerland) 1µg RNA was reverse-transcribed to cDNA (ThermoScript reverse transcription PCR system; Invitrogen, Basel, Switzerland). PCR amplification was performed with the ABI Prism Sequence Detector System using TaqMan gene expression assays. Results are illustrated as fold induction relative to the 18s ribosomal RNA transcription.

Western Blotting

After protein isolation from different treated cancer cell suspensions, the protein concentration was measured using a DC Protein Assay Reagent Package (Bio-Rad, Hercules, CA, USA). Protein aliquots were separated by SDS-PAGE electrophoresis and blotted using a V3 Western Workflow system by BioRad (Hercules, CA, USA). PVDF membranes were blocked with TBST (containing 5% BSA) and incubated with the primary Cyclin A1 antibody (Abcam, clone ab53699) overnight at 4°C. Protein expression was measured by densitometry and illustrated relative to α-Tubulin as a reference protein.

Flow cytometry

Cells were detached from culture plates and transferred to FACS tubes. Cells washed with PBS. The single cell-suspension was stained with surface antibody cocktail for 30 minutes at 4°C. After staining, the samples were washed with PBS and then fixed with 4% formaldehyde and stored at 4°C. For intracellular cytokine staining (ICS), Brefeldin A was added 5h prior to block vesicular transport. For ICS first cells were stained with surface antibodies, later cells were washed with PBS, fixed with 4% PFA for 5 minutes and then permeabilized with 1% Saponin-PBS solution for 5 minutes. Cells were subsequently stained with antibodies against cytokines for 2h at 4°C. The samples were analyzed either on the BD FACS Canto II or BD Fortessa (BD Biosciences, LSR II Fortessa 4L). Data analysis was carried out in FlowJo (V10.7.1, BD, Ashland, OR, USA).

Statistics

The CD8+ T-cell counts were normalized to their respective area as shown in the figure legends. Due to limited availability of paired samples, non-parametric Wilcoxon-test was used for analysis with categorical values. To define high versus low CD8+ T-cells count, a linear regression of the normalized CD8+ T-cell count to the intraepithelial area and the overall survival was performed. Due to its strong correlation, the median of the normalized CD8+ T-cell count was used to define the groups. A CD8+ T-cell count ≥ the median was defined

as the high CD8+ T-cell group and < the median as the low CD8+ T-cell group. Based on these two groups, disease-free survival (DFS) and overall survival (OS) were compared and the log-rank test was performed to determine significance between the groups. The stromal content was calculated by the division of stromal area to the annotated area. The cut-off calculation of loose versus dense stroma was performed by a ROC-curve analysis including the CD8+ T-cell high and low group. The cut-off value of 0.6 with the highest likelihood ratio was taken and applied for the further statistical analysis. Disease-free and overall survival of loose versus dense stroma were also compared using log-rank statistical analysis. The data of normalized CD8+ T-cell counts and stromal content were used to distinguish between stromal dense and CD8+ T-cell high or low and stromal loose and CD8+ T-cell high or low groups. No patient was in the stromal dense/CD8+ T-cell high group. The disease-free and overall survival data of three groups were compared and a log-rank test was performed. GraphPad prism (version 9.3.1) and SPSS (IBM, version 26) were used to calculate statistical differences.

Results

Patient characteristics

Due to limited availability of paired samples, we included 19 samples from patients with PM originating from CRC for the analysis of CD8+ quantities and compartment distribution. 19 PM lesions were collected during CRS before the HIPEC treatment. In case of synchronous disease, tumor tissue from the primary tumor was sampled during CRS. If the PM occurred metachronous, the primary tumor was resected before CRS/HIPEC. The majority of patients had a T4 stage colorectal cancer with nodal metastasis (**Table 1**).

19 patients with PM from CRC	
Age (median, IQR)	46y (39y – 60y)
Gender (f/m)	10 (52.6%)/9 (47.4%)
DFS (median/range)	14 months (range 1 – 47 months)
OS (median/range)	39 months (range 7 – 105 months)
T-stage of the primary tumor	
T3	5 (26.3%)
T4	14 (73.7%)
N-stage of the primary tumor	
N0	3 (15.8%)
N1	8 (42.1%)
N2	8 (42.1%)
G-stage/histological grading	
G1	6 (31.5%)
G2	9 (47.4%)
G3	4 (21.1%)
Histological subtype	
Adenocarcinoma NOS	16 (84.2%)
Signet cell differentiation	3 (15.8%)

PCI (median, IQR)	8 (6-16)
CC-score	0 (100%)
HIPEC regimen	
MitomycinC/Doxorubicin	15 (78.9%)
Oxaliplatin	4 (21.1%)
PM occurence	12 (63.2%) synchronous
	7 (36.8%) metachronous
RAS mutations	
Wild-type	10 (52.6%)
KRAS mutation	7 (36.8%)
NRAS mutation	2 (10.5%)
MSS	19 (100%)
No systemic chemotherapy prior CRS/HIPEC	5 (26.3%)
Neoadjuvant chemotherapy	14 (73.7%)
Douplet drug combination (FOLFOX/FOLFIRI)	11 (57.9%)
Triplet drug combination (FOLFOXIRI)	3 (15.8%)
Adjuvant/additive chemotherapy	
First line	**18 (94.7%)**
Single drug (Capecitabine)	4 (21.1%)
Douplet drug combination (FOLFOX/FOLFIRI)	5 (26.3%)
+ VEGF antagonist	5 (26.3%)
+ EGFR antagonist	2 (10.5%)
Triplet drug combination + VEGF antagonist (FOLFOXIRI)	2 (10.5%)
No of cycles (median, IQR)	6 (3 – 9)

***Table 1: Patient characteristics**, NOS: not otherwise specified, CC-Score: Completeness of Cytoreduction Score (0 stands for completed tumor resection).*

All patients had a MSS type of the colon cancer. The extent of the disease had a median PCI of eight, matching to preferred criteria to qualify for the CRS/HIPEC treatment. The majority of patients was treated with Mitomycin C/Doxorubicin HIPEC. All patients were radically resected with complete cytoreduction-score (CC-Score) of zero. The two groups based on the CD8+ T-cell number normalized to intraepithelial area of the PM lesion into high versus low had no significant differences in PCI or driver mutations (K-Ras or N-Ras) (**Table 2**). Further, the adjuvant systemic chemotherapy regimen was similar between the groups in terms of number of cycles and drug combinations.

	CD 8+ T-cell high (n=9)	CD 8+ T-cell low (n=10)	p-value
Age (median, IQR)	42y (37.5y – 58y)	50.5y (42.5y – 61y)	0.27
Histological subtype			
Signet cell differentiation	2 (22.2%)	1 (10%)	0.58
PCI (median, IQR)	10 (5.5 – 12.5)	7.5 (5.75 – 17.25)	0.78
PM occurence	7 (77.8% synchronous)	5 (50%) synchronous	0.35
	2 (36.4%) metachronous	5 (50%) metachronous	
Hematogenous metastasis	2 (22.2%)	8 (80%)	0.023
(liver and lung)			
RAS mutations			0.21
KRAS mutation	2 (22.2%)	5 (50%)	
NRAS mutation	1 (11.1%)	1 (10%)	
No systemic chemotherapy prior CRS/HIPEC	4 (44.4%)	1 (10%)	0.14
Neoadjuvant chemotherapy	5 (55.6%)	9 (90%)	
Douplet drug combination	4	7	1
Triplet drug combination	1	2	
Adjuvant/additive chemotherapy			

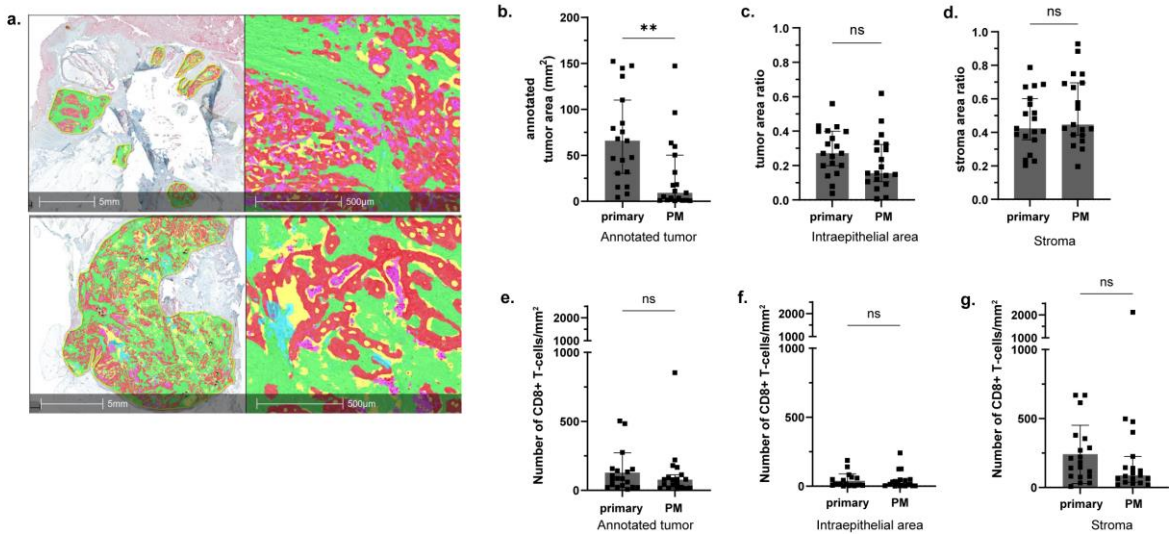
First line	**8 (88.9%)**	**10 (100%)**	0.88
Single drug	2 (22.2%)	2 (20%)	
Douplet drug combination	3 (33.3%)	2 (20%)	
+ VEGF antagonist	1 (11.1%)	4 (40%)	0.35
+ EGFR antagonist	1 (11.1%)	1 (10%)	1
Triplet drug combination + VEGF antagonist	1 (11.1%)	1 (10%)	
No of cycles (median, IQR)	6 (4.5 – 7.5)	5 (3 – 12)	0.73

Table 2: Comparison of patient characteristics between the two groups CD8+ T-cells high and low *(normalized to the intraepithelial area of PM).*

CD8+ T-cells within the Pan CK+ intraepithelial area of PM lesions is associated with prolonged patient survival

To assess CD8+T cells in the whole tumor area of the primary and the corresponding PM lesion we scanned the slides as shown in **Supplementary Figure 1a**. The yellow line marks the border of the annotated tumor area. The zoomed pictures show the different areas of the tumor (green: stromal area, violet: necrotic area, red: intraepithelial area, yellow: white space area). We noticed that the analyzed annotated tumor area was significantly larger for the primary tumor than for the PM lesions (p=0.0018) (**Supplementary Figure 1b**). However, intraepithelial and stromal area within annotated tumor area were similar between the primary and the PM lesion (**Supplementary Figure 1c and 1d**). Interestingly, the number of CD8+ T-cells normalized to the corresponding area (within annotated tumor area, intraepithelial and stroma) were also similar between primary tumor and PM lesions (**Supplementary Figures 1e, 1f, and 1g**).

Supplementary Figure 1



Supplementary Figure 1: The composition and the CD8+ T-cell accumulation of the primary tumor and the corresponding PM lesions. (a)*The scanned histological slide of the primary and the corresponding PM lesion. The yellow line marks the border of the annotated tumor area. The zoomed pictures show the different areas of the tumor (green: stroma, violet: necrosis, red: intraepithelial, yellow: white space).* ***(b – d)*** *bar graphs illustrates the annotated tumor area and the intraepithelial ratio as well as the stromal ratio in primary tumors and PM lesions.* ***(e – f)*** *the bar graphs show the number of CD8+ T-cells normalized to the corresponding area. The error bars represent the median and the lines the interquartile range. Each dot represents a patient. **** = p≤0.0001, *** = p≤0.001, ** = = p≤0.01, * = p≤0.05, ns = p>0.05.*

We noticed that compared to intraepithelial area, the stromal area harbored significantly higher CD8+ T cells in both primary tumors and PM lesions (**Figure 1a and 1b**). We then first classified primary tumors based on the presence of CD8+ T cells within intraepithelial area allowing creation of CD8+ T-cell high and low groups (**Supplementary Figure 2a, dotted line shows the median**). We notice that intraepithelial CD8+ T-cells infiltration in primary tumors has no impact on DFS and OS between CD8 high and CD8 low groups (p=<0.0001, respectively p=0.0001) (**Supplementary Figures 2b and 2c**). However, patients with high CD8+ T cells numbers in the stroma of primary tumors showed significantly longer DFS than those with low stromal CD8+ T cell numbers, but the OS was similar in between CD8 high and CD8 low groups (**Supplementary Figures 2d – 2f**).

Figure 1

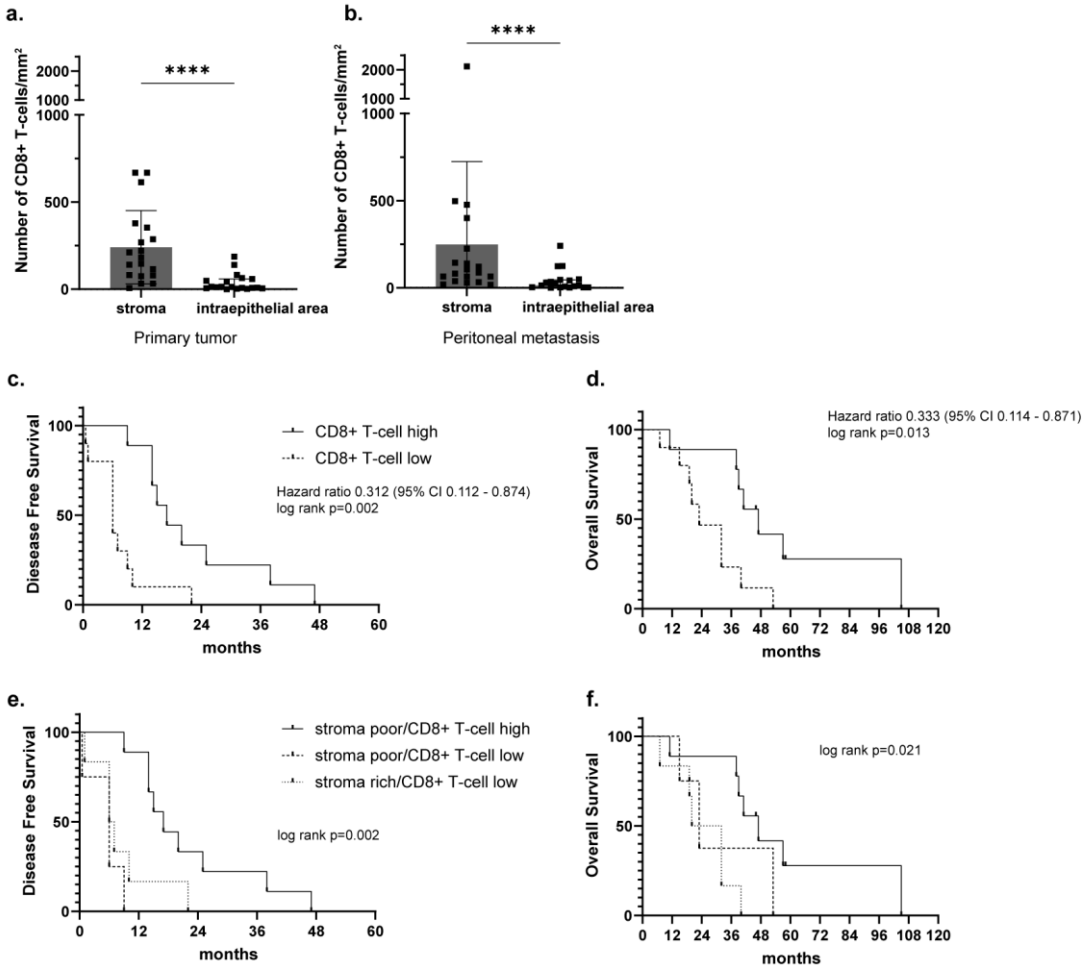
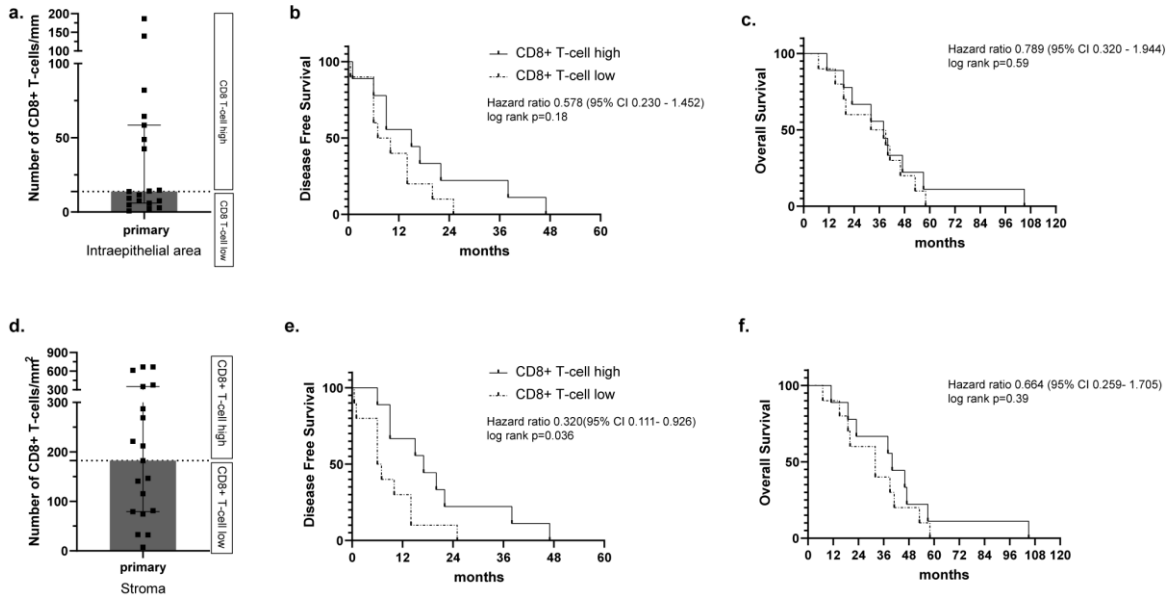


***Figure 1: Assessment of CD8+T cells in patient samples**. **(a and b**) analysis of CD8+ T cells in stroma and epithelium of the paired primary tumor and the corresponding PM lesions of 19 patients. The graphs illustrate the number of CD8+ T-cells normalized to the corresponding area of stroma or epithelium. DFS **(c)** and OS **(d)** based on intraepithelial CD8+ T-cell counts of PM lesions. 9 patients belong to the CD8+ T-cell high group and 10 patients to the CD8+ T-cell low group. DFS **(e)** and OS **(f)** based on the stroma content and CD8+ T-cell distribution. The 9 patients with a CD8+ T-cell high PM lesion were associated with low stromal content (continuous line), 6 patients with a CD8+ T-cell low PM-lesion had a dense stroma (fine dotted line) and 4 patients with a CD8+ T-cell low PM-lesion had a poor stroma (dotted line). Error bars represent the median and the lines the interquartile range. Each dot represents a patient. **** = p≤0.0001, *** = p≤0.001, ** = = p≤0.01, * = p≤0.05, ns = p>0.05*

Supplementary Figure 2: CD8+ T-cells in the primary tumors. (a) *Shows the number of intraepithelial CD8+ T-cells in the primary tumors and the dotted horizontal line marks the median. The median allows the creation of a CD8+ T-cell high*

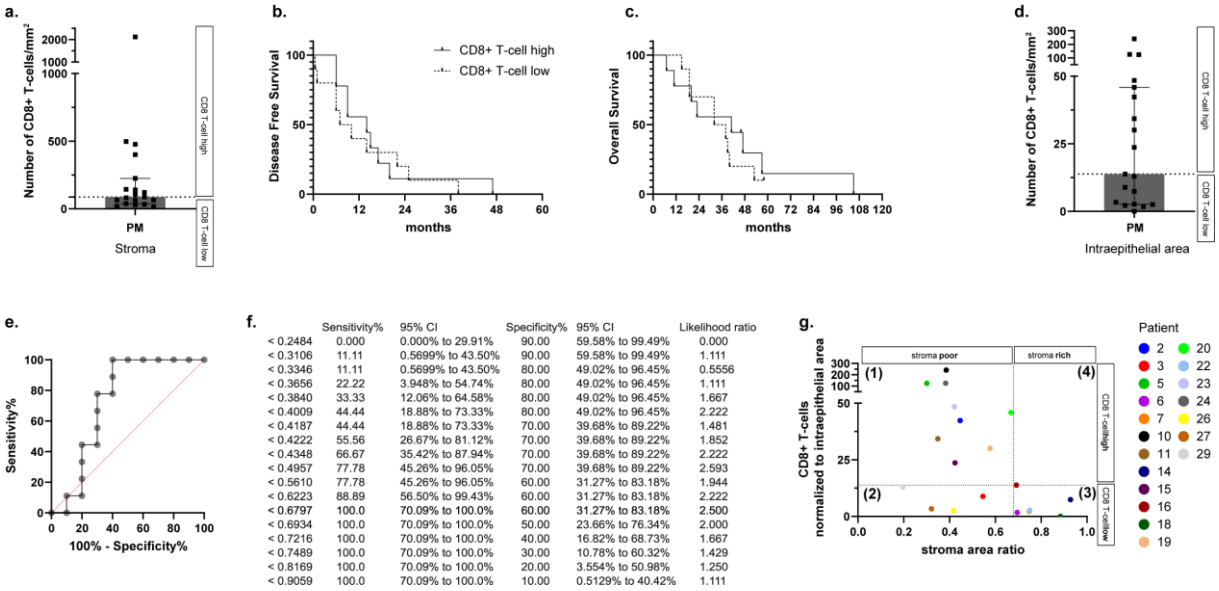
Supplementary Figure 2



and low groups. ***(b)*** *illustrates the DFS and* ***(c)*** *the OS.* ***(d)*** *shows the CD8+ T-cells located in the stroma normalized to the stromal area.* ***(e)*** *The CD8+ T-cell high group presents a significant longer DFS than the low group.* ***(f)*** *no influence on the OS of the patients. Bars represent the median and the lines the interquartile rang. Each dot represents a patient.*

Assessment of PM lesions based on CD8 high and CD8 low groups revealed that the number of stromal CD8+ T-cells in PM lesions did not influence DFS or OS (**Supplementary Figures 3a – 3c**). Conversely, comparing CD8 high and CD8 low groups (**Supplementary Figure 3d**) in the intraepithelial area of PM lesions significantly influenced DFS (log rank p=0.002) as well as OS (log rank p=0.013) (**Figures 1c and 1d**). These results suggest that the presence of intraepithelial CD8+ T cells of the PM lesions positively influence the outcome of these patients. Since stromal density is also known to influence outcome for patients with colorectal cancer[67] we added this additional prognostic factor in our assessment.

Supplementary Figure 3



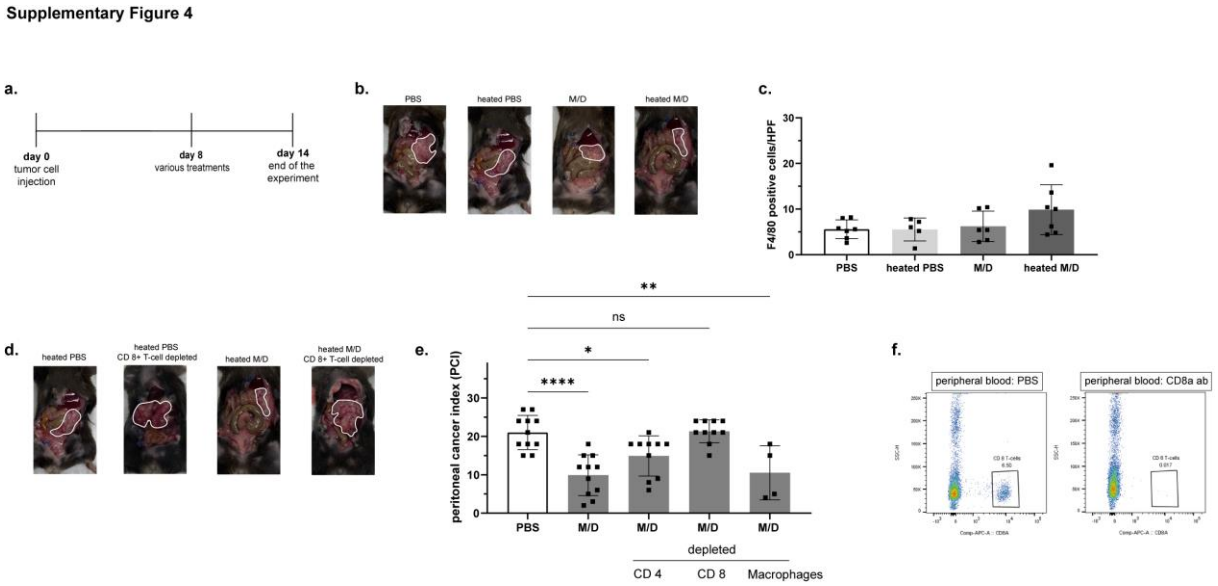
Supplementary Figure 3: The distribution of CD8+ T-cells in PM lesions. (a) *The distribution of CD8+ T-cells in the stroma is shown.* ***(a – c)*** *illustrate survival based on stromal CD8+ T-cells distribution in PM lesions* ***(d)*** *The bar graph shows the distribution of intraepithelial CD8+ T-cells normalized by area in PM-lesions among the 19 patients. The dotted line indicates the median and divide the cohort into CD8+ T-cell high and low.* ***(e)*** *Shows the ROC curve to determine the cut-off value of the stromal content.* ***(f)*** *Presents the sensitivity and specificity for each potential cut-off value.* ***(g)*** *Illustrates the CD8+ T-cell number normalized to intraepithelial area (y-axis) against the stromal content (x-axis). The horizontal dotted line represents the median and cut-off of CD8+ T-cells and the vertical dotted line the cut-off value of 0.6797 for the stromal area ratio. Bars represent the median and the lines the interquartile range . Each dot represents a patient.*

The cut-off value for the stroma poor and stroma rich group was determined according to the ROC curve illustrated in **Supplementary Figures 3e and 3f**. Thus, dual assessment of stromal density with CD8+ T-cells allowed the creation of four groups namely ***(1)*** CD8+ T-cell high/stroma poor group (n=9), ***(2)*** CD8+ T-cell low/stroma poor group (n=4), ***(3)*** CD8+ T-cell low/stroma rich group (n=6) and ***(4)*** CD8+ T-cell high/stroma rich group (n=0) (**Supplementary Figure 3g**). The comparison of these groups showed significantly longer DFS and OS for group 1 (CD8+ T-cell high/stroma loose group, **Figures 1e and 1f.** The median DFS for groups 1-3 was 17, 6 and 6.5 months, respectively, whereas the OS for groups 1-3 was 47, 23, and 25 months, respectively.

Heated chemotherapy prevented growth of PM lesions in a CD8+ T-cell dependent manner in a PM mouse model

Our patient data revealed the importance of CD8+ T-cells in human peritoneal tumor tissues and their influence on patient survival. As it is not possible to study the impact of HIPEC treatment on CD8+T cell-mediated immunity in patients, we decided to discern this aspect using a PM mouse model. We intraperitoneally injected MC-38-Ova (murine colon cancer cells) cells in C57BL/6 mice to establish microscopic PM lesions. Eight days

after the cells injection, mice were treated in four different conditions PBS, heated PBS, M/D, heated M/D at day 8 (**Supplementary Figure 4a**).



Supplementary Figure 4: HIPEC treatment in PM mouse model. (a) *time-line of the experiment.* ***(b)*** *The effect of different treatments on PM lesions. The white line marks PM lesions.* ***(c)*** *Quantification of Macrophages in treated PM lesions.* ***(d)*** *PM lesions of treated mice with and without CD8+ T-cells.* ***(e)*** *PCI of mice with and without CD8, CD4 and Macrophages.* ***(f)*** *shows depletion of CD8+ T-cells depletion in the peripheral blood of the mice. Each dot represents one mouse. Error bars illustrate the mean +/-SD. **** = p≤0.0001, *** = p≤0.001, ** = = p≤0.01, * = p≤0.05, ns = p>0.05*

Compared to the other treatment groups, the peritoneal tumor load, measured using murine PCI (peritoneal cancer index), was significantly reduced after heated M/D treatment, **(Figures 2a and Supplementary Figure 4b**). Mice that received heated M/D treatment showed significantly more CD8+ T-cells and Granzyme B+ cells in PM lesions (**Figures 2b– 2d**), while other immune cells such as macrophages did not change (**Figure 2b and Supplementary Figure 4c**). Furthermore, depletion of CD8+ T-cells (**Supplementary Figure 4f**) before heated M/D treatment abrogated its protective effect (**Figure 2e and Supplementary Figures 4d and 4e**). This data suggests that CD8+T cells are crucial to control growth of PM lesions after heated M/D treatment. Moreover, the depletion of CD4+T cells and macrophages did not abrogate protective effects of heated M/D, suggesting these cells are not important for therapeutic effects.

Interestingly, we were able to include a patient who received CRS/HIPEC treatment two times allowing us to analyze PM tumor tissue collected during first CRS and again after 17 months during second CRS (**Figure 2f**). Compared to PM tissue collected during first CRS, a massive increase in intraepithelial CD8+ T cells was noticed in the tumor area of PM lesions collected during second CRS (**Figure 2g**.). This is an important finding, as this patient had an extraordinary long-term survival of 102 months (8.5 years since the first HIPEC) (**Figures 2h and 2i**).

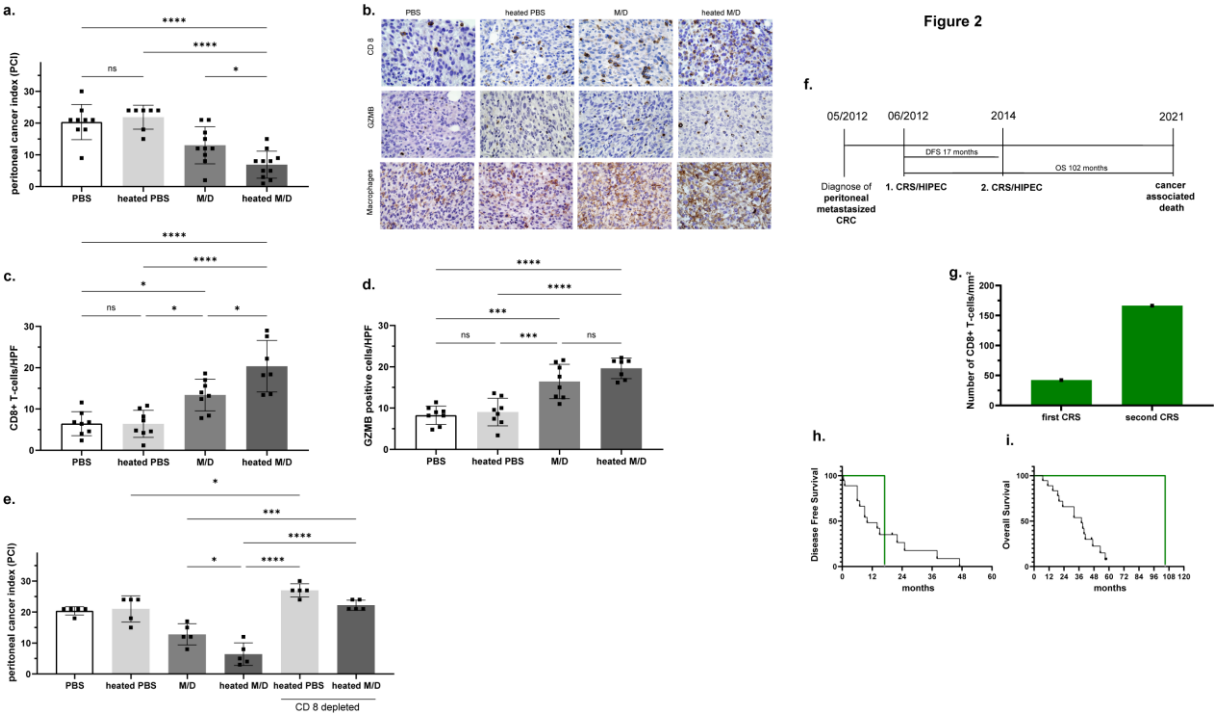


Figure 2: Impact of HIPEC treatment in PM mouse model. (a) *measurement of peritoneal tumor load as PCI. mice were treated with PBS (n=9) or heated PBS (n=7) or with M/D (n=10) or with heated M/D (n=11).* ***(b)*** *Staining of tumor tissues for the presence of CD8+T cells, Granzyme B+ cells and macrophages.****(c and d)*** *Quantification of CD8+ T cells and GZMB+ cells.* ***(e)*** *PCI of the treated mice with and without CD8+ T-cells. Each dot represents one mouse.* ***(f)*** *Demonstrates the treatment time-line of a single patient with PM from CRC.* ***(g)*** *The CD8+ T-cell number normalized to intraepithelial area is shown after the first CRS and 17 months after the first HIPEC.* ***(h)*** *DFS of this patient compared to 18 other patients with PM from CRC.* ***(i)*** *Presents the OS of this patient (green line), which was 102 months. Error bars show the mean +/- SD. **** = p≤0.0001, *** = p≤0.001, ** = = p≤0.01, * = p≤0.05, ns = p>0.05.*

Heated chemotherapy treatment induces immunogenic changes in human cancer cells and patient derived tumor organoids

While we could discern the role of CD8+ T cells in controlling PM lesions, the direct impact of heated chemotherapies on tumor cells leading to protective immunity was not clear. Therefore, to understand effects of HIPEC treatment on cancer cells, we carried out in-vitro experiments, where human cancer cells (HCT-8) were exposed to short-term treatment with heated chemotherapy mimicking HIPEC in patients. We treated colorectal cancer cell-lines with PBS, heated PBS, chemotherapy (Oxaliplatin 300mg/l or M/D 15mg/l) and short-term (30 minutes) heated chemotherapy, respectively. After treatment, cells were washed to remove

dead cells due to direct cytotoxicity by chemotherapy (not shown). Cells were examined for immunogenic changes after 48-72 hours. We noticed that surviving cancer cells showed enhanced expression of MHC-class I molecules (**Figure 3a and Supplementary Figure 5a**). In addition, a panel of nine Cancer Testis Antigens (CTAs), frequently expressed in immunogenic cancers[68,69] was screened with RT-PCR (**Figure 3b and Supplementary Figure 5b**). H Heated chemotherapy with Oxaliplatin or M/D enhanced the expression of the CTA Cyclin A1 and SSX-4 at mRNA and Cyclin A1 at protein levels (**Figures 3c - 3f and Supplementary Fig 5c and 5d**). We also noticed similar changes in Cyclin A1 and SSX-4 expression in another colorectal cancer cell line (HT-29, **Supplementary Figure 5e**).

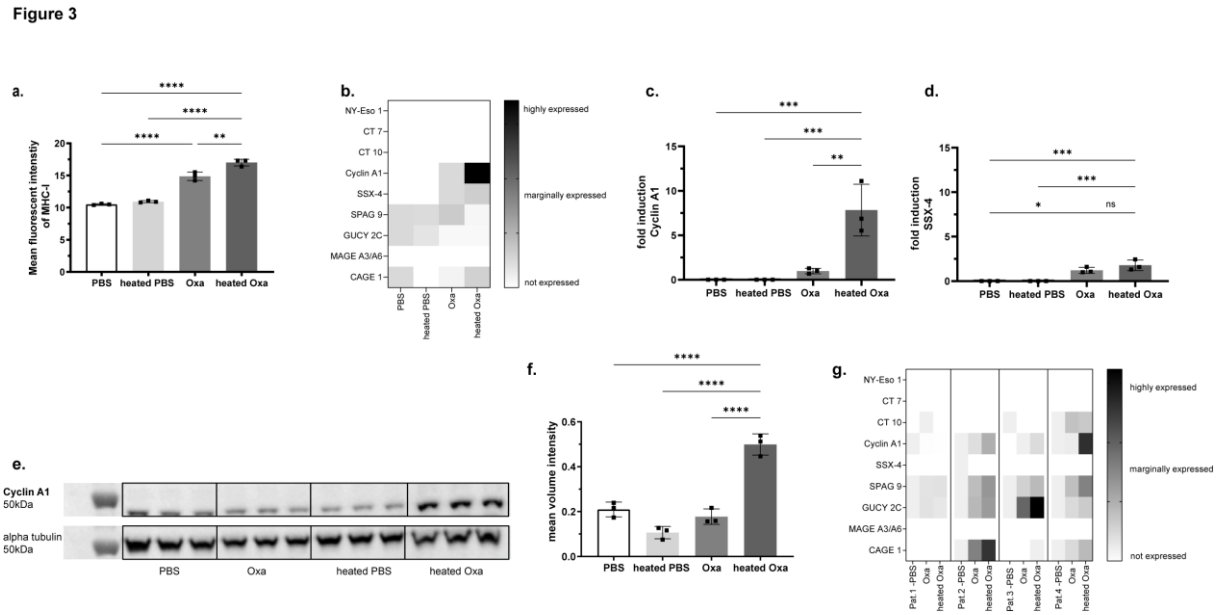
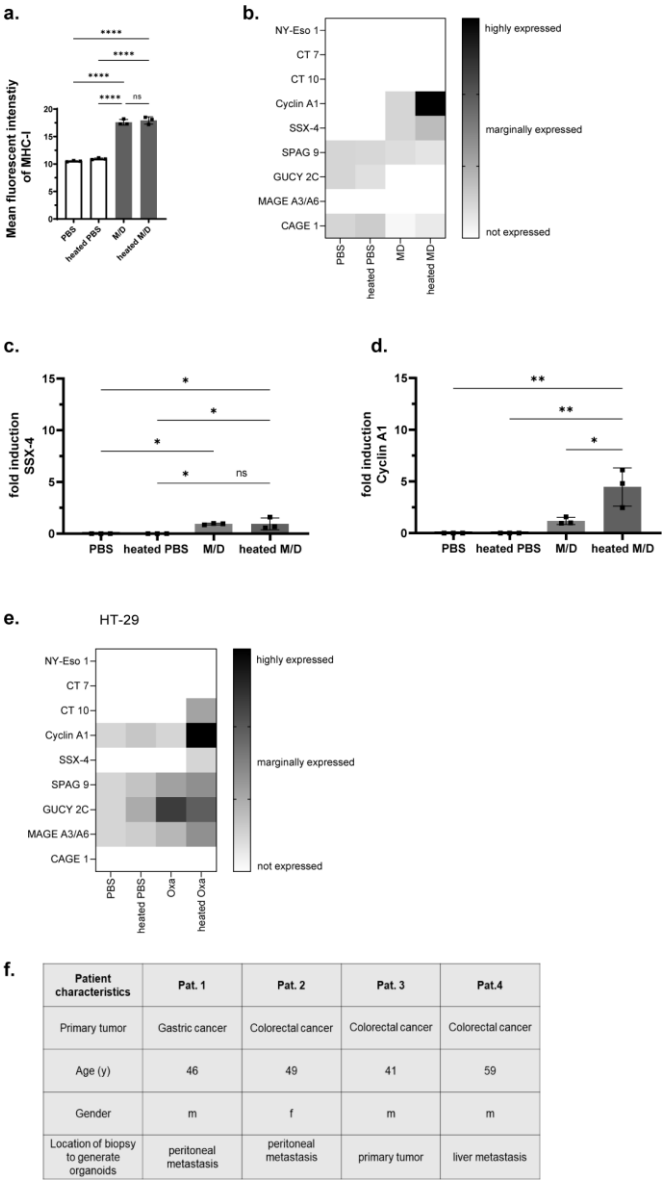


Figure 3: The effects of hyperthermic chemotherapy on colorectal cancer cell-lines and on patient derived tumor organoids. (a) *MHC-I expression on treated HCT-8 cells.* ***(b)*** *The heat-map shows the expression profile of nine different CTAs of untreated and treated HCT-8 cell-line samples.* ***(c and d)*** *The fold induction of Cyclin A1 and SSX-4 expression.* ***(e and f)*** *Western Blot for Cyclin A1 and the quantification of the protein expression.* ***(g)*** *CTA expression of four different patient derived tumor organoids after oxaliplatin treatment. The experiments were performed in triplicates. One representative experiment out of three is shown. Error bars show the mean +/-SD. **** = p≤0.0001, *** = p≤0.001, ** = = p≤0.01, * = p≤0.05, ns = p>0.05.*

Furthermore, to understand the effects of heated chemotherapy directly on primary patient material, we utilized patient-derived tumor organoids (for patient information please see **Supplementary Figure 5f**) and treated tumor organoids as we treated cells in the experiments above. Similar to cell lines data, tumor organoids from colorectal cancer patients (2 to 4) depicted higher expression of CyclinA1 upon treatment with heated chemotherapy while patient 1 with a gastric tumor did not show any change in CTA expression (**Figure 3g**). These observations are interesting, as they reflect differences in CTA expression after treatments probably

due to their primary tumor location. The number of induced CTAs also differed between patient tumor organoids and might reflect the individual immunogenic reaction towards the treatments.

Supplementary Figure 5



f.

Patient characteristics	Pat. 1	Pat. 2	Pat. 3	Pat.4
Primary tumor	Gastric cancer	Colorectal cancer	Colorectal cancer	Colorectal cancer
Age (y)	46	49	41	59
Gender	m	f	m	m
Location of biopsy to generate organoids	peritoneal metastasis	peritoneal metastasis	primary tumor	liver metastasis

Supplementary Figure 5: Immunogenic effects on colorectal cancer cells after treatment with heated chemotherapy. (a) *heated M/D increases the expression of MHC-I molecules compared other treatments.* ***(b)*** *the expression pattern of CTA`s mediated by heated M/D.* ***(c and d)*** *Expression of SSX-4 and cyclin A1 after different treatments.* ***(e)*** *Comparable expression pattern of CTA on HT-29 cell line.* ***(f)*** *table of patient characteristics from whom patient derived tumor organoids were prepared. One representative experiment out of three is shown. Error bars show the mean +/-SD.*

Heated chemotherapy elicits protective immunity

To confirm data obtained from our PM mouse model and to understand functional consequences of immunogenic changes on human cancer cells and patient-derived tumor organoids via heated

chemotherapies, we created a multistep in-vitro setup shown in **Figures 4a, 4c and 4e**This co-culture setting allows to study how immune cells can recognize treatment-induced immunogenic changes on cancer cells.

Figure 4

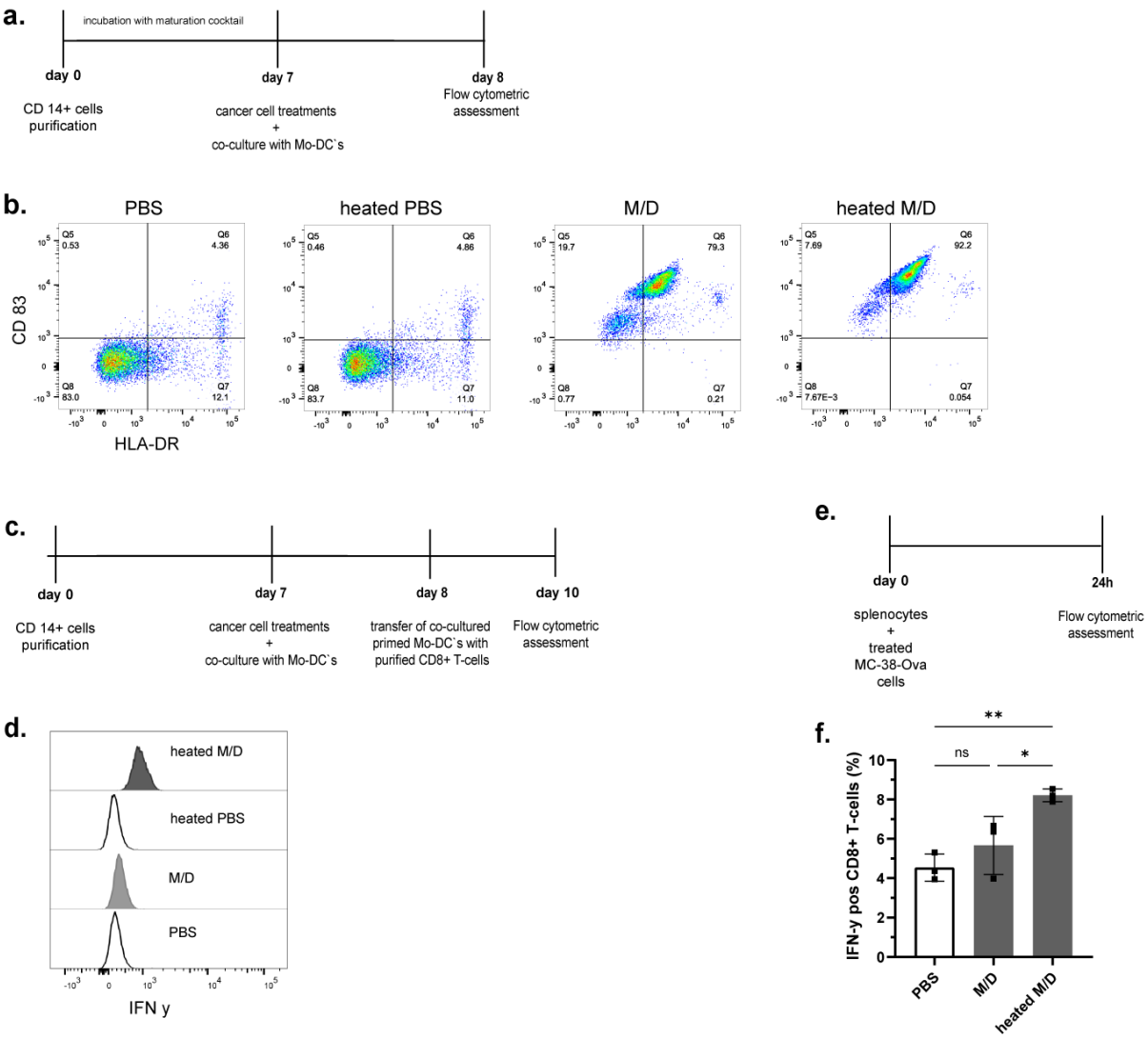
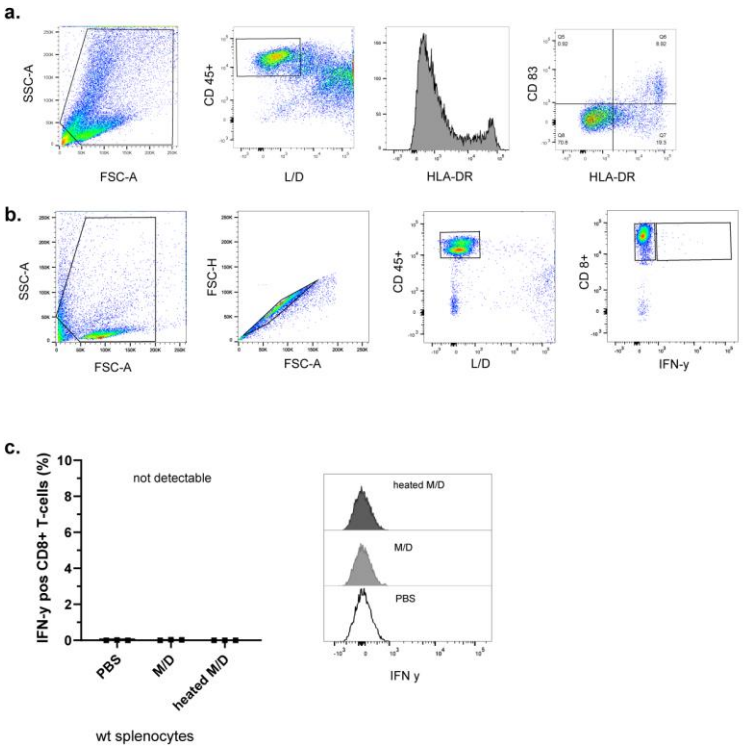


Figure 4: Induction of antigen-specific CD8+T cells via heated chemotherapy. (a) *Time-line of the experiment.* ***(b)*** *FACS data on the maturation state of Mo-DC`s depending on the cancer-cell treatment.* ***(c)*** *Time-line of the experiment with additional co-culture of CD8+ T-cells.* ***(d)*** *Shows FACS data of CD8+ T-cells and their IFN-γ production depending on the cancer-cell treatment.* ***(e)*** *Shows the time-line of a similar experiment using splenocytes from OT-I mice, which have a specific TCR for the ovalbumin.* ***(f)*** *Presents the ratio of IFN-γ positive CD8+ T-cells after co-culturing with PBS, or M/D or heated M/D treated MC-38-Ova cancer cells with splenocytes from a OT-I mouse. Error bars show the mean +/-SD. **** = p≤0.0001, *** = p≤0.001, ** = = p≤0.01, * = p≤0.05, ns = p>0.05*

First, we MACS-purified CD14+ monocytes and then initiated their differentiation into monocytic-DC (Mo-DC) as described in Methods. Seven days later, Mo-DCs were added to cancer cells that were pretreated with either PBS, or heated PBS or chemotherapy alone (M/D) or heated chemotherapy (M/D). Before adding MO-DC to treated cancer cells, multiple washing steps were performed to avoid any direct impact of chemotherapy

agents on MO-DCs. We then assessed activation/maturation of Mo-DC`s by analyzing expression of CD83 and HLA-DR molecules using flow cytometry (see gating strategy in **Supplementary figure 6a**). As shown in **Figure 4b**, we noticed marked expression of HLA-DR and CD83 on MO-DCs with heated chemotherapy when compared to chemotherapy without heat, while treatment with PBS± heat failed to enhance expression of these molecules. These results suggest that immunogenic changes following chemotherapy treatment are able to mature dendritic cells. To assess, whether matured DCs are able to activate purified autologous CD8 +T cells, we carefully collected MO-DCs from cancer cells co-cultures and then added them to MACS-purified autologous CD8 +T cells (**Figure 4c).** After 48 hours, we collected cells from co-cultures for flow cytometry and stained them for CD8 and for intracellular IFNγ. Gated CD8+ T cells (please see gating strategy in **Supplementary Figure 6b**) showed high levels of IFNγ when co-cultured with Mo-DCs that were primed with cancer cells treated with heated chemotherapy (**Figure 4d**). Interestingly, chemotherapy without heat induced significant MO-DCs maturation (**Figure 4b**) but did not activate CD8+ T cells to produce IFNγ.

Supplementary Figure 6



Supplementary Figure 6: Gating strategy (a) *gating strategy for the Mo-DC maturation experiment (exclusion of debris, gating on living immune cells, histogram for HLA-DR expression, gating for CD 83 against HLA-DR).* ***(b)*** *gating strategy for the intracellular IFN-γ production by CD8+ T-cells (exclusion of debris, gating on single cells, gating on living immune cells, gating on CD8+ against IFN -γ).* ***(c)*** *Co-culture experiment between different treated MC-38-Ova cancer cells and splenocytes from wt mice. No IFN-γ producing CD8+ T-cells could be detected.*

Furthermore, to elaborate whether CD8+ T cells responded in antigen-specific manner, we utilized an artificial antigen-specific in-vitro model as we lacked human T cells clones that would recognize an antigen (such as Cyclin A1) on treated cancer cells. In this setup, murine MC-38 OVA cancer cells were co-cultured with Ova-specific OT-1 CD8 + T cells. We used single cell suspension from whole spleen and did not purify CD8+T cells, so other cells within spleen could act as antigen presenting cells. In flow cytometry assessments, we noticed that gated CD8+ T-cells from spleen of OT-1 mice responded best when treated with heated M/D (**Figure 4f**)**,** while wild-type CD8+ T cells completely failed to respond to MC-38-Ova cells in all treatment conditions (**Supplementary Figure 6c**). This is expected as CD8+T cells from WT spleens were not exposed to Ova antigen before. Overall, such an in-vitro setup allowed us to show that compared to unheated chemotherapies, heated chemotherapy is able to induce more potent tumor-specific immunity.

Discussion

A subset of patients with CRC PM after CRS/HIPEC treatment show long-term survival. What causes the long-term survival of PM patients is largely unknown. We therefore hypothesized that the cellular composition of the peritoneal tumor microenvironment may be prognostic. Furthermore, we aimed to explore if HIPEC enables favorable protective immunological changes, which would explain the long-term survival observed in some patients. For this reason, we focused on examining the immune cell composition of PM lesions and particularly focused on the assessment of CD8+T-cells in PM lesions. We co-stained primary tumors and PM lesions for CD8+ T cells and pan-cytokeratin (CK) to mark intraepithelial areas. With a sophisticated method, we were able to divide the whole tumor area into three regions: stromal area, intraepithelial area, and necrotic area. Furthermore, the CD8+ T-cell count was analyzed for the whole (annotated) tumor region and each subarea, which turned out to be crucial, because the CD8+ T-cell count per annotated tumor area and stroma were not discriminative. We noticed that the CD8+ T-cell infiltration into the epithelium of PM lesions was crucial as it was associated with prolonged disease free and the overall survival of patients with PM originating from colorectal cancer. Patients with a higher intraepithelial CD8+ T-cell infiltration further presented a rather low stromal content, an observation also favoring prolonged survival. These observations from tissues of PM patients led us to test immunological benefits post HIPEC in-vitro and in murine models.

After optimizing an appropriate in-vitro system to mimic HIPEC-like conditions using heated chemotherapy, we could show immunological changes (CTA upregulation and MHC-I expression) happening on cancer cells and on patient derived tumor organoids. These results seemingly suggest that HIPEC may affect immunogenicity of the cancer cells leading to an efficient immune reaction providing long-term control of PM

lesions in patients. To understand such a process, we utilized our established PM mouse model[65,70]. We treated mice harboring micro-PM lesions locally with heated chemotherapies and relevant control treatments. We noticed that heated chemotherapy controlled growth of PM lesions. We found higher infiltration of CD8+ T cells within PM lesions of those mice that received heated chemotherapies. In the absence of CD8+ T cells, protective effects of heated chemotherapies were lost confirming the role of CD8+ T cells in control of PM lesions after treatment with heated chemotherapies. Further in-vitro experiments using ova-antigen specific CD8+T cells confirmed antigen specific response of CD8+ T cells. Thus, we suggest that HIPEC treatment can mount a local antigen-specific CD8+T cells response.

CD8+ T-cells are known to control tumor growth in the primary tumor [67,71,72] particularly from MSI type colorectal cancer [45,73] . In addition, data is available from hematogenous metastasis, where the CD8+ T-cell infiltration in liver oligometastasis from colorectal cancer was analyzed and a higher CD8+/CD3+ T-cell ratio correlated with a significant longer recurrence-free and overall survival [46]. In the peritoneal cavity however, the role of CD8+ T cells is poorly described. Our observations confirm the critical role of CD8+ T cells also in the peritoneum. However, the mere presence of CD8+ T cells within PM lesions only partially explains favorable outcomes and the functional state of CD8+ T cells is likely an additional decisive factor[74]. CD8+ T cell activation can be enhanced either via immunotherapies or by modulating the immunosuppressive tumor microenvironment. In the context of this study, we did not thoroughly explore the role of other cell types. For example, one study compared immune cell infiltrations between non-paired primary tumors and peritoneal metastasis [47]. This study found more NK cells in PM lesions whereas the primary tumor contained primarily CD8+ T-cells. A profound analysis of the PM-microenvironment may help to understand how the specific components interact and possibly attenuate the immune reaction in the peritoneum.

Our study observed that HIPEC can induce CD8+ T cell mediated tumor control in the mouse model. However, the molecular mechanism of HIPEC induced T-cell immunity within PM lesions remains to be explored. Induction of immunogenic cell death or a boost of a pre-existing immune reaction by cytotoxic drugs are potential mechanisms [36]. A recent study has shown that Mitomycin C in combination with hyperthermia triggers an immune reaction via Hsp 90 in a subcutaneous tumor mouse model [35]. While in-depth mechanisms remain to be elucidated, our data indicates that HIPEC does not only act through drug mediated tumor cytotoxicity but is able to induce immunogenic changes. This better explains the impressive impact on survival, observed in selected patients, and highlights the need for research with a different perspective. So far, cytoreductive surgery is seen as a purely tumor ablative procedure and drugs for intraperitoneal treatment are selected based on their cytotoxic profile. Increasing cytotoxicity however, may not improve the effect as observed by several clinical studies [75] but come at the price of increased postoperative complications [59]. In conclusion, our

data highlights that the presence of CD8+ T cells within PM lesions correlates with prolonged survival of human patients. In addition, we show that heated chemotherapies induce immunogenic changes on cancer cells leading to protective CD8+ T cells mediated immunity. Overall, we conclude that induction of protective CD8+ T-cell immunity may contribute to improved survival rates observed after multimodal treatment, including HIPEC. This study opens the door for further experimental and clinical research toward an immunomodulating role of locoregional intraperitoneal therapies.

4. Discussion

PM arising from CRC is considered a death sentence to patients. Patients with PM are often diagnosed very late, where limited treatment options are available. Most of the patients are given systemic treatments, which isn`t a curative treatment option. Due to lack of the fundamental research, very little is known about the pathophysiology of PM lesions thus PM-specific treatments are not available. In order to offer better treatment options to a patient with PM some of the clinicians started considering radical surgeries and local treatment. Paul Sugarbaker treated patients with PM with the so called CRS/HIPEC approach for the first time in the 90s[64]. During the cytoreductive surgery (CRS) procedure, the macroscopic/visible tumor lesions are removed. This surgical procedure is associated with an operation time for several hours and multiple resections of organs for example the spleen or a bowel resections are performed. In case of a synchronous presentation, the primary tumor will also be resected during CRS. Often microscopic metastatic lesions remain, for this reason hyperthermic intraperitoneal chemotherapy is performed with heated chemotherapy to eradicate remnant cancer cell or cancer cell clusters. Studies provide evidence, that patient selection is the key to achieve best outcomes for these patients. For examples, the extent of the disease (PCI), the grading of the tumor (G-status), the nodal status, the RAS/RAF mutation status, the performance status, the nutritional status, and comorbidities are essential to include the patients, that might profit from such a treaement[28]. Because the morbidity can range between 12% to 52%[76,77] and the mortality rate from 0.9% to 5.8%[76,77]. Even though, the development and standardization of the procedure and the training of surgeons has reduced morbidity and mortality but chances of surgical complications remain very high. Nevertheless, patients treated with CRS/HIEPC show mOS of 50 months compared to roughly 6 months OS without any treatment[30]. The current limitation of the treatment approach is the recurrence of the disease. The majority of patients will develop a recurrence in the peritoneal cavity, which is associated with an impaired survival compared to a recurrence in lung or liver[30]. The reason for the phenomenon is not yet understood. If the different tumor microenvironment or a different responsiveness to systemic chemotherapy might be the reason, could be explored in the future. The peritoneal recurrence is suspected to be due to an inefficient HIPEC treatment. Cancer cells or even clusters will remain in the peritoneal cavity and depending on the additive systemic chemotherapy treatment and the capability of the anticancer immune response, these will then form the peritoneal recurrence. As many unknown clinical facts in this field, induction of anticancer immune response remains an assumption requiring further clinical and basic research. To highlight again, patients treated with CRS/HIPEC normally receive systemic chemotherapy before the surgery as neoadjuvant treatment and are almost always treated with systemic chemotherapy after CRS/HIPEC, depending on the recovery status from CRS/HIPEC.

As the treatment is in clinical practice for a long time, no one doubted the effect of the HIPEC treatment, Although, no randomized controlled trial was ever performed. Surgeons just had a better feeling, to “clean” the abdomen with heated chemotherapy, even though systemic toxicity of the HIPEC treatment was reported. The first randomized controlled trial (RCT) on the effectiveness of HIPEC in the treatment of PM from ovarian cancer was published by a group from the Netherlands[78]. Van Driel et al. described a prolonged progression free and overall survival in the CRS + HIPEC group compared to CRS only. Furthermore, the side effects rate was not higher in the CRS + HIPEC group. In parallel, the French clinicians conducted the first RCT treating patients, almost 30 years after the start of this treatment approach, with PM from CRC also known as PORODIGE-7 trial[59]. The patients were either treated with CRS alone or CRS + HIPEC. HIPEC was only performed with Oxaliplatin. Unfortunately, this RCT turned out to be negative. HIPEC was not beneficial in terms of overall survival. They even detected more late postoperative complications with the combination treatment. These results were discouraging for the clinicians who were treating PM patients. However, the negative result is not astonishing, nor convincing, because the sample size calculation was based on a too high effect of the HIPEC treatment with around 11 months prolonged overall survival. No newly developed chemotherapeutic agent or even immunotherapies are in general able to achieve such a prolonged OS. Therefore, the included patient of n=265 were simply too less. Because the real effect was overestimated. Therefore, effectiveness of HIPEC is still debatable. The effect of complete resection during CRS is out of the question[79,80] More recently, French PM study group has already started a clinical trial investigating the influence of checkpoint inhibition after CRS/HIPEC, which may end up with a negative outcome as the role of CD8+ T-cells in PM lesions is so far unknown.

Currently, in the field of PM many clinical observational studies are out there but there is hardly any basic research lab that have explored the biology of PM lesions and mechanisms operating within the microenvironment of PM lesions. Therefore, we have very limited data available to assess exact impact of previous and upcoming data. Our laboratory is one of the pioneer laboratory that has both clinical and experimental model based mechanistic studies with a focus to develop PM specific treatments.

Therefore, based on our own observation in Zurich cohort, where few patients showing survival of up to 8 years after CRS/HIPEC, in this thesis I particularly focused on assessing the role of CD8+ T-cells on PM development. Moreover, I explored that HIPEC might contribute to that via activating an anticancer immune response.

We determined the CD8+ T-cells in CRC-PM patients with a sophisticated artificial intelligence based method. This allowed discerning the number of CD8+ T-cells in different areas of the tumor (annotated, stromal, intraepithelial). The intraepithelial location of CD8+ T-cells made the difference and elucidated the influence

on the survival of patients. Moreover, the study on the cohort of 19 -paired patient samples (primary versus PM) revealed that patients with a high number of intraepithelial CD8+ T-cells is correlated with a loose stroma and vice versa. The stroma rich and CD8+ T-cell low phenotype, similar to the CMS 4 subtype of colorectal cancer, is associated with a worse DFS and OS[67]. Thus, we demonstrate for the first time the clear influence of CD8+ T-cells on disease-free and overall survival. These results go along the findings of Seebauer et al[47], where they studied for the first time the tumor microenvironment of PM lesions and the primary tumor (non-paired) with regard to immune cell infiltration such as CD8+ T-cells, CD4+ T-cells, NK cells, B cells and also the proliferation capacity, as well as neovascularization. They report a functional reorganization of the PM tumor microenvironment with significant increased numbers of cytotoxic active NK cells, lower proliferation rates and senescent cancer cells, whereas the primary tumor presented more CD8+ T-cells. Unfortunately, this study lacked any correlation to survival. Overall, previously published and data included in this thesis strengthen the role of CD8+ T cells within PM lesions and warrants for future studies characterizing CD8+ T cells deeply within PM lesions in a large cohort of patients and analyzing their effects on patient survival.

Zunino B. et al described that HIPEC might induce an anticancer immune response [35]. Their study showed the induction of an immune response via the exposure of heat shock protein 90 (Hsp 90). They injected HIPEC pretreated cells in the mouse subcutaneously, which is not ideal as cells may have entirely different phenotype after in-vitro HIPEC and such an approach would also not show any direct on organs and cells that may come in contact with heated chemotherapies when performed in-vivo. In contrast to his experiments, In order to assess impact of HIPEC on the tumor microenvironment and tumor-specific immunity, I performed HIPEC in-vivo in a PM mouse model, which mimics the patients situation the best. To do so, I had to further optimize our PM mouse model[70]. The PM lesions should be at the time-point of HIPEC treatment visible, but still very small to mimic the clinical situation after CRS. Unfortunately, a complete CRS procedure would not have been technically possible in a mouse model. Our PM bearing mice were treated with Mitomycin C/Doxorubicin – based HIPEC, which resulted in a significant higher intratumoral CD8+ T-cell count and also significantly reduced tumor load. The assessment of the peritoneal tumor load turned out to be a real challenge. After assessing the peritoneal tumor load with in vivo imaging system (IVIS), which was not accurate, we decided to apply the murine PCI[65,81]. Survival, as used on our previous study by Lehmann et al[70]. was not allowed anymore for these kind of experiments by the cantonal ethics committee. Nevertheless, the PCI is a good instrument to assess the tumor load, but it certainly has limitations such as researcher experience, that may affect reproducibility and accuracy. In case of tumor microenvironment studies not related to treatment effects, the model development should go in a direction of spontaneous PM development[82]. Because the intraperitoneal injection of cancer cells doesn`t mimic the process of peritoneal metastasis. Mechanistically my

data revealed that CD8+ T cells were important to enhance tumor-specific immunity. To my knowledge I am the first who has used in-vivo HIPEC in PM mouse model to study impact on CD8+ T cells and their functions. As there was no mechanistic literature available how CD8+ T cells can be functionally primed by HIPEC, we followed literature coming from other cancer immunity replated publications and decided to analyze cancer testis antigens (CTA`s) as readout for immunogenicity after HIPEC. CTAs are interesting because their expression is restricted to tumors and germ-line cells and could therefore potentially serve as antigens for antigen directed immunotherapies[83,84]. We carefully selected 9 most frequent CTAs expressed in other cancers and analyzed their expression in HIPEC treated cancer cells and patient tumor organoids. Cyclin A1, a CTA expressed in acute myeloid leukemia (AML) patients[85] and also ovarian cancer patients[86], could be induced in two colon cancer cell-lines and patient derived tumor organoids upon treatment with heated chemotherapy in vitro. Due to lack of Cyclin A1 specific T-cells clones, we could not assess T-cell-mediated killing of Cyclin A1+ cancer cells. However, using MC-38-OVA cells that were treated with chemotherapies ± heat, we could show enhanced IFNγ production via OT-1 cells when they were in contact with those MC-38-OVA cells that were treated with heated chemotherapies.

Although, the effect of HIPEC is debatable or not yet completely understood, the procedure is routinely performed in the clinics. As CRS/HIPEC is an extreme invasive procedure may involve post treatment complications it is important to understand physiological reactions of the HIPEC treatment to make it safer for future use. The most common complications after CRS/HIPEC are infectious such as surgical site infections, gastrointestinal leakage, fistulas, intraabdominal abscesses or pulmonary infections[32]. Therefore, an early diagnosis of complication is crucial, because major morbidity has an impact on the outcome[31]. Up to now, mainly prognostic factors have been discussed in connection with infectious complications after CRS/HIPEC, which have an influence on the occurrence of infectious complications. For example the nutritional score, the PCI, the performance status and large bowel resections correlate with higher risks for infectious complications[87]. The postoperative monitoring of these patients consists of daily clinical examination and daily blood measurements of infectious parameters, renal function and blood count. An anastomotic leak is normally observed between postoperative day 4 to 6 after a bowel resection. Changes of inflammatory parameters can be early signs for such a complication. In our first clinical study, we described a significant CRP elevation between postoperative day 5 and 8. Exactly the time-point, when we would expect surgical infectious complications. Even though, the HIPEC procedure is considered to be a local treatment, systemic effects are reported. For example, Cisplatin can induce severe nephrotoxicity, Oxaliplatin can cause hemorrhagic complications and Mitomycin C influences WBC counts negatively in up to 39% of the patients[88-91]. Interestingly, these secondary CRP increase could only be detected after the application of a prolonged HIPEC

protocol and not after Oxaliplatin-based HIPEC. Other HIPEC protocols are performed with Mitomycin C/Doxorubicin or Cisplatin. Cisplatin is indicated for mesotheliomas or PM originating from ovarian cancer. We hypothesized as underlying mechanism for the secondary CRP increase a bacterial translocation from the small intestine; thus, detected significantly more bacterial DNA in the blood of these patients and an elevated pancreatic stone protein (PSP), as indirect signs for bacterial translocation. The description of that phenomenon is very important, which is illustrated in our second clinical study[34]. But beside the impact on the clinical monitoring, it would have been interesting to study, if this "unspecific" inflammatory response after the application of the prolonged protocol has an impact on disease free or overall survival. This we have not addressed in that study.

The consequence of this secondary CRP increase without an infectious complication requiring treatment is a reduced specificity in the diagnosis of an infectious complication. In contrast to our findings, Amroun K. et al. report that CRP levels are the best predictors of postoperative infectious complications after CRS/HIPEC[77]. Interestingly, they do not mention details on the HIPEC procedure, it can be assumed that they used Oxaliplatin for HIPEC as they included only colorectal cancer patients from a single center at France. The patients in our cohort were treated with 3 different HIPEC protocols, which allowed a comparison. Due to the secondary CRP increase after the prolonged protocol (Mitomycin C/Doxorubicin and Cisplatin), the specificity of CRP was dramatically reduced. After the Oxaliplatin-based protocol, CRP served as an accurate prognostic factor also in our study. Furthermore, we detected a dampened reaction of WBC counts after the application of a prolonged protocol in case of an infectious complication. This is ultimately reflected in a low sensitivity and illustrates systemic toxic effects of HIPEC treatment[92]. The conclusion of our findings is that the used HIPEC protocol is essential in the interpretation of inflammatory marker during the postoperative management of CRS/HIPEC patients. A CRP increase, especially between postoperative day 5 and day 8, after the prolonged protocol can either be a "physiological" inflammatory response or an infectious complication. To increase the accuracy of CRP, we recommend to additionally determine procalcitonin (PCT). PCT is produced by the C cells of the thyroidal gland upon a bacterial infection[93]. It has a high specificity in the diagnosis of bacterial infection and sepsis.

In this last section, I would like to highlight two other important facts studying PM patients and treatment related effects on treatment outcomes: the patient cohort is heterogenous and the HIPEC treatment itself consists of different variables. The patient cohort may differ in regard to primary tumor, extent of the disease, neoadjuvant treatment, additive treatment, occurrence of hematogenous metastasis and mutational status of the tumor. The HIPEC treatment includes variables such as the chemotherapeutic drug, the duration of the lavage and the applied temperature. A clinical study can never take into account all factors and if one tries to balance

cofounders, one might end up with a very small patient cohort. This is sometime quite frustrating and on the other hand it is a reality. Therefore, I can very well understand the surgeons, who continue performing HIPEC just with the Mitomycin C/Doxorubicin combination instead of Oxaliplatin. Because the PRODIGE 7 trial allows no conclusion for HIPEC effectiveness in general and the HIPEC benefit remains debatable. Performing further mechanistic studies on PM development and treatment response may help in refining HIPEC treatment regime offering more effective treatment with less side effects hopefully prolonging the survival of patients suffering with PM.

The experimental tools described in this thesis is just a beginning for further clinical and experimental research that will help in fully characterizing PM tumor microenvironment and in developing novel treatments. Overall, work performed in this thesis provides evidences that CD8+ T cells can control development of PM lesions; however, a direct clinical evidence is missing but mouse studies do show that CD8+ T cells control growth of PM lesions; Furthermore, CD8+ T cells in mouse model show that HIPEC induces protective immunity. Therefore, it is tempting to suggest the use of immunotherapies after HIPEC treatment, but we lack experimental studies to support such a proposal. As mentioned before, this is the first study that have used HIPEC in PM mouse to study immune responses; hopefully, future mechanistic studies will use improved HIPEC protocols directed towards mounting an efficient immune reaction. The clinical studies that became part of this thesis adds new ways of assessing early complications and infections occurring post CRS/HIPEC. Early detection of these is crucial as many of the patients die or suffer for a long time due to these complications. In conclusion, as a surgeon-scientist, I think this work will provide survival benefits to patients suffering with PM by early detection of postoperative complications and by further refinement of HIPEC protocols in the future.

5. Acknowledgements

First, I would like to thank Prof. Dr.med. K. Lehmann and PD Dr. A. Gupta for the opportunity to pursue my doctoral studies as the first PhD student in their research group. During the last years, I experienced interesting, challenging and encouraging discussions in the group and could finish a small part of our studies with the Master degree in Medical Biology and after that continue the studies as PhD student.

Second, but not least, I must thank Linda Russo, my PhD colleague and good friend. Without her, I would not have been able to finish my doctoral studies being a mom. She was not only helping me in performing most mouse experiments, but she also became a close friend and inspiring person in my life. Discussing and interpreting results from current experiments added an additional important value to my work.

Furthermore, I would like to thank Prof. Dr. Rolf Graf, who initially motivated me to do a research fellowship in the lab of him and Prof. Dr.med. Pierre-Alain Clavien. I also need to express a big thank to Theresia Reding-Graf, his wife. She was not only the good soul in the lab, she was/is also a talented teacher in biology and biological techniques. I could learn so much from you, many thanks Theresia.

I would also like to thank all the colleagues from the lab. I liked to work, laugh, cry and discuss with you.

Moreover, I would like to acknowledge my thesis committee, Prof. Dr. Maries van den Broek, Prof. Dr.med. Achim Weber and Prof. Dr.med. Michael Scharl for the support, thoughtful advice and helpful discussion during my PhD thesis.

Furthermore, I would also like to thank Prof. Dr.med. Chantal Pauli, she introduced me and our research group in the handling of patient-derived organoids and also donated us the organoids, we used to perform our experiments. Furthermore, she connected me to Prof. Dr.med. Viktor Koelzer. He was a very important and inspiring person I got to know during my PhD thesis. He introduced me to the world of artificial intelligence in analysing histological slides. The enthusiastic way to work with him and to discuss my results led to an important milestone of my project. Thanks a lot Viktor, it was great to learn from you.

A big thank you goes to my family, who supported me unconditionally throughout the whole time and, above all, always believed in me. And of course I would like to thank my husband Pascal for his support and the critical discussions that have undoubtedly made me what I am today. And last but not least, I thank Emil, our son, who joined us during the PhD studies. It's wonderful to have you and it was a great decision that I now get to spend a lot of time with you.

I thank all those who have been positive, supportive and also critical.

6. Curriculum vitae

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7. Figures and table

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Table 1	**The current TNM classification from colon cancer** Adapted from: URL: https://www.researchgate.net/figure/American-Joint-Committee-on-Cancer-AJCC-staging-system-for-colorectal-cancer-29_tbl1_236636422 Rodriguez-Fraile, M. et al. FDG/CT in colorectal cancer. Rev Esp Nuc. Imagen Mol. 2020

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