UNIVERSITÀ DEGLI STUDI DI PALERMO

Dottorato di ricerca in Oncologia e Chirurgia Sperimentali
Dipartimento di Medicina di Precisione in Area Medica, Chirurgica e Critica
(Me.Pre.C.C.)

CHARACTERIZATION OF TUMOR IMMUNE MICROENVIRONMENT IN PERITONEAL CARCINOMATOSIS TREATED WITH CYTOREDUCTIVE SURGERY AND HIPEC

Doctoral dissertation of:
Selene Sammataro

Tutor:
Prof. Claudio Tripodo

The Chair of the Doctoral Program:
Prof. Antonio Russo

2024 - Cycle 36

INDEX

1. ABSTRACT

Background: Peritoneal carcinomatosis is characterized by the dissemination of tumor cells within the peritoneal cavity. The immune microenvironment in peritoneal carcinomatosis is notably intricate and exhibits significant inter-patient variability. Influencing factors of the immune response in the peritoneal cavity include cancer type and stage, the presence of immunosuppressive cells, and the expression of immune checkpoint molecules. Despite the recognized complexity, there remains a paucity of research dedicated to comprehensively characterizing the immune microenvironment in this context.

Methods: Histopathological evaluation and immunohistochemical analyses were conducted on pre-CRS + HIPEC and post-CRS + HIPEC biopsies obtained from 12 patients. Quantitative analysis of signals was performed using Halo Image software (Indica Labs).

Aim: The objective of this study was to characterize the immune infiltrate in patients with peritoneal carcinomatosis secondary to gynecologic cancers and Pseudomyxoma Peritonei, who underwent Cytoreductive Surgery (CRS) in conjunction with Hyperthermic Intraperitoneal Chemotherapy (HIPEC), the primary therapeutic strategy for this condition.

Results: Our analysis revealed an enrichment of T and B cells in pre-CRS + HIPEC samples. Moreover, in 50% we detected a tendency to form microaggregates resembling tertiary lymphoid structures within the stroma. Additionally, we observed increased expression of PD1 and PD-L1 within these structures.

In post-CRS + HIPEC biopsies we observed a decrease of cellular density of immune cells, except for pro-tumoral M2 macrophages.

Conclusion: Further studies involving a larger cohort of cases are needed to investigate the immune microenvironment in peritoneal carcinomatosis in order to identify a potential prognostic and predictive biomarkers in both gynecologic cancers and Pseudomyxoma Peritonei.

2. SUMMARY

2.1. PERITONEAL CARCINOMATOSIS

2.1.1 ***Peritoneal carcinomatosis development***

Peritoneal carcinomatosis refers to the spread of tumor cells within the peritoneal cavity. This condition can arise primarily from the peritoneum, such as in mesothelioma and pseudomyxoma peritonei, or secondarily as metastases from intraperitoneal cancers (ovarian, gastric, colon) and extraperitoneal cancers (lung, breast, renal).

The peritoneum is a large serous membrane of mesodermal origin, divided into:

Parietal peritoneum: Covers the anterior and posterior abdominal walls.

Visceral peritoneum: Covers the organs.

Both types of peritoneum share analogies in term of composition; indeed they result constituted by glycocalyx, mesothelial cells, basal lamina, sub-mesothelial stroma, and elastic lamina. The primary role of the peritoneum is to maintain homeostasis in the abdominal cavity through peritoneal fluid, which allows molecular exchange with plasma. Additionally, it secretes glycosaminoglycans and surfactants, inhibits inflammation, facilitates leukocyte migration, presents antigens, and aids tissue repair[1].

The cancer cells implantation could be explained with the *Seed and Soil theory*: cancer cells (seeds) attaches prefentially to a specific microenvironment (soil)[2].

Cancer cell implantation in peritoneal carcinomatosis can be explained by the Seed and Soil theory, where cancer cells (seeds) preferentially attach to a specific microenvironment (soil).

1.Detachment: Cancer cells acquire a mobile phenotype through epithelial-to-mesenchymal transition (EMT). Downregulation of E-cadherin leads to detachment and the formation of multicellular clusters in ascites. Accidental surgical cutting into the tumor site can also facilitate malignant cell detachment.

2. Dissemination: A common symptom of peritoneal carcinomatosis is the accumulation of ascites. Cancer cells circulate and survive in the abdominal cavity by forming apoptosis-resistant clusters. In vitro studies show that clustered epithelial ovarian cancer (EOC) cells activate Akt kinase, preventing apoptosis and promoting survival by inhibiting Caspase-3 .

3. Adhesion: Multiple adhesion molecules facilitate interactions between cancer cells and the peritoneum. α5β1 integrin on cancer cells binds to fibronectin on mesothelial cells. In vitro, cancer cells adhere to mesothelial cells via CD43 expression . Inflammatory mediators promote ICAM-1 and VCAM-1 expression, ligands for integrins. Molecules like E-selectin, P-selectin, and CD44 are also involved in adhesion. Mesothelial cells can acquire a myofibroblastic phenotype, amplifying attachment. The omentum is a preferential site for attachment, likely due to fatty acid release from adipose cells and the presence of milky

spots—highly vascularized lymphoid aggregates providing chemokines and growth factors

4. Invasion: Cancer cells bypass the intact mesothelial layer by invading intercellular spaces or by inducing mesothelial cell removal/retraction. Milky spots near lymphatic stomata enhance adhesion through VEGF. Inflammatory cytokines like TNF-α and IL-1β cause mesothelial cell retraction, exposing the stroma. Cancer cells can induce mesothelial apoptosis via FAS-L/FAS interaction. Inhibition of myosin prevents mesothelial cell migration and removal beneath tumor spheroids, but does not affect spheroid adhesion. Once cancer cells breach the mesothelial layer and basal lamina, they access the underlying stroma, which supports survival, proliferation, and invasion. EOC cells express CXCR4, interacting with SDF-1. CXCR4 inhibition reduces peritoneal metastases growth and spread in mouse models, highlighting the SDF-1-CXCR4 axis's importance. The endothelial lining of lymph and blood vessels recruits immune cells, platelets, and possibly circulating stem cells, creating a tumor microenvironment that promotes tumor growth and invasion. Cancer-associated fibroblasts (CAFs) in the stroma produce cytokines and VEGF, remodel the ECM, and enhance tumor progression . Immune cells also contribute to peritoneal carcinomatosis progression .

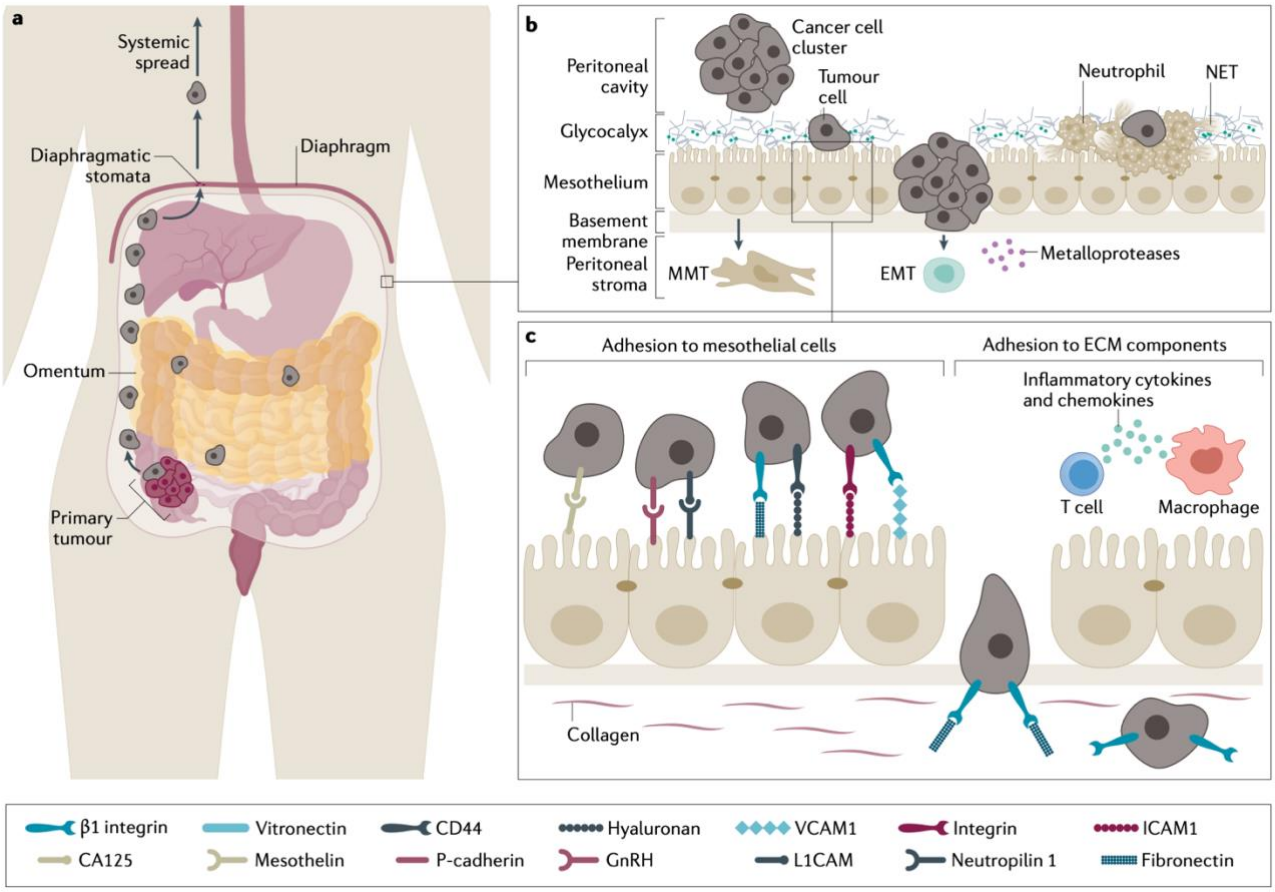
4 Invasion: Two mechanisms have been identified through which cancer cells circumvent the intact mesothelial cell layer that lines the peritoneal cavity: intercellular invasion and mesothelial clearance.

Intercellular invasion: cancer cells invade intercellular spaces between mesothelial cells. Milky spots[15], often located near lymphatic stomata (small gaps between mesothelial cells with direct lymphatic system connections), enhance adhesion through VEGF. Inflammatory cytokines like TNF-α and IL-1β, released by cancer cells, cause mesothelial cell retraction, exposing the underlying stroma[16].

Mesothelial Clearance: Cancer cells induce mesothelial cell apoptosis via FAS-L/FAS interaction[17]. Iwanicki et al. showed that downregulation or inhibition of myosin prevents the migration and removal of mesothelial cells underneath tumor spheroids[18]. Interestingly, spheroid adhesion to mesothelial cells was unaffected by myosin inhibition or blocking antibodies against α5-integrin. In a xenograft model, α5β1 integrin generated contractile force driving mesothelial cell retraction, promoting stromal invasion [19,20].

Once cancer cells breach the mesothelial cell layer and basal lamina, they gain access to the underlying stroma, which supports survival, proliferation, and invasion. EOC cells often express CXCR4, which interacts with SDF-1. In mouse models, CXCR4 inhibition reduced peritoneal metastasis growth and improved survival, indicating the SDF-1-CXCR4 axis's importance[20].

The endothelial epithelium recruits immune cells (monocytes, leukocytes, lymphocytes), platelets, and possibly circulating stem cells, creating a tumor microenvironment that promotes tumor growth and invasion. Cancer-associated fibroblasts (CAFs) in the stroma produce cytokines, VEGF, and remodel the ECM, facilitating tumor progression by enhancing proliferation, invasion, and metastasis[21]. Immune cells also contribute to peritoneal carcinomatosis progression, tumor growth, and invasion. [22]



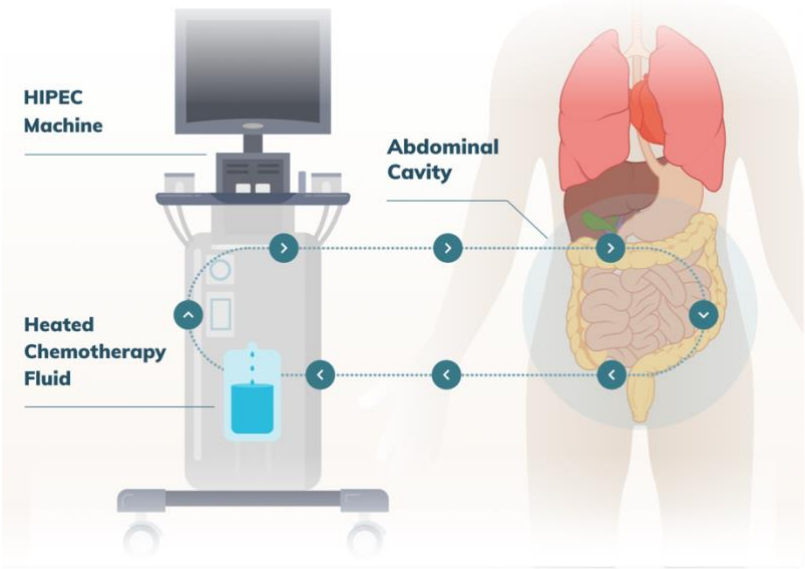
Peritoneal carcinomatosis development*. Cortés-Guiral, Delia et al. "Primary and metastatic peritoneal surface malignancies." Nature reviews. Disease primers vol. 7,1 91. 16 Dec. 2021, doi:10.1038/s41572-021-00326-6.*

2.1.2 *Cytoreductive surgery and HIPEC*

Peritoneal carcinomatosis poses signficant treatment challenges due to its advanced stage and the widespread distribution of tumor nodules throughout the peritoneal cavity. The most effective therapeutic approach currently available is cytoreductive surgery (CRS) followed by hyperthermic intraperitoneal chemotherapy (HIPEC)[23].

Cytoreductive surgery is designed to excise as much visible tumor tissue as possible from the peritoneal cavity. During this procedure, the surgeon conducts an extensive exploration and resection of tumor nodules from various abdominal regions. The primary objective is to achieve a complete or near-complete removal of all visible macroscopic tumors. The extent of the surgery is determined by the location and extent of the disease.[24]

HIPEC involves the administration of heated chemotherapeutic agents directly into the peritoneal cavity. This technique aims to eradicate any microscopic residual disease while minimizing the systemic side effects commonly associated with conventional chemotherapy.



Schematic representation of HIPEC machine. https://www.mesothelioma.com/treatment/chemotherapy/hipec/

This procedure improves the effectiveness of chemotherapy through several mechanisms including:

- **Thermal enhancement:** hyperthermia increases the membrane permeability and the drug cytotoxicity
- **Inhibition of repair mechanisms:** cells are less able to repair the damage caused by chemotherapeutic drugs

- **Activation of lysosomal enzymes:** hyperthermia can activate lysosomal enzymes.
- **Improved vascular flow in normal cells:** hyperthermia selectively favour blood flow in normal cells, reducing the toxic effects of chemotherapy on healthy tissues[25].

The heated chemotherapy solution is circulated throughout the abdomen for a specific duration, typically ranging from 1 to 2 hours.

Two different techniques for chemoperfusate administration are described and reported below.

The first technique is the *open technique* where the abdominal wall is lifted to create a funnel-like space. Inflow and outflow lines, connected to a pump and heating unit, are inserted into this space. The chemoperfusate is then circulated through these lines, allowing for a more even distribution within the abdominal cavity. Surgeons often prefer this technique because it allows them to perform anastomoses after HIPEC, minimizing the risk of compromising their integrity. However, there are some disadvantages to the open technique: heat dissipation can occur, potentially affecting the efficacy of the treatment and there is a risk of personnel exposure to the toxic chemotherapeutic agents.

The second technique is *closed technique.* In this method, the inflow and outflow lines are inserted through distinct incisions, and the abdominal wall is subsequently closed before the initiation of Hyperthermic Intraperitoneal Chemotherapy (HIPEC). This approach mitigates the risk of personnel exposure to antineoplastic drugs and facilitates superior temperature regulation. Nonetheless, a limitation of the closed technique is its association with uneven distribution of the chemoperfusate within the peritoneal cavity[26].

2.1.3 ***The clinical trials challenge***

Nowadays many challenges are associated with cytoreductive surgery followed by HIPEC administration.

The first challenge is related to the patient selection before the operation, which requires careful consideration potential benefits against risks including extent of disease, General Health Status, adequate organ function and Performance Status

Each patient should be evaluated individually, considering the specific cancer type, disease stage, overall health, and patient preferences, to determine the suitability of HIPEC as a treatment option[27,28]. Ultimately, the decision to proceed with HIPEC requires a multidisciplinary approach involving surgeons and oncologists, as well as input from other specialists, to ensure comprehensive patient care.

One useful tool for patient selection could be the analysis of quantitative prognostic indicators, such as the *"Peritoneal Cancer Index"* proposed by Sugarbaker. This index divides the peritoneal cavity into 13 regions, and a numerical score based on the size and extent of tumor involvement is assigned[29]. This method could serve as a prognostic factor because it allows for the estimation of the completeness of cytoreduction.

The definition of a specific value indicating complete cytoreduction is still controversial and varies according to the primary tumor. For example, in colorectal peritoneal carcinomatosis, a CCR-0 score is required for complete cytoreduction, while in pseudomyxoma peritonei, complete cytoreduction may involve both CCR-0 and CCR-1[30].

Clinical trials play a crucial role in evaluating the safety and effectiveness of CRS and HIPEC treatment, but the results are controversial and still debated within the medical community.

Various clinical trials have yielded diverse findings regarding the efficacy of Cytoreductive Surgery (CRS) and Hyperthermic Intraperitoneal Chemotherapy (HIPEC) across different cancer types.

In colon cancer, Verwaal et al. demonstrated improved overall survival (OS) with CRS + HIPEC treatment[31]; the French PRODIGE 7 trial did not show a significant OS benefit in patients treated with CRS and oxaliplatin-based HIPEC[32].

The COLOPEC trial highlighted that HIPEC, used as a prophylactic treatment for recurrent disease, did not improve disease-free survival compared to adjuvant chemotherapy alone.[33]

Similarly, in ovarian cancer, meta-analyses showed that CRS + HIPEC improved the 5-year overall survival rate compared to CRS and chemotherapy alone, but only in patients with primary ovarian cancer. However, this survival benefit was not observed in patients with recurrent ovarian cancer. [34]

Zhang et al. demonstrated that HIPEC correlated with better prognosis in patients with primary ovarian cancers, but for recurrent cancers, HIPEC only improved overall survival (OS) without a significant impact on progression-free survival (PFS)[35].

The OVHIPEC trial showed that interval debulking surgery + HIPEC improved PFS and OS compared to surgery alone[36].

In gastric cancer, meta-analyses suggest a general improvement in prognosis with CRS + HIPEC, especially in locally advanced cases[37], and prophylactic HIPEC has been associated with improved OS[38]. However, ongoing trials continue to provide further insights into the efficacy of CRS + HIPEC. Notably, in pseudomyxoma peritonei, retrospective analyses have indicated improved overall survival with CRS + HIPEC, though extensive disease is linked to higher postoperative mortality rates, underscoring the importance of meticulous patient selection.[39-41]

In summary, findings from trials assessing CRS + HIPEC demonstrate promise in specific scenarios. Nonetheless, it's crucial to acknowledge that the absence of standardized protocols presents challenges in constructing robust trials and reaching definitive conclusions.

2.2 TUMOR IMMUNE MICROENVIRONMENT

Tumor microenvironment is the complex ecosystem and dynamic milieu surrounding cancer cells within a tumor. It encompasses various cellular and non-cellular components, including stromal cells, immune cells, blood vessels, extracellular matrix, and signaling molecules. It plays a crucial role in tumor growth, invasion, and response to therapy. Immune microenvironment tumor-associated have a pivotal role in neoplastic progression in solid and haematological cancers[42].

Based on the level of immune activity within the tumor, the tumor microenvironment can be broadly classified into "hot" and "cold" categories. "Hot" microenvironment is characterized by a robust infiltration of immune cells, such as T cells, dendritic cells, and macrophages.

This heightened immune response is often accompanied by increased expression of programmed cell death ligand 1 (PD-L1) on tumor cells and programmed cell death protein 1 (PD-1) on cytotoxic T lymphocytes (CTLs) infiltrating the tumor. The interaction between PD-L1 and PD-1 suppresses the activity of CTLs, allowing tumor cells to evade immune surveillance. Immunotherapies targeting the PD-L1/PD-1 pathway can disrupt this interaction, unleashing the anti-tumor immune response and leading to tumor regression. Therefore, the presence of high PD-L1 expression on tumor cells and PD-1 expression on CTLs serves as a biomarker for predicting response to immunotherapy in tumors with a "hot" microenvironment.

Conversely, a "cold" microenvironment is characterized by a lack of immune cell infiltration and immunosuppressive factors, allowing tumors to evade immune detection and destruction. In cold tumors immune activity is restricted and a low expression of PD-L1 and PD-1 on tumor cells and CTLs respectively is detected [43].

The interaction between immune system and developing tumor is called “cancer immunoediting” and it consists of three phases:

1. **Elimination:** the immune system recognizes and eliminates tumor cells before they become clinically detectable. This phase involves immune cells, such as cytotoxic T cells and natural killer cells, attacking and destroying tumor cells through various mechanisms, including the release of cytotoxic molecules and induction of cell death.
2. **Equilibrium:** development of a dynamic equilibrium between tumor and immune system; in this phase, immune cells exert selective pressure on the cancer cells, favoring the growth of less immunogenic variants.
3. **Escape:** tumor acquire genetic and epigenetic changes that allow it to evade immune detection and destruction. These changes can include mutations that alter antigen presentation, downregulation of immune recognition molecules, or recruitment of

immunosuppressive cells to the tumor microenvironment. As a result, the tumor cells can proliferate and progress without being effectively targeted by the immune system[44].

In the tumor microenvironment, both innate and adaptive immune cells play a role in responding to cancer and their functions can have either pro-tumoral or anti-tumoral effects based on their cell type and signaling pathways.

Among the adaptive immune cells, cytotoxic T lymphocytes ($CD8^+$) exert anti-tumoral properties through various mechanisms. They can directly kill target cells through cytotoxicity, inducing apoptosis via FasL-mediated pathways, or by secreting molecules such as IFN-γ and TNF-α; furthermore, they directly recognize tumor cells and can be activated by dendritic cells and T- helper cells ($CD4^+$) cells through co-stimulatory molecules[45].

Regulatory T cells ($Foxp3^+$) are a specific subset of $CD4^+$ lymphocytes that contribute to immune evasion by suppressing the activity of other T cells through the secretion of molecules like IL-10 and TGF-β, which have immunosuppressive effects[46].

B cells can exhibit a dual role, with both anti-tumoral and pro-tumoral activities. Indeed B cells are able to produce antibodies against tumor antigens, thereby facilitating the recognition and elimination of cancer cells.

Conversely, a specific subset of B cells known as regulatory B cells can exert pro-tumoral effects. These regulatory B cells express molecules such as programmed death-ligand 1 (PD-L1) and produce immunosuppressive cytokines like interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β)[47].

Recent studies showed that in response to chronic inflammation or tumoral immune activation, inflammatory infiltrate composed of T cells, B cells, dendritic cells, organized into microaggregates known as tertiary lymphoid structures (TLS). TLSs are ectopic organized lymphoid aggregates sharing similarities with secondary lymphoid organs. Once matured, they consist of B-cell follicles, T-cell zones, and specialized antigen-presenting cells. Tertiary lymphoid structures contribute to the local immune response by facilitating the maturation and activation of B and T cells, resulting in antibody production and the generation of effector T-cell responses against the persistent antigen.

These structures facilitate the interaction between B-cells and T-cells, leading to the activation of the latter[48, 49].

Natural Killer (NK) cells, characterized by the CD3-CD56+ phenotype, exhibit features of both the innate and adaptive immune systems and play a crucial role in tumor

immunosurveillance and immune defense mechanisms. NK cells can be recruited to the tumor site through chemokines released by dendritic cells (DCs) and other immune cells and kills target cells through perforins and granzymes secretion[49].

Several types of innate immune cells, including dendritic cells, macrophages, and neutrophils, have crucial roles in the immune response against tumors.

Dendritic cells (DC cells), as mentioned above, act as antigen-presenting cells capable of activating CD8+, NK, and B cells[50].

Neutrophils in cancer can be categorized into two distinct phenotypes, named as N1 and N2. N1 neutrophils exert anti-tumoral functions through cytotoxicity, antibody-dependent cell-mediated cytotoxicity (ADCC), and activation of T cells.

On the other hand, N2 neutrophils exhibit strong immunosuppressive and tumor-promoting activity by releasing various factors such as HGF, oncostatin M, reactive oxygen species, reactive nitrogen species, MMPs, and neutrophil elastase[51].

Similarly, macrophages can be classified into M1 and M2 phenotypes.

M1 polarized macrophages release pro-inflammatory cytokines and oxygen nitrogen molecules that are crucial for eliminating tumor cells.

In contrast, M2 macrophages produce immunosuppressive molecules like IL-10 and TGF-β. They also contribute to angiogenesis and tissue remodeling through the secretion of VEGF and MMPs[52].

Lastly, myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of myeloid cells that significantly impact cancer progression and immune suppression. They inhibit the function of CTLs, NK cells, and DCs, while also promoting angiogenesis, tissue remodeling, and the development of pre-metastatic niches. These factors facilitate the spread of cancer cells to distant sites[53].

2.2.1 *Cytoreductive surgery and HIPEC effects on peritoneal immune microenvironment*

It has been noted that the tumor immune microenvironment in metastatic foci may differ from that observed in primary tumors due to signaling that is dependent on the tissue[54]. Given that the peritoneum is a common site of metastasis from abdominal cancer, comprehending the immune microenvironment in patients undergoing Cytoreductive Surgery (CRS) and Hyperthermic Intraperitoneal Chemotherapy (HIPEC) is essential for optimizing therapeutic approaches. Although the effects of hyperthermia on the peritoneal immune environment have not been extensively studied, the mechanisms described below are not specific to HIPEC.

Intraperitoneal heated chemotherapy induces the expression of Hsp90 on the surface of dying cells, leading to the activation of dendritic cells and T cells[55].

In mouse models of colorectal peritoneal metastasis, HIPEC resulted in increased infiltration of CD4+, CD8+, $CD68^+$, and $CD20^+$ cells into the omental and visceral peritoneum, along with overexpression of Hsp90 mRNA[56].

In the same model, combining HIPEC with anti-PD-1 treatment improved survival compared to HIPEC alone[57].

Mild hyperthermia enhances the recruitment of immune cells by stimulating vascular perfusion and upregulating vascular adhesion molecules such as ICAM-1, which promote immune cell infiltration[58].

Ovarian cancer patients presented an increased expression of PD-1 on $CD8^+$ cells and upregulation of Hsp genes after HIPEC treatment, indicating their activation. Additionally, immune pathways were upregulated[59, 60]. Hyperthermia also enhances the cytotoxic activity of $CD8^+$ cells, improving the expression of effector molecules such as perforin and granzymes.[61]

Regarding HIPEC following cytoreduction, analysis of peritoneal fluid in colorectal cancer patients showed a high CD4/CD8 ratio in patients with extensive cytoreduction and a borderline Peritoneal Cancer Index (PCI) score[62].

Current data suggests that heated IP treatment may have a biological impact, as evidenced by transcriptomic and proteomic changes that could serve as predictive or prognostic biomarkers.

Nowadays peritoneal carcinomatosis is a complex disease, and currently, our knowledge about the role of the immune system in this context and its related therapeutic effects remain limited.

Further studies are needed to better characterize the tumor microenvironment in the context of peritoneal carcinomatosis. to formulate efficacious treatment approaches.

3. AIM

The objective of this project was to investigate the infiltration of immune cells within the peritoneal microenvironment of patients diagnosed with peritoneal carcinomatosis, originating from various primary tumors. The study aimed to characterize the immunological microenvironment before and after Hyperthermic Intraperitoneal Chemotherapy (HIPEC) and assess any alterations following treatment.

4.MATERIAL AND METHODS

4.1 Patients and sample collection

According with the ethical standards of the Helsinki declaration 12 pre- and post-cytoreductive surgery + HIPEC peritoneal biopsies have been collected from patients treated at the departments of Gynecologic Oncology and Oncologic Surgery of the Hospital ARNAS Civico, Di Cristina and Benfratelli in Palermo. All patients presented peritoneal carcinomatosis derived from High Grade Serous Ovarian Cancer (n = 7), Endometrial cancer (n = 1), and Pseudomyxoma Peritonei (n = 4). Details are showed in the table below.

ID	AGE	DIAGNOSIS	NEOADJUVANT CHEMOTHERAPY	CRS + HIPEC
Pz1	61	HGSOC	YES	YES
Pz2	73	ENDOMETRIAL CANCER	YES	YES
Pz3	53	HGSOC	YES	YES
Pz4	42	HGSOC	YES	YES
Pz5	68	HGSOC	YES	YES
Pz6	54	HGSOC	YES	YES
Pz7	47	HGSOC	YES	YES
Pz8	54	HGSOC	YES	YES
Pz9	44	PSEUDOMYXOMA PERITONEI	NOT	YES
Pz10	65	PSEUDOMYXOMA PERITONEI	NOT	YES
Pz11	53	PSEUDOMYXOMA PERITONEI	NOT	YES
Pz12	70	PSEUDOMYXOMA PERITONEI	NOT	YES

4.2 HIPEC

After cytoreduction, HIPEC was administered using the "Closed Technique" with the PRS System (A.C.T.A Group, Naples). Cannulas were inserted into the abdominal cavity and the incision was closed. A preheated volume of Physiological Solution circulated for 5-10 minutes to raise the cavity temperature to 42°. Once the temperature was stabilized, the administration of chemotherapy drugs began. The therapy lasted for 90 minutes, during which the drug was infused along with CO_2, creating a bubbling effect that increased pressure within the peritoneal cavity. This ensured better coverage and thermal homogeneity. Upon completion of the therapy, the cavity was emptied and final wash with Physiological Solution at room temperature (using the same initial filling volume) was performed for 5-10 minutes. After the wash the cannulas were removed, and the abdomen was definitively closed.

4.3 H&E staining

Samples were fixed in 10% neutral buffered formalin overnight (3800604, Leica Biosystems), washed in water and subsequently preserved in 70% ethanol before undergoing paraffin fixation. Four-micrometer-thick tissue sections were deparaffinized and rehydrated. Slides were stained using hematoxylin and eosin (Harrys' hematoxylin for Histology and Eosin Y alcoholic solution, Bio-Optica, Milano Spa). Slides were examined under a Zeiss Axioscope-A1 optical microscope (Zeiss, Germany), and microphotographs were captured at a magnification of 10x using an Axiocam 503 Color digital camera equipped with the ZEN2 imaging software (Zeiss, Germany).

4.4 Quantitative immunolocalization analyses

Immunohistochemical staining were carried out using the BOND-III Fully Automated IHC and ISH Staining System from Leica. According to the manufacturer's protocol, sections were unmasked with Epitope Retrieval Solution at pH6 or pH9 (RE7113-CE or RE7119-CE, Leica Biosystems). The sections were incubated with the following primary antibodies: mouse anti-human CD8 (4B11 clone, Leica Biosystems), CD4 (4B12 clone, Leica Biosystems), Foxp3 (236A/E7 clone, Leica Biosystems), CD163 (10D6 clone, Leica Biosystems), and CD20 (L26 clone, Leica Biosystems), rabbit anti-human PD-1 (CAL20 clone, Leica Biosystems) and PD-L1 (7310 clone, Leica Biosystems). Immunohistochemistry was developed using BOND Polymer Refine Detection Kit (DS9800, Leica Biosystems), which includes peroxidase block, post-primary reagent, polymer reagent, DAB (3,3′-Diaminobenzidine) chromogen, and Hematoxylin. Finally, the slides were dehydrated and mounted using Surgipath Micromount mounting medium (3801731, Leica Biosystems). For mouse anti-human CD21 (2G9 clone, Leica Biosystems), manual immunohistochemical staining was performed by unmasking using Epitope Retrieval Solution pH9 and, after primary antibody the signal was revealed using Signal Stain Boost IHC Detection Reagent AP Mouse (31926, Cell Signaling) and Vulcan Fast Red Chromogen Kit 2 (901-FR805-100923, Biocare Medical). All the sections were analyzed under Zeiss AxioScope A1 optical microscope (Zeiss, Germany) and microphotographs were collected at 10x and 20x magnification using an Axiocam 503 Color digital camera with the ZEN2 imaging software (Zeiss Germany). Quantitative analyses were performed by calculating the average percentage of positive cells in five non overlapping fields (200x magnification) using

the HALO Image analysis software (v3.2.1851.229, Indica Labs) and the output was expressed as the “percentage of positive cells”.

4.5 Statistical analysis

Graphics design and statistical analysis were conducted using GraphPad Prism (v9.1.1, GraphPad Software, San Diego, CA, USA).

5. RESULTS

5.1 T-cell in peritoneal carcinomatosis

To assess T cell infiltration in peritoneal carcinomatosis, we conducted immunohistochemistry for CD3 on biopsies collected from patients prior to cytoreductive surgery (CRS) and HIPEC.

Our analysis revealed the presence of $CD3^+$ cells in both the peritumoral and intratumoral areas, indicating a strong T cells recruitment.

Subsequently, we performed a quantitative analysis of the signal, confirming the initial findings. Specifically, by calculating the percentage of positive cells, we detected CD3 values ranging from 10% to 33% (Figure 1A-E).

Furthermore, we investigated the presence and distribution of two T lymphocyte subtypes: T-helper and T-cytotoxic cells. Immunohistochemistry and quantification analyses for CD4 and CD8 were performed (Figure 2A-E and 3A-E), showing a higher expression of $CD4^+$ cells compared to $CD8^+$ cells. We observed CD4 levels ranging from 11% to 47%, and CD8 levels ranging from 4% to 24%. The higher percentage of CD4 was probably due to the fact that this marker is also expressed by macrophages.

To assess the predominance of T-helper cells over T-cytotoxic cells, we calculated the CD4/CD8 fractions, which confirmed our results (Figure 4).

Additionally, we aimed to investigate potential differences in T-helper and T-cytotoxic cells between patients with peritoneal carcinomatosis originating from Gynecological cancers and those with Pseudomyxoma Peritonei. Significantly higher levels of $CD4^+$ and $CD8^+$ cells average percentages were observed in Gynecological cancers compared to Pseudomyxoma Peritonei (see Graphs in Figure 5A-B).

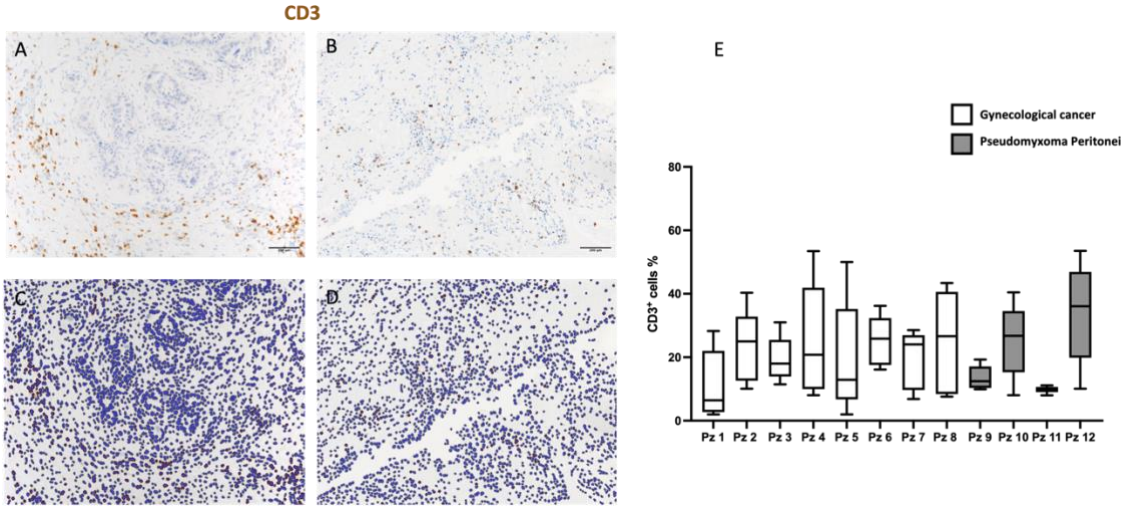


Figure 1. Representative microphotographs of $CD3^+$ cells in pre-CRS + HIPEC peritoneal carcinomatosis at 100x magnification; scale bar 200 μm (**A and B)**. Segmentation analysis output **(C and D).** Quantitative analysis of CD3 positive cells percentage **(E).** Values are represented as mean ± sd.

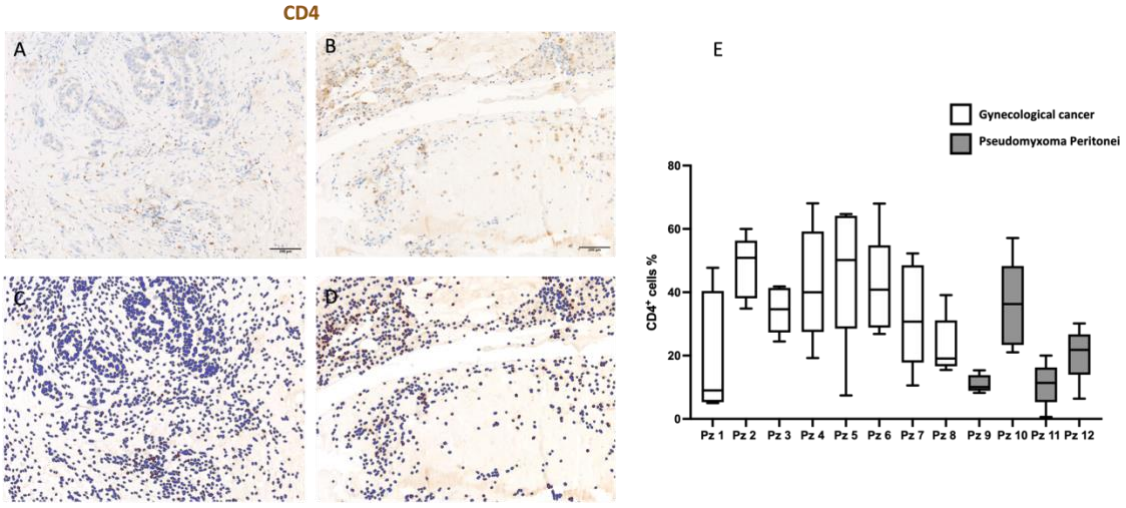


Figure 2. Representative microphotographs of $CD4^+$ cells in pre-CRS + HIPEC peritoneal carcinomatosis at 100x magnification; scale bar 200 μm (**A and B)**. Segmentation analysis output **(C and D).** Quantitative analysis of CD4 positive cells percentage **(E).** Values are represented as mean ± sd.

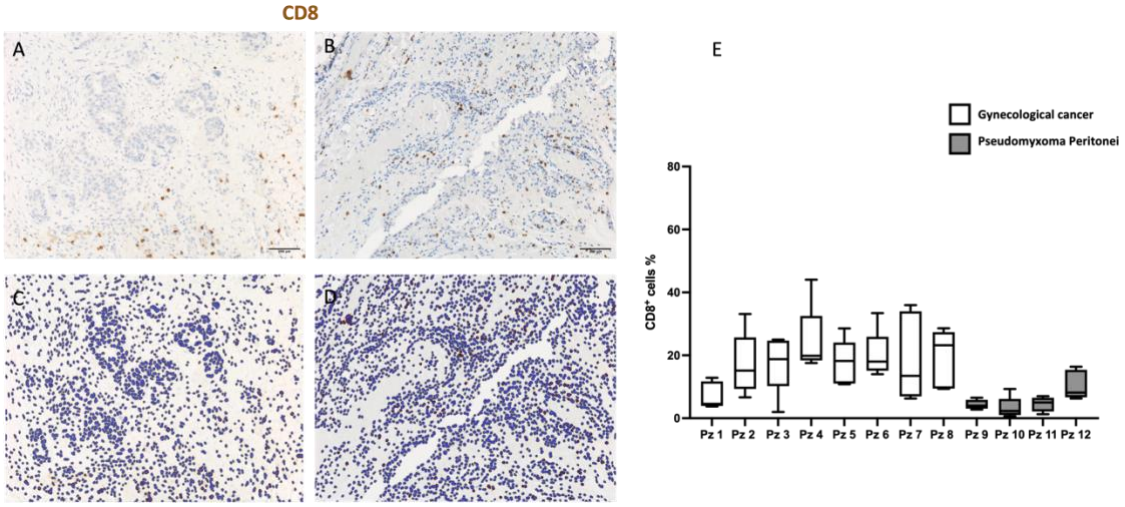


Figure 3. Representative microphotographs of $CD8^+$ cells in pre-CRS + HIPEC peritoneal carcinomatosis at 100x magnification; scale bar 200 μm (**A and B)**. Segmentation analysis output **(C and D).** Quantitative analysis of CD8 positive cells percentage **(E).** Values are represented as mean ± sd.

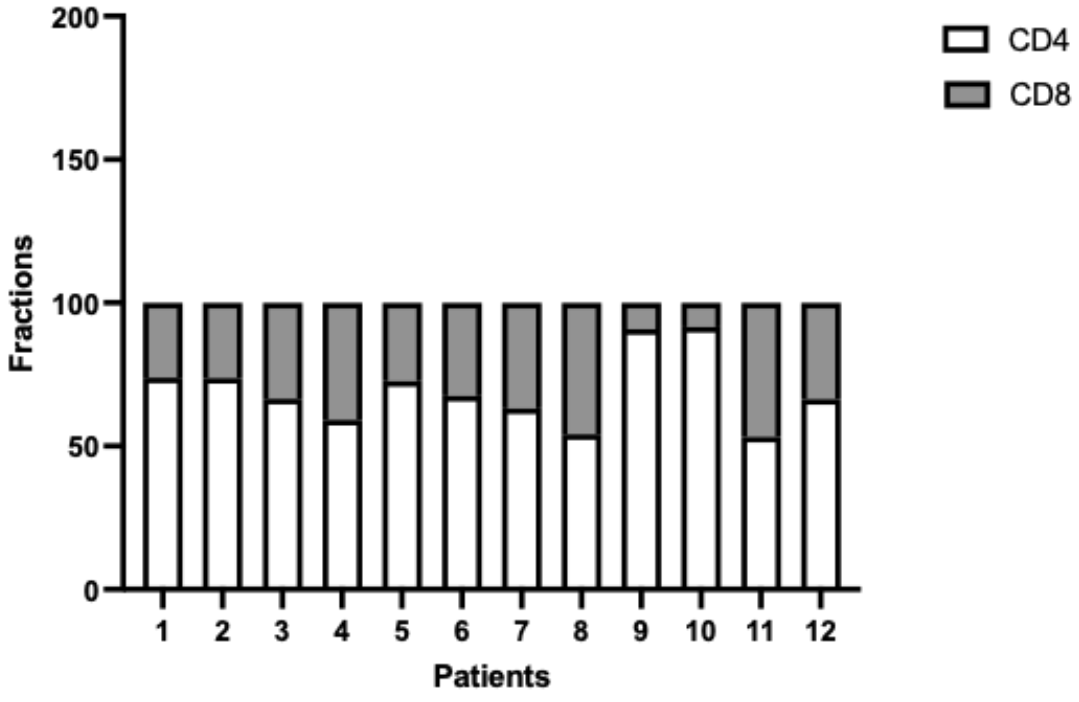


Figure 4. Representative graph of CD4/CD8 fractions.

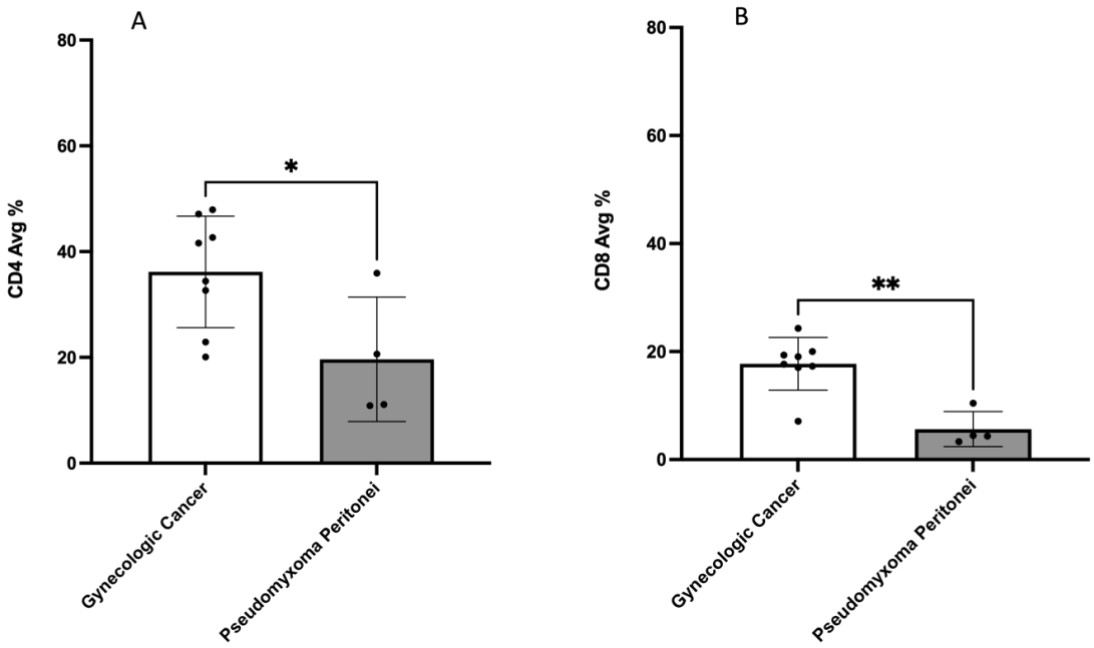


Figure 5. Comparative analysis of CD4 positive cells average percentage in patients with Peritoneal Carcinomatosis from Gynecologic Cancer vs patients with Peritoneal carcinomatosis from Pseudomyxoma Peritonei (unpaired t-test, p value <0,05) **(A)**. Comparative analysis of CD8 positive cells average percentage in patients with Peritoneal Carcinomatosis from Gynecologic Cancer vs patients with Peritoneal carcinomatosis from Pseudomyxoma Peritonei (unpaired t-tests, p value <0,05) **(B).** Values are represented as mean ± sd.

5.2 Immune suppressive cells in peritoneal carcinomatosis

We investigated the infiltration and distribution of M2 macrophages and T_{reg} cells in peritoneal carcinomatosis by performing immunohistochemistry for CD163 (M2 macrophage marker) and Foxp3 (T_{reg} cell marker) on pre-CRS + HIPEC samples.

Figure 6A-E highlight a robust M2 macrophages infiltrate in both the peritumoral and intratumoral areas; quantitative analysis confirmed these findings, demonstrating $CD163^+$ cells percentages ranging from approximately 17-37%.

To assess the predominance of this immunosuppressive cell population relative to cytotoxic T cells, we calculated the CD163/CD8 fractions, which confirmed that M2 macrophages were more abundant than $CD8^+$ cells (Figure 7).

T_{reg} infiltration was observed in both the peritumoral and intratumoral compartments and quantitative analyses revealed percentages of $Foxp3^+$ cells ranging from 0.05% to 1.5% (Figure 8A-E).

Finally, the comparative analysis of $CD163^+$ and $Foxp3^+$ cells average percentages did not reveal any significant variation between the Gynecological cancer group and the Pseudomyxoma Peritonei group (Figure 9A-B).

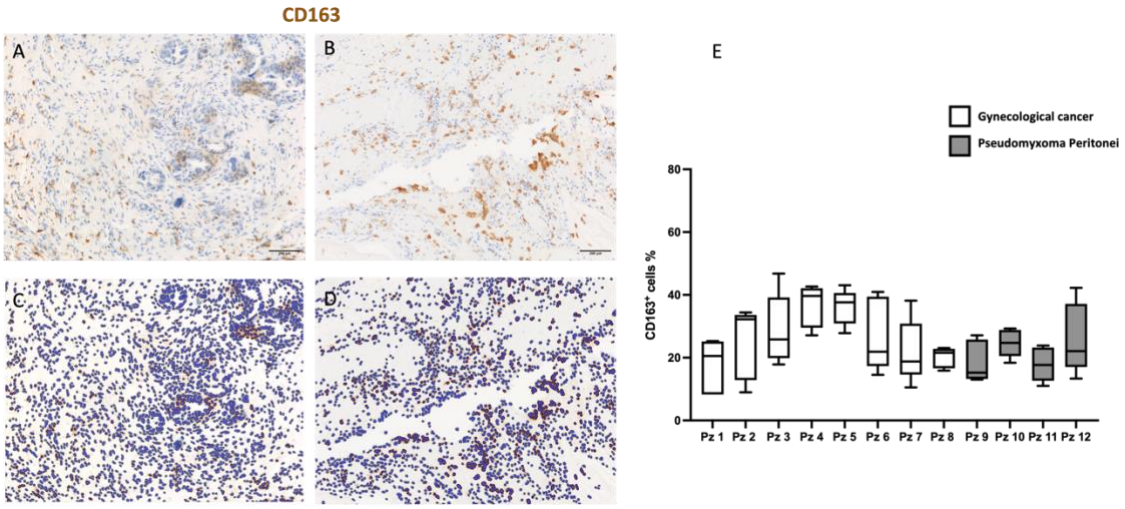


Figure 6. Representative microphotographs of CD163^{+} cells in pre-CRS + HIPEC peritoneal carcinomatosis at 100x magnification; scale bar 200 μm (**A and B)**. Segmentation analysis output **(C and D).** Quantitative analysis of CD163 positive cells percentage **(E).** Values are represented as mean ± sd.

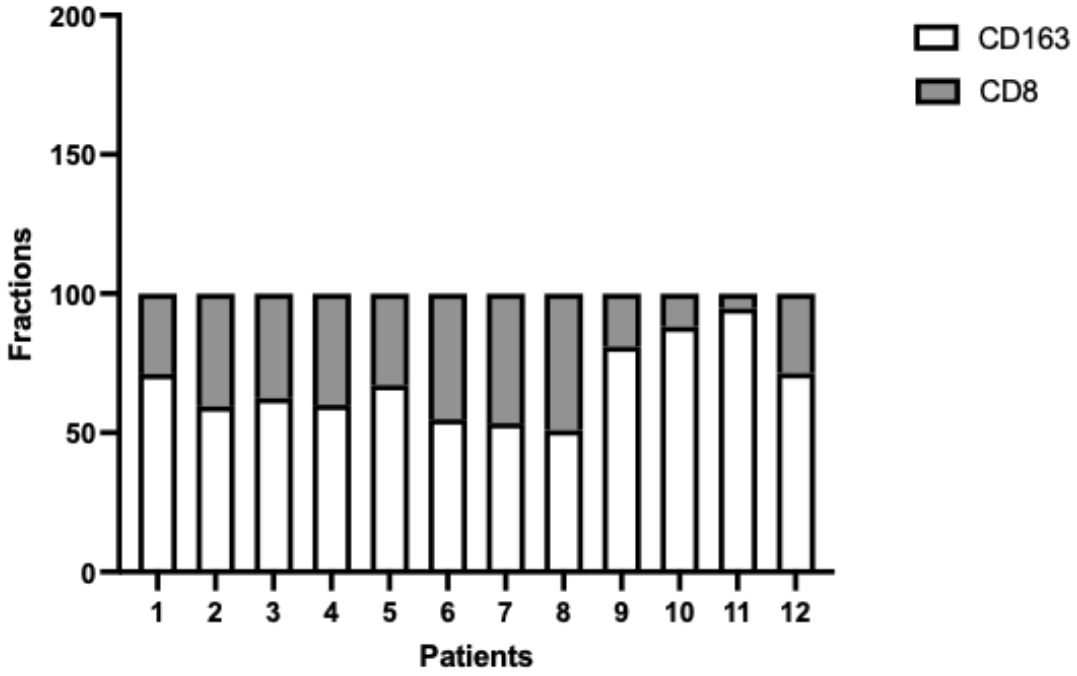


Figure 7. Representative graph of CD163/CD8 fractions.

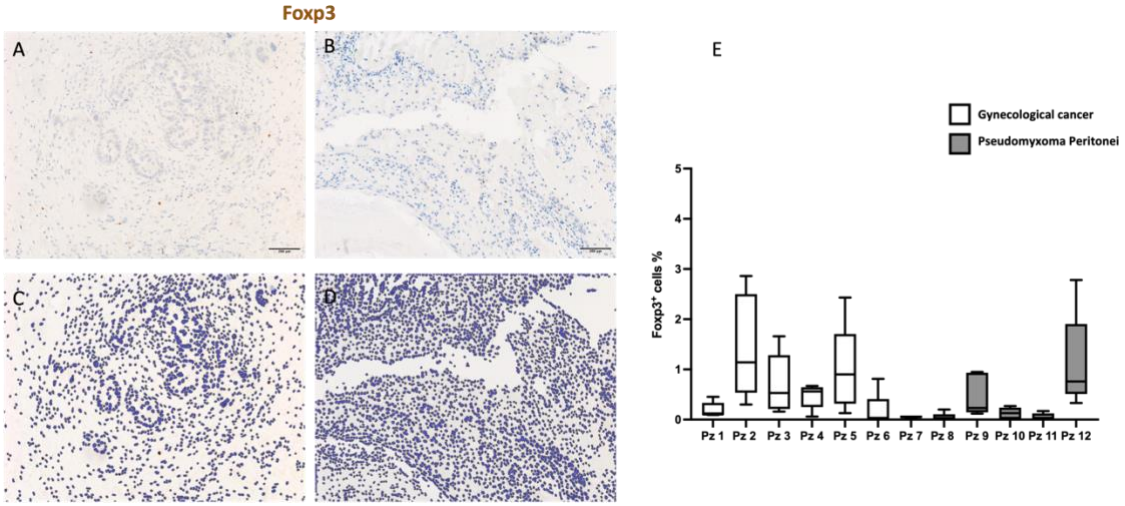


Figure 8. Representative microphotographs of Foxp3^{+} cells in pre-CRS + HIPEC peritoneal carcinomatosis at 100x magnification; scale bar 200 μm (**A and B)**. Segmentation analysis output **(C and D).** Quantitative analysis of Foxp3 positive cells percentage **(E).** Values are represented as mean ± sd.

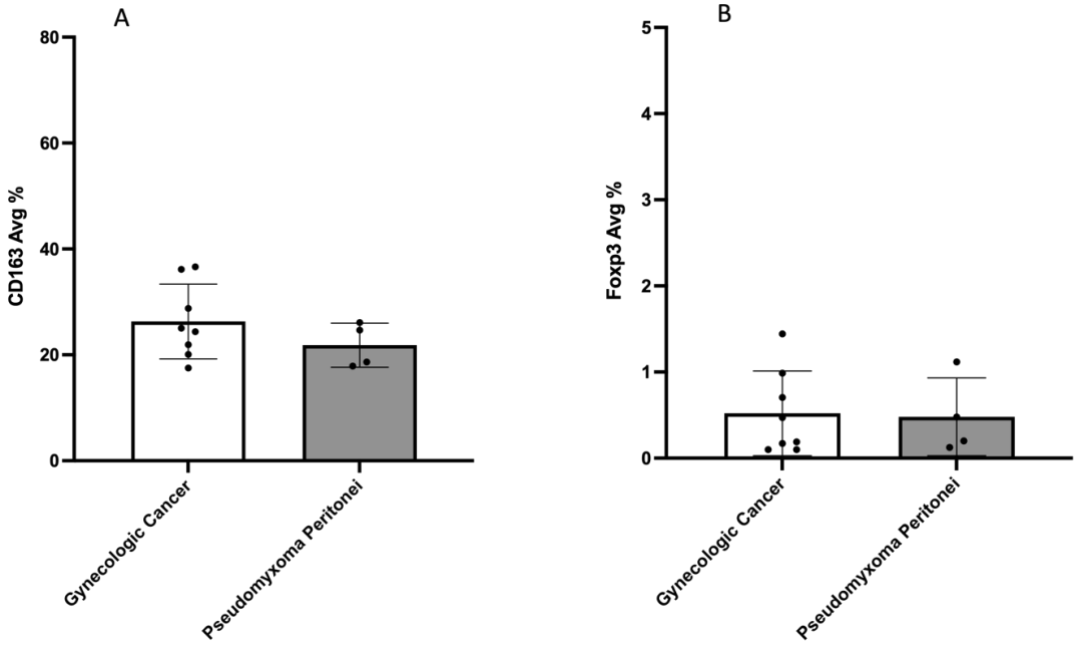


Figure 9. Comparative analysis of CD163 positive cells average percentage in patients with Peritoneal Carcinomatosis from Gynecologic Cancer vs patients with Peritoneal carcinomatosis from Pseudomyxoma Peritonei **(**unpaired t-test, p value >0,05) **(A)**. Comparative analysis of Foxp3 positive cells average percentage in patients with Peritoneal Carcinomatosis from Gynecologic Cancer vs patients with Peritoneal carcinomatosis from Pseudomyxoma Peritonei unpaired t-test, p value >0,05) **(B).** Values are represented as mean ± sd.

5.3 B-cells and immune microaggregates in peritoneal carcinomatosis

To detect B cells infiltration in peritoneal carcinomatosis, we conducted immunohistochemistry for CD20 in pre-CRS + HIPEC samples.

As depicted in Figure 10A-E, we identified a consistent population of B cells which were weakly distributed in intratumoral areas but tended to form microaggregates in the surrounding stroma. In the same figure is shown the quantification of $CD20^+$ cells percentage showed values around 10-39%.

Notably, no statistically significant differences were observed in the B cell average percentage between patients diagnosed with Gynecological malignancies and those with Pseudomyxoma Peritonei, as depicted in Figure 11.

Since the histological staining already highlighted the presence of cellular microaggregates with various shapes and sizes, we further investigated the presence of follicular dendritic cells by performing immunohistochemistry for CD21.

As demonstrated in the panels and table of Figure 12, approximately 50% of the patient samples harbored $CD21^+$ aggregates. These aggregates also expressed CD20, CD3, CD4, and CD8, leading us to consider them as potential forming tertiary lymphoid structures.

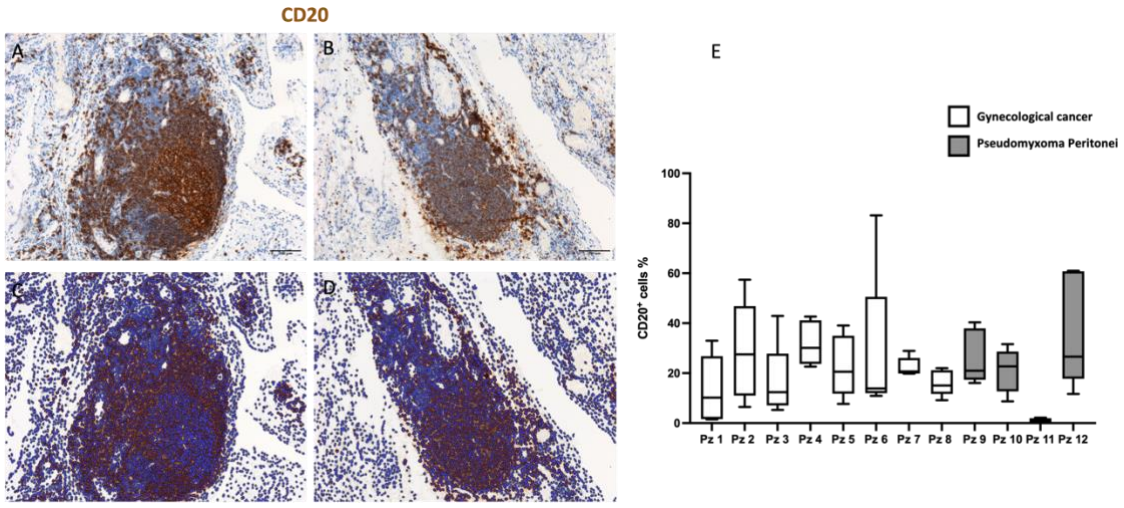


Figure 10. Representative microphotographs of $CD20^{+}$ cells in pre-CRS + HIPEC peritoneal carcinomatosis at 100x magnification; scale bar 200 μm (**A and B)**. Segmentation analysis output **(C and D).** Quantitative analysis of CD20 positive cells percentage **(E).** Values are represented as mean ± sd.

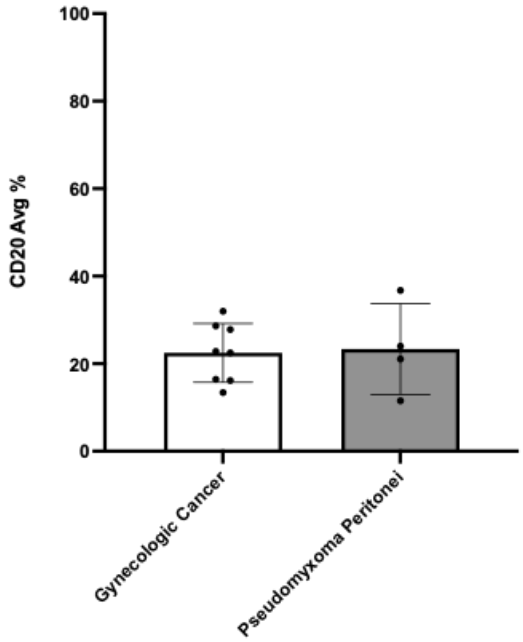
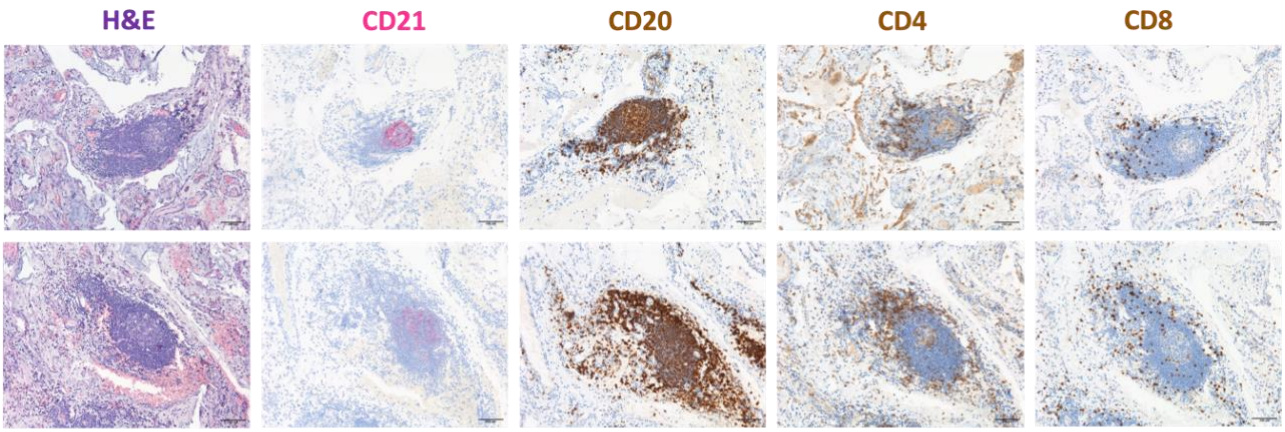


Figure 11. Comparative analysis of CD20 positive cells average percentage in patients with Peritoneal Carcinomatosis from Gynecologic Cancer vs patients with Peritoneal carcinomatosis from Pseudomyxoma Peritonei **(**unpaired t-test, p value >0,05**).** Values are represented as mean ± sd.



ID	Total Lymphoid aggregates	CD21 positive	CD21 negative
Pz1	0	0	0
Pz2	1	1	0
Pz3	5	0	5
Pz4	3	1	2
Pz5	6	0	6
Pz6	10	8	2
Pz7	2	2	0
Pz8	0	0	0
Pz9	6	4	2
Pz10	0	0	0
Pz11	3	0	3
Pz11	0	0	0
Pz12	49	43	6

Figure 12. Representative panel and count of lymphoid microaggregates in pre-treatment peritoneal carcinomatosis samples at 100x magnification; scale bar 200 μm.

5.4 PD1 and PD-L1 in peritoneal carcinomatosis

To investigate immune checkpoint expression in peritoneal carcinomatosis, we performed immunohistochemistry for PD-1 and PD-L1 in pre-CRS + HIPEC samples. Figures 14A-E and 15A-E illustrate a notable expression of these immune checkpoint proteins within lymphoid microaggregates. The corresponding quantitative analysis revealed variable percentages of these markers among patients, ranging from approximately 0.02% to 3% for PD-1 and 0.02% to 0.2% for PD-L1.

Furthermore, we conducted a comparative analysis between patients diagnosed with Gynecological malignancies and those with Pseudomyxoma Peritonei. This analysis revealed higher average percentages of PD-1 and PD-L1 proteins in the latter group of patients, as depicted in Figure 16.

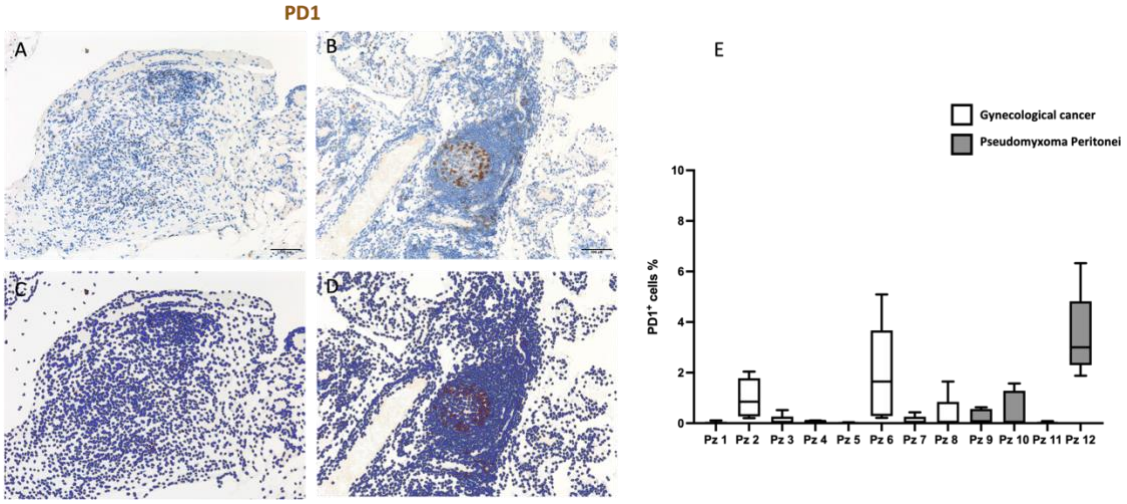


Figure 14. Representative microphotographs of PD1^{+} cells in pre-CRS + HIPEC peritoneal carcinomatosis at 100x magnification; scale bar 200 μm (**A and B)**. Segmentation analysis output **(C and D).** Quantitative analysis of PD1 positive cells percentage **(E).** Values are represented as mean ± sd.

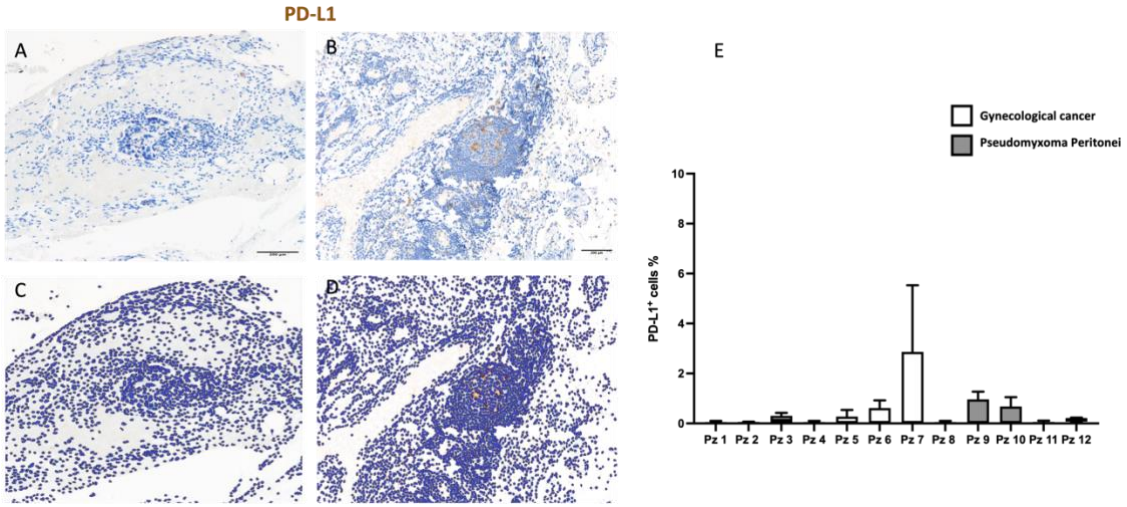


Figure 15. Representative microphotographs of PD-L1^{+} cells in pre-CRS + HIPEC peritoneal carcinomatosis at 100x magnification; scale bar 200 μm (**A and B)**. Segmentation analysis output **(C and D).** Quantitative analysis of PD-L1 positive cells percentage **(E).** Values are represented as mean ± sd.

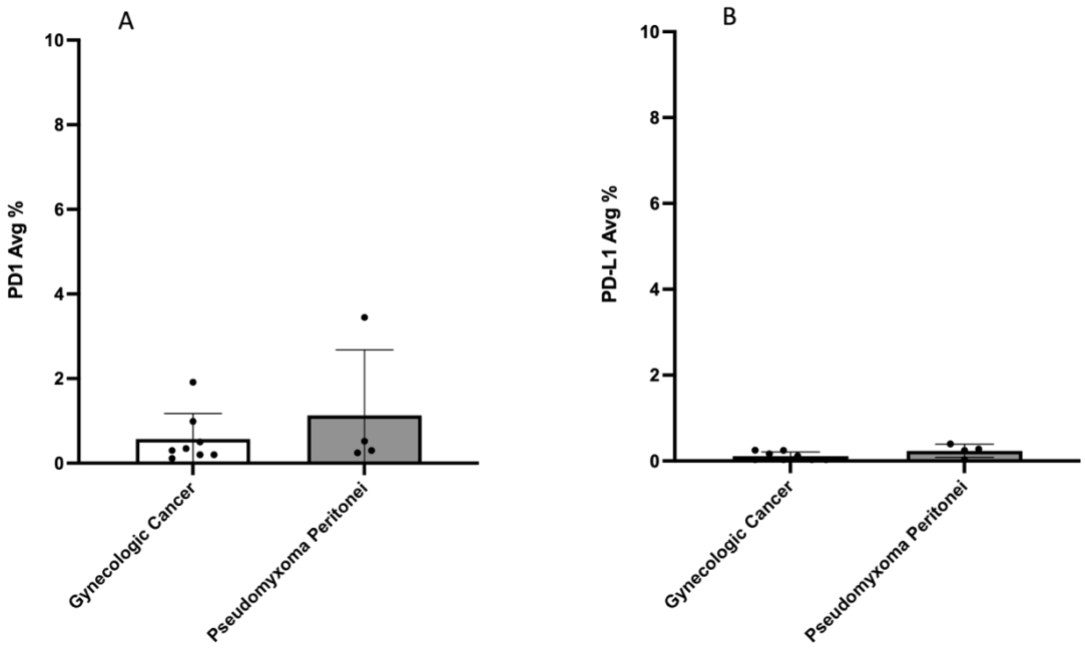


Figure 16. Comparative analysis of PD1 positive cells average percentage in patients with Peritoneal Carcinomatosis from Gynecologic Cancer vs patients with Peritoneal carcinomatosis from Pseudomyxoma Peritonei **(**unpaired t-test, p value >0,05) **(A).** Comparative analysis of PD-L1 positive cells average percentage in patients with Peritoneal Carcinomatosis from Gynecologic Cancer vs patients with Peritoneal carcinomatosis from Pseudomyxoma Peritonei unpaired t-test, p value >0,05) **(B).** Values are represented as mean ± sd.

5.5 Cytoreductive surgery and HIPEC effects on immune cells

The last goal of this study was to investigate the impact of cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) treatment on the immune cell infiltrate within the tumor microenvironment. To this end, we conducted immunohistochemical and quantitative analyses on post-CRS + HIPEC biopsies.

We specifically assessed the expression of CD3 (Figure 17A-E), CD4 (Figure 18A-E), CD8 (Figure 19A-E), CD163 (Figure 20A-E), Foxp3 (Figure 21A-E), CD20 (Figure 22A-E), PD1 (Figure 23A-E), and PD-L1 (Figure 24A-E).

The analyses were performed on a total of 7 post-CRS + HIPEC tumor biopsies, comprising 3 cases of Gynecologic cancer and 4 cases of Pseudomyxoma Peritonei.

Immunohistochemistry revealed the absence of cancer cells and lower levels of all the evaluated markers, except for M2 macrophages.

Quantitative analysis confirmed low T cells (1-8% for total T cells, 10-20% for T helper cells and 3-6% for T cytotoxic cells), B cells (1-14%) and T_{reg} cells (0.1-0.6%), as well as decreased levels of the immune checkpoint molecules PD-1 (0.02-0.2%) and PD-L1 (0.02-0.7%); however, the average percentage of M2 macrophage infiltration remained high, ranging from 14% to 40%.

To further validate these results, we conducted a comparative analysis between the post-treatment biopsies and their respective pre-treatment biopsies. The graphs in Figure 25A-H illustrate an overall reduction in all markers percentages, with significant results observed for total T cells ($CD3^+$) and B cells ($CD20^+$) cells; no differences were found in the percentages of M2 macrophages ($CD163^+$), which remained high after treatment.

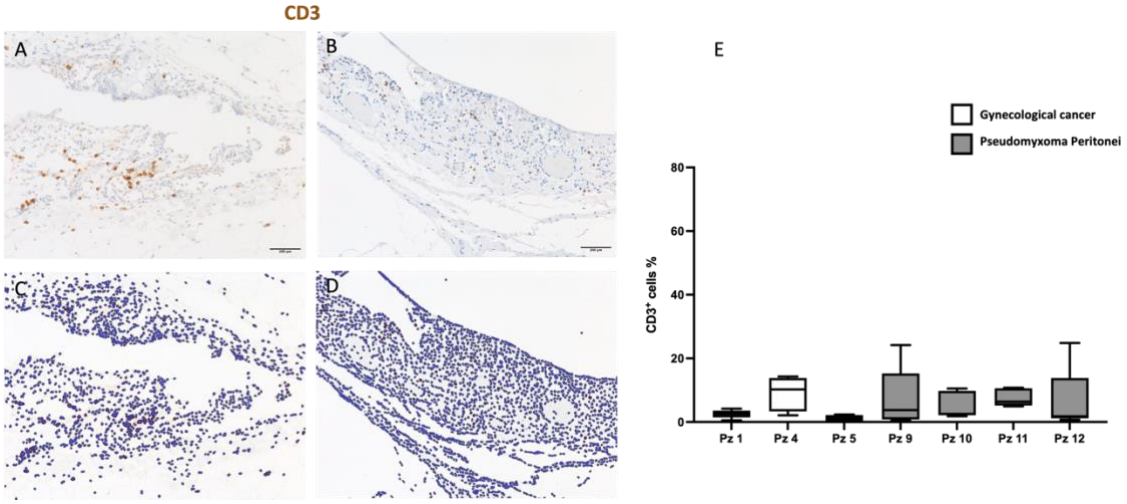


Figure 17. Representative microphotographs of $CD3^+$ cells in post-CRS + HIPEC peritoneal carcinomatosis at 100x magnification; scale bar 200 μm (**A and B)**. Segmentation analysis output **(C and D).** Quantitative analysis of CD3 positive cells percentage **(E).** Values are represented as mean ± sd.

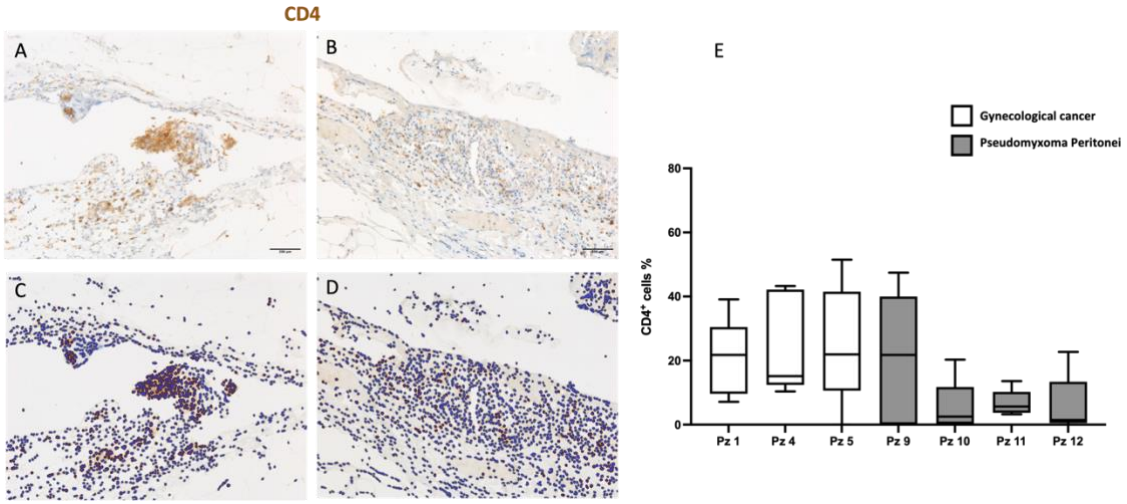


Figure 18. Representative microphotographs of $CD4^+$ cells in post-CRS + HIPEC peritoneal carcinomatosis at 100x magnification; scale bar 200 μm (**A and B)**. Segmentation analysis output **(C and D).** Quantitative analysis of positive cells percentage **(E).** Values are represented as mean ± sd.

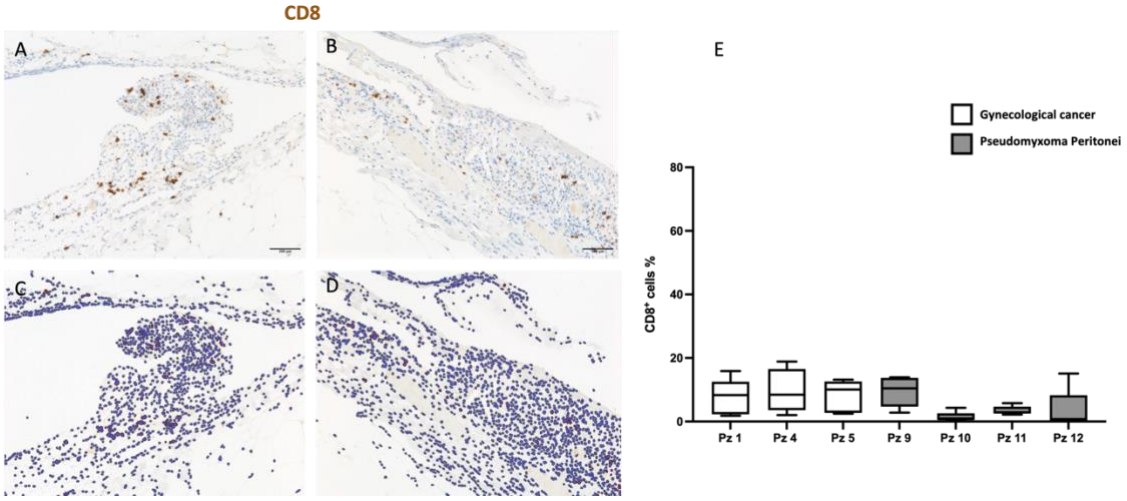


Figure 19. Representative microphotographs of $CD8^+$ cells in post-CRS + HIPEC peritoneal carcinomatosis at 100x magnification; scale bar 200 μm (**A and B)**. Segmentation analysis output **(C and D).** Quantitative analysis of CD8 positive cells percentage **(E).** Values are represented as mean ± sd.

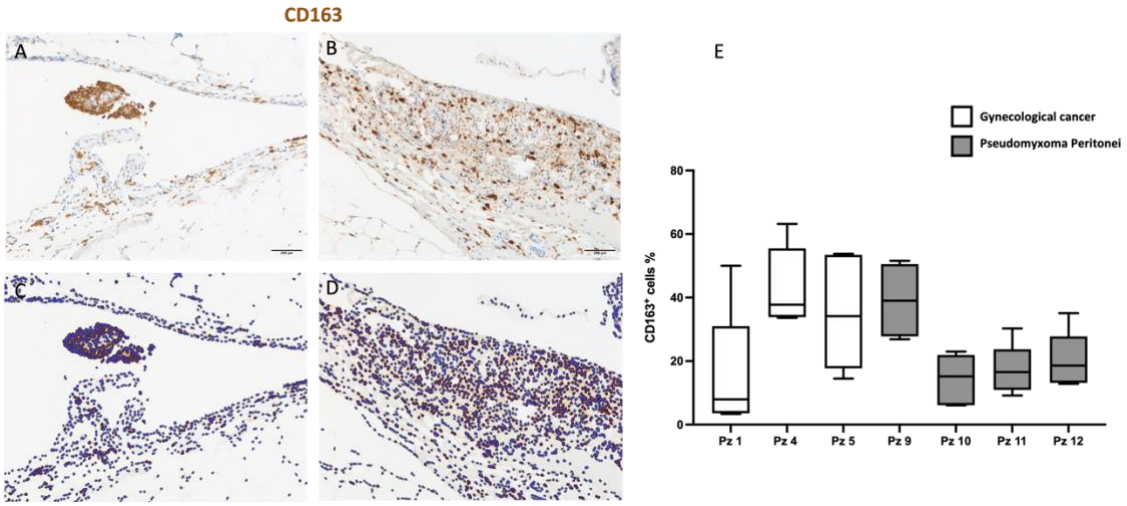


Figure 20. Representative microphotographs of $CD163^+$ cells in post-CRS + HIPEC peritoneal carcinomatosis at 100x magnification; scale bar 200 μm (**A and B)**. Segmentation analysis output **(C and D).** Quantitative analysis of CD163 positive cells percentage **(E).** Values are represented as mean ± sd.

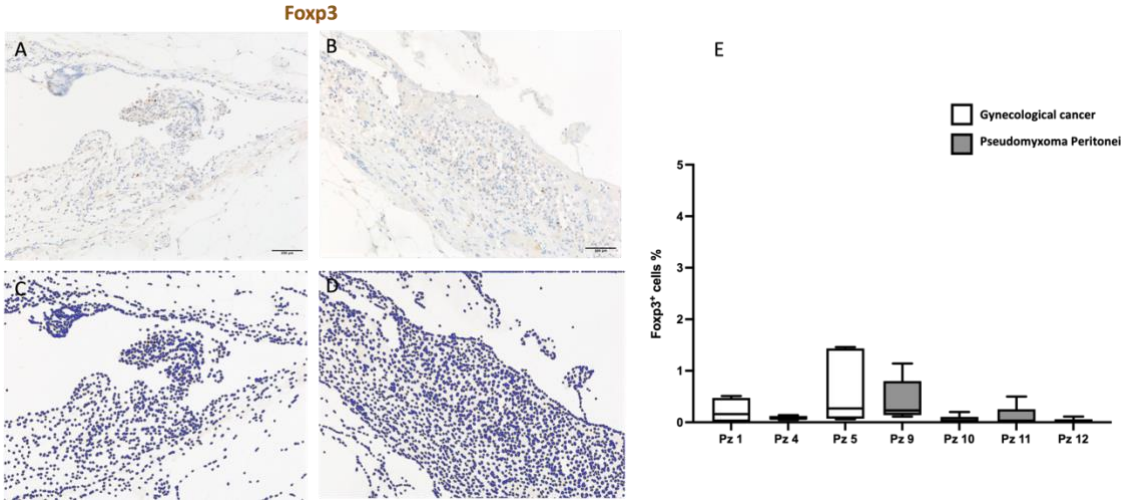


Figure 21. Representative microphotographs of Foxp3^{+} cells in post-CRS + HIPEC peritoneal carcinomatosis at 100x magnification; scale bar 200 μm (**A and B)**. Segmentation analysis output **(C and D).** Quantitative analysis of Foxp3 positive cells percentage **(E).** Values are represented as mean ± sd.

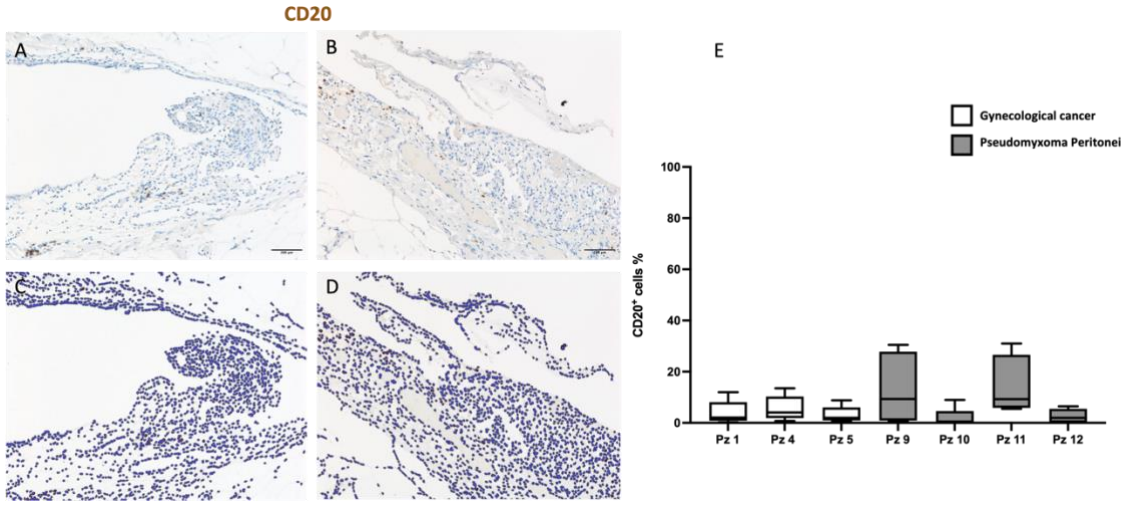


Figure 22. Representative microphotographs of CD20^{+} cells in post-CRS + HIPEC peritoneal carcinomatosis at 100x magnification; scale bar 200 μm (**A and B)**. Segmentation analysis output **(C and D).** Quantitative analysis of CD20 positive cells percentage **(E).** Values are represented as mean ± sd.

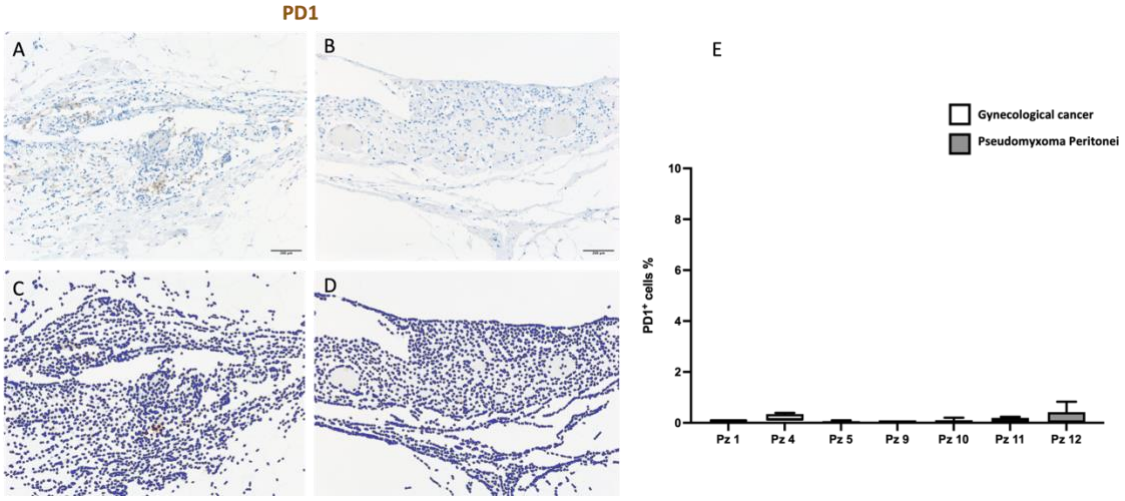


Figure 23. Representative microphotographs of PD1^{+} cells in post-CRS + HIPEC peritoneal carcinomatosis at 100x magnification; scale bar 200 μm (**A and B)**. Segmentation analysis output **(C and D).** Quantitative analysis of positive cells percentage **(E).** Values are represented as mean ± sd.

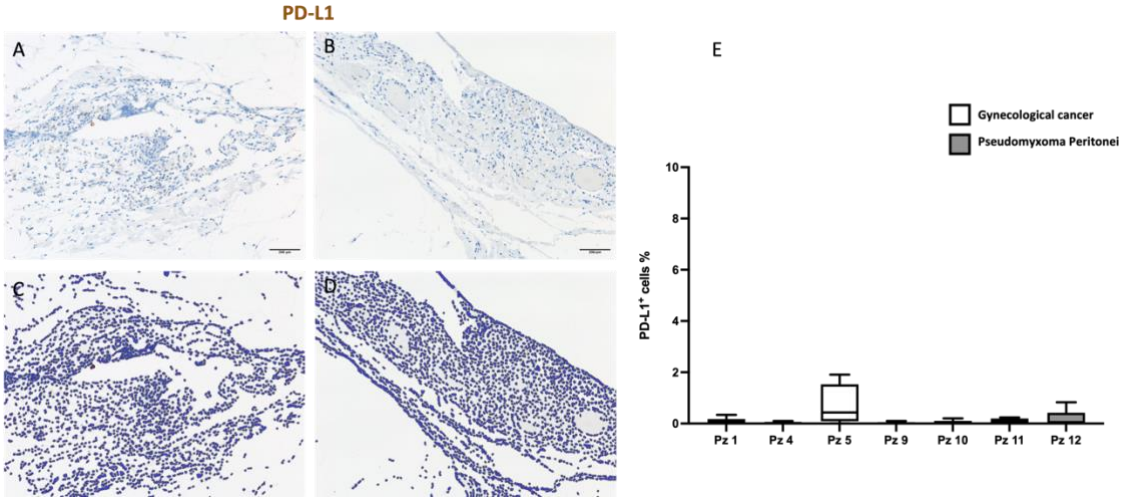


Figure 24. Representative microphotographs of PD-L1^{+} cells in post-CRS + HIPEC peritoneal carcinomatosis at 100x magnification; scale bar 200 μm (**A and B)**. Segmentation analysis output **(C and D).** Quantitative analysis of PD-L1 positive cells percentage **(E).** Values are represented as mean ± sd

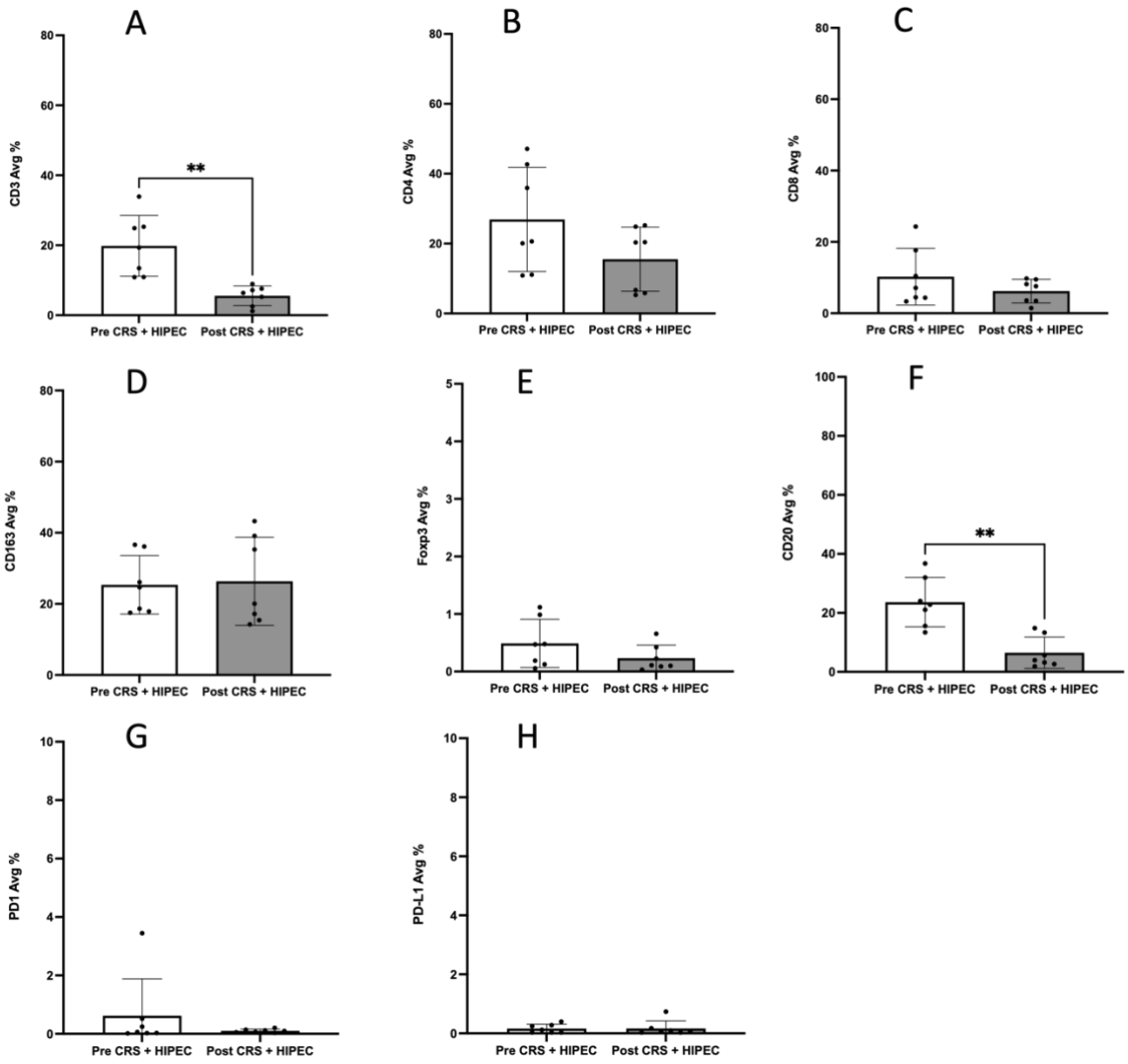


Figure 25. Comparative analysis between pre and post-CRS + HIPEC samples. CD3 positive cells average percentage (paired t-test, p value <0,05) **(A)**; CD4 positive cells average percentage (paired t-test, p value >0,05) **(B)**; CD8 positive cells average percentage (paired t-test, p value >0,05) **(C)**; CD163 positive cells average percentage (paired t-test, p value >0,05) **(D)**; Foxp3 positive cells average percentage (paired t-test, p value >0,05) **(E)**; CD20 positive cells average percentage (paired t-test, p value <0,05) **(F)**; PD1 positive cells average percentage (paired t-test, p value >0,05) **(G)**; PD-L1 positive cells average percentage (paired t-test, p value >0,05) **(H)**. Values are represented as mean ±

6. DISCUSSION

Peritoneal carcinomatosis often signifies an advanced stage of primary abdominal tumors and is associated with a poor prognosis[1]. The treatment typically involves cytoreductive surgery to remove the tumor mass, followed by HIPEC, which entails administering heated chemotherapy directly into the abdominal cavity to eradicate any microscopic cancer residuals and prevent recurrence[23]. However, the lack of standardized criteria for patient selection and treatment application modalities poses challenges for designing appropriate clinical studies.

Tumor microenvironment is a complex and dynamic system consisting of various components: cancer cells, extracellular matrix, blood vessels, immune cells, and signaling molecules[42].

The primary players in the anti-tumor immune response are $CD3^+$ T lymphocytes, which can be categorized into two major groups: $CD3^+CD4^+$ T-helper cells and $CD3^+CD8^+$ cytotoxic T cells. The presence of these cells is associated with a favorable prognosis in many cancer types[45].

Conversely, specific cell types can be activated by signaling molecules released by cancer cells, thereby exerting pro-tumoral activity. Examples include $CD163^+$ M2 macrophages and $Foxp3^+$ T regulatory cells, whose presence is associated with a poorer prognosis in various cancer types.[46, 52]

Another significant population within the tumor microenvironment is comprised of $CD20^+$ B-cells, which, upon activation, can generate antibodies against tumor cell antigens.

Inflammatory signals within cancer can also trigger the development of tertiary lymphoid structures. These structures are ectopic organized lymphoid aggregates which share similarities with secondary lymphoid organs. Tertiary lymphoid structures enhance the local immune response by facilitating the maturation and activation of B and T cells, resulting in antibody production and the generation of effector T-cell responses against the persistent antigens[47 - 49].

The peritoneum represents a unique microenvironment that influences deeply the neoplastic progression, promoting the implantation of cancer cells[1]. Nowadays, there are few in vitro and in vivo studies investigating the role of the immune system in peritoneal carcinomatosis and the effects of treatments on it.

The aim of this project was to dissect the presence and the distribution of the immune infiltrates in patients with peritoneal carcinomatosis secondary to gynecologic cancers and Pseudomyxoma Peritonei, who underwent Cytoreductive Surgery (CRS) in conjunction with Hyperthermic Intraperitoneal Chemotherapy (HIPEC).

Our findings revealed a strong presence of T-helper and T-cytotoxic cells in both peritumoral and intratumoral areas, indicating a significant T-cell response, with $CD4^+$ T-helper cells comprising the majority, as per data obtained from flow cytometry conducted by Franko et. al[63].

When comparing patients with peritoneal carcinomatosis associated with Gynecological cancer to those with peritoneal carcinomatosis from Pseudomyxoma Peritonei, we detected a higher level of $CD4^+$ and $CD8^+$ cell infiltration in the former group. This difference could potentially be attributed to the administration of neoadjuvant chemotherapy in gynecologic patients.

Alongside the aforementioned findings, we observed a significant infiltration of M2 macrophages and T regulatory cells with no significant differences between Gynecological and Pseudomyxoma Peritonei peritoneal carcinomatosis. Notably, the number of macrophages was higher than that of cytotoxic cells, suggesting a prevailing immunosuppressive activity.

Furthermore, we identified a consistent presence of B-cell infiltrate. Interestingly, unlike the previously mentioned cell types, B-cells were less abundant in the intratumoral areas but tended to form microaggregates within the surrounding stroma. These aggregates displayed diverse shapes and sizes and many of them were also positive for follicular dendritic cells ($CD21^+$), T-helper cells ($CD4^+$) and T-cytotoxic cells ($CD8^+$) . Based on these observations, we hypothesize that these aggregates represented the formation of tertiary lymphoid structures.

Given the current focus on immune checkpoint studies, our other goal was to investigate the expression of PD1 and PD-L1 revealing a strong expression in patients with immune aggregates.

By comparing Gynecological patients with Pseudomyxoma Peritonei patients, we observed a higher percentage of expression in the latter group.

Finally, we focused our analysis on immune infiltrates in residual peritoneal tissue biopsies following cytoreductive surgery and HIPEC treatment.

Despite the limitations represented by the small size and paucicellularity, we performed immunohistochemical analyses on only 7 samples. Overall, we highlighted a reduction expression of all markers analyzed, except for CD163, which remained higher.

The maintenance of high levels of expression of CD163 expression could be attributed to peritoneal resident macrophages or be a direct consequence of the HIPEC treatment.

This project had a significant limitation linked to the restricted number of patients due to the infrequent utilization of HIPEC, for this reason we were no able to obtain association with clinical outcomes.

7. CONCLUSIONS

Peritoneal carcinomatosis is a complex disease, and the composition of its immune microenvironment can vary among patients with different primary tumors.

Our analyses revealed a robust recruitment of T and B cells, alongside the presence of immunosuppressive cells populations and expression of immune checkpoint molecules. Treatment involving cytoreduction and HIPEC resulted in an overall decrease of immune cells infiltrates, with the exception of macrophages.

A notable finding was the identification of forming tertiary lymphoid structures, which could be associated with improved prognosis.

Therefore, it would be advantageous to conduct further investigations involving a larger patient cohort to establish correlations between the assessed immunological markers and clinical outcomes.

8. REFERENCES

1. Cortés-Guiral, D., Hübner, M., Alyami, M., Bhatt, A., Ceelen, W., Glehen, O., Lordick, F., Ramsay, R., Sgarbura, O., Van Der Speeten, K., Turaga, K. K., & Chand, M. (2021). Primary and metastatic peritoneal surface malignancies. *Nature reviews. Disease primers*, *7*(1), 91. https://doi.org/10.1038/s41572-021-00326-6.
2. Paget S. (1989). The distribution of secondary growths in cancer of the breast. 1889. *Cancer metastasis reviews*, *8*(2), 98–101.
3. Sawada, K., Mitra, A. K., Radjabi, A. R., Bhaskar, V., Kistner, E. O., Tretiakova, M., Jagadeeswaran, S., Montag, A., Becker, A., Kenny, H. A., Peter, M. E., Ramakrishnan, V., Yamada, S. D., & Lengyel, E. (2008). Loss of E-cadherin promotes ovarian cancer metastasis via alpha 5-integrin, which is a therapeutic target. *Cancer research*, *68*(7), 2329–2339. https://doi.org/10.1158/0008-5472.CAN-07-5167
4. Hansen, E., Wolff, N., Knuechel, R., Ruschoff, J., Hofstaedter, F., & Taeger, K. (1995). Tumor cells in blood shed from the surgical field. *Archives of surgery (Chicago, Ill. : 1960)*, *130*(4), 387–393. https://doi.org/10.1001/archsurg.1995.01430040049007.
5. Zajac, O., Raingeaud, J., Libanje, F., Lefebvre, C., Sabino, D., Martins, I., Roy, P., Benatar, C., Canet-Jourdan, C., Azorin, P., Polrot, M., Gonin, P., Benbarche, S., Souquere, S., Pierron, G., Nowak, D., Bigot, L., Ducreux, M., Malka, D., Lobry, C., … Jaulin, F. (2018). Tumour spheres with inverted polarity drive the formation of peritoneal metastases in patients with hypermethylated colorectal carcinomas. *Nature cell biology*, *20*(3), 296–306. https://doi.org/10.1038/s41556-017-0027-6.
6. Casey, R. C., Burleson, K. M., Skubitz, K. M., Pambuccian, S. E., Oegema, T. R., Jr, Ruff, L. E., & Skubitz, A. P. (2001). Beta 1-integrins regulate the formation and adhesion of ovarian carcinoma multicellular spheroids. *The American journal of pathology*, *159*(6), 2071–2080. https://doi.org/10.1016/s0002-9440(10)63058-1
7. Carduner, L., Picot, C. R., Leroy-Dudal, J., Blay, L., Kellouche, S., & Carreiras, F. (2014). Cell cycle arrest or survival signaling through αv integrins, activation of PKC and ERK1/2 lead to anoikis resistance of ovarian cancer spheroids. *Experimental cell research*, *320*(2), 329–342. https://doi.org/10.1016/j.yexcr.2013.11.011.
8. Casey, R. C., Burleson, K. M., Skubitz, K. M., Pambuccian, S. E., Oegema, T. R., Jr, Ruff, L. E., & Skubitz, A. P. (2001). Beta 1-integrins regulate the formation and adhesion of ovarian carcinoma multicellular spheroids. *The American journal of pathology*, *159*(6), 2071–2080. https://doi.org/10.1016/s0002-9440(10)63058-1.

9. Ziprin, P., Alkhamesi, N. A., Ridgway, P. F., Peck, D. H., & Darzi, A. W. (2004). Tumour-expressed CD43 (sialophorin) mediates tumourmesothelial cell adhesion. *Biological chemistry*, *385*(8), 755–761. https://doi.org/10.1515/BC.2004.092.
10. Klein, C. L., Bittinger, F., Skarke, C. C., Wagner, M., Köhler, H., Walgenbach, S., & Kirkpatrick, C. J. (1995). Effects of cytokines on the expression of cell adhesion molecules by cultured human omental mesothelial cells. *Pathobiology : journal of immunopathology, molecular and cellular biology*, *63*(4), 204–212. https://doi.org/10.1159/000163953.
11. Ween, M. P., Oehler, M. K., & Ricciardelli, C. (2011). Role of versican, hyaluronan and CD44 in ovarian cancer metastasis. *International journal of molecular sciences*, *12*(2), 1009–1029. https://doi.org/10.3390/ijms12021009.
12. van Baal, J. O. A. M., van Noorden, C. J. F., Nieuwland, R., Van de Vijver, K. K., Sturk, A., van Driel, W. J., Kenter, G. G., & Lok, C. A. R. (2018). Development of Peritoneal Carcinomatosis in Epithelial Ovarian Cancer: A Review. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*, *66*(2), 67–83. https://doi.org/10.1369/0022155417742897.
13. Nieman, K. M., Kenny, H. A., Penicka, C. V., Ladanyi, A., Buell-Gutbrod, R., Zillhardt, M. R., Romero, I. L., Carey, M. S., Mills, G. B., Hotamisligil, G. S., Yamada, S. D., Peter, M. E., Gwin, K., & Lengyel, E. (2011). Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nature medicine*, *17*(11), 1498–1503. https://doi.org/10.1038/nm.2492.
14. Meza-Perez, S., & Randall, T. D. (2017). Immunological Functions of the Omentum. *Trends in immunology*, *38*(7), 526–536. https://doi.org/10.1016/j.it.2017.03.002.
15. Gerber, S. A., Rybalko, V. Y., Bigelow, C. E., Lugade, A. A., Foster, T. H., Frelinger, J. G., & Lord, E. M. (2006). Preferential attachment of peritoneal tumor metastases to omental immune aggregates and possible role of a unique vascular microenvironment in metastatic survival and growth. *The American journal of pathology*, *169*(5), 1739–1752. https://doi.org/10.2353/ajpath.2006.051222.
16. Yonemura, Y., Endou, Y., Nojima, M., Kawamura, T., Fujita, H., Kaji, M., Ajisaka, H., Bandou, E., Sasaki, T., Yamaguchi, T., Harada, S., & Yamamoto, H. (1997). A possible role of cytokines in the formation of peritoneal dissemination. *International journal of oncology*, *11*(2), 349–358. https://doi.org/10.3892/ijo.11.2.349.
17. Heath, R. M., Jayne, D. G., O'Leary, R., Morrison, E. E., & Guillou, P. J. (2004). Tumour-induced apoptosis in human mesothelial cells: a mechanism of peritoneal

invasion by Fas Ligand/Fas interaction. *British journal of cancer*, *90*(7), 1437–1442. https://doi.org/10.1038/sj.bjc.6601635.

18. Iwanicki, M. P., Davidowitz, R. A., Ng, M. R., Besser, A., Muranen, T., Merritt, M., Danuser, G., Ince, T. A., & Brugge, J. S. (2011). Ovarian cancer spheroids use myosin-generated force to clear the mesothelium. *Cancer discovery*, *1*(2), 144–157. https://doi.org/10.1158/2159-8274.CD-11-0010.
19. Mitra, A. K., Sawada, K., Tiwari, P., Mui, K., Gwin, K., & Lengyel, E. (2011). Ligand-independent activation of c-Met by fibronectin and α(5)β(1)-integrin regulates ovarian cancer invasion and metastasis. *Oncogene*, *30*(13), 1566–1576. https://doi.org/10.1038/onc.2010.532.
20. Kajiyama, H., Shibata, K., Terauchi, M., Ino, K., Nawa, A., & Kikkawa, F. (2008). Involvement of SDF-1alpha/CXCR4 axis in the enhanced peritoneal metastasis of epithelial ovarian carcinoma. *International journal of cancer*, *122*(1), 91–99. https://doi.org/10.1002/ijc.23083.
21. Sandoval, P., Jiménez-Heffernan, J. A., Rynne-Vidal, Á., Pérez-Lozano, M. L., Gilsanz, Á., Ruiz-Carpio, V., Reyes, R., García-Bordas, J., Stamatakis, K., Dotor, J., Majano, P. L., Fresno, M., Cabañas, C., & López-Cabrera, M. (2013). Carcinoma-associated fibroblasts derive from mesothelial cells via mesothelial-to-mesenchymal transition in peritoneal metastasis. *The Journal of pathology*, *231*(4), 517–531. https://doi.org/10.1002/path.4281.
22. Balkwill, F., & Mantovani, A. (2001). Inflammation and cancer: back to Virchow?. *Lancet (London, England)*, *357*(9255), 539–545. https://doi.org/10.1016/S0140-6736(00)04046-0.
23. Al-Shammaa HA, Li Y, Yonemura Y. Current status and future strategies of cytoreductive surgery plus intraperitoneal hyperthermic chemotherapy for peritoneal carcinomatosis. World J Gastroenterol. 2008 Feb 28;14(8):1159-66. doi: 10.3748/wjg.14.1159. PMID: 18300340; PMCID: PMC2690662.
24. Sugarbaker P. H. (1995). Peritonectomy procedures. *Annals of surgery*, *221*(1), 29–42. https://doi.org/10.1097/00000658-199501000-00004.
25. González-Moreno, S., González-Bayón, L. A., & Ortega-Pérez, G. (2010). Hyperthermic intraperitoneal chemotherapy: Rationale and technique. *World journal of gastrointestinal oncology*, *2*(2), 68–75. https://doi.org/10.4251/wjgo.v2.i2.68.
26. Halkia, E., Tsochrinis, A., Vassiliadou, D. T., Pavlakou, A., Vaxevanidou, A., Datsis, A., Efstathiou, E., & Spiliotis, J. (2015). Peritoneal carcinomatosis: intraoperative

parameters in open (coliseum) versus closed abdomen HIPEC. *International journal of surgical oncology*, *2015*, 610597. https://doi.org/10.1155/2015/610597

27. Roviello, F., Caruso, S., Marrelli, D., Pedrazzani, C., Neri, A., De Stefano, A., & Pinto, E. (2011). Treatment of peritoneal carcinomatosis with cytoreductive surgery and hyperthermic intraperitoneal chemotherapy: state of the art and future developments. *Surgical oncology*, *20*(1), e38–e54. https://doi.org/10.1016/j.suronc.2010.09.002.
28. Piso, P., Glockzin, G., von Breitenbuch, P., Sulaiman, T., Popp, F., Dahlke, M., Esquivel, J., & Schlitt, H. J. (2009). Patient selection for a curative approach to carcinomatosis. *Cancer journal (Sudbury, Mass.)*, *15*(3), 236–242. https://doi.org/10.1097/PPO.0b013e3181a58f30.
29. Jacquet, P., & Sugarbaker, P. H. (1996). Clinical research methodologies in diagnosis and staging of patients with peritoneal carcinomatosis. *Cancer treatment and research*, *82*, 359–374. https://doi.org/10.1007/978-1-4613-1247-5_23.
30. Al-Shammaa, H. A., Li, Y., & Yonemura, Y. (2008). Current status and future strategies of cytoreductive surgery plus intraperitoneal hyperthermic chemotherapy for peritoneal carcinomatosis. *World journal of gastroenterology*, *14*(8), 1159–1166. https://doi.org/10.3748/wjg.14.1159.
31. Verwaal, V. J., van Ruth, S., de Bree, E., van Sloothen, G. W., van Tinteren, H., Boot, H., & Zoetmulder, F. A. (2003). Randomized trial of cytoreduction and hyperthermic intraperitoneal chemotherapy versus systemic chemotherapy and palliative surgery in patients with peritoneal carcinomatosis of colorectal cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, *21*(20), 3737–3743. https://doi.org/10.1200/JCO.2003.04.187.
32. Quénet, F., Elias, D., Roca, L., Goéré, D., Ghouti, L., Pocard, M., Facy, O., Arvieux, C., Lorimier, G., Pezet, D., Marchal, F., Loi, V., Meeus, P., Juzyna, B., de Forges, H., Paineau, J., Glehen, O., & UNICANCER-GI Group and BIG Renape Group (2021). Cytoreductive surgery plus hyperthermic intraperitoneal chemotherapy versus cytoreductive surgery alone for colorectal peritoneal metastases (PRODIGE 7): a multicentre, randomised, open-label, phase 3 trial. *The Lancet. Oncology*, *22*(2), 256–266. https://doi.org/10.1016/S1470-2045(20)30599-4
33. Zwanenburg, E. S., El Klaver, C., Wisselink, D. D., Punt, C. J. A., Snaebjornsson, P., Crezee, J., Aalbers, A. G. J., Brandt-Kerkhof, A. R. M., Bremers, A. J. A., Burger, P. J. W. A., Fabry, H. F. J., Ferenschild, F. T. J., Festen, S., van Grevenstein, W. M. U., Hemmer, P. H. J., de Hingh, I. H. J. T., Kok, N. F. M., Kusters, M., Musters, G. D.,

Schoonderwoerd, L., … COLOOPEC Collaborators Group (2024). Adjuvant Hyperthermic Intraperitoneal Chemotherapy in Patients With Locally Advanced Colon Cancer (COLOPEC): 5-Year Results of a Randomized Multicenter Trial. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, *42*(2), 140–145. https://doi.org/10.1200/JCO.22.02644.

34. Huo, Y. R., Richards, A., Liauw, W., & Morris, D. L. (2015). Hyperthermic intraperitoneal chemotherapy (HIPEC) and cytoreductive surgery (CRS) in ovarian cancer: A systematic review and meta-analysis. *European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology*, *41*(12), 1578–1589. https://doi.org/10.1016/j.ejso.2015.08.172
35. Desiderio, J., Chao, J., Melstrom, L., Warner, S., Tozzi, F., Fong, Y., Parisi, A., & Woo, Y. (2017). The 30-year experience-A meta-analysis of randomised and high-quality non-randomised studies of hyperthermic intraperitoneal chemotherapy in the treatment of gastric cancer. *European journal of cancer (Oxford, England : 1990)*, *79*, 1–14. https://doi.org/10.1016/j.ejca.2017.03.030.
36. Zhang, G., Zhu, Y., Liu, C., Chao, G., Cui, R., & Zhang, Z. (2019). The prognosis impact of hyperthermic intraperitoneal chemotherapy (HIPEC) plus cytoreductive surgery (CRS) in advanced ovarian cancer: the meta-analysis. *Journal of ovarian research*, *12*(1), 33. https://doi.org/10.1186/s13048-019-0509-1.
37. Granieri, S., Bonomi, A., Frassini, S., Chierici, A. P., Bruno, F., Paleino, S., Kusamura, S., Germini, A., Facciorusso, A., Deraco, M., & Cotsoglou, C. (2021). Prognostic impact of cytoreductive surgery (CRS) with hyperthermic intraperitoneal chemotherapy (HIPEC) in gastric cancer patients: A meta-analysis of randomized controlled trials. *European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology*, *47*(11), 2757–2767. https://doi.org/10.1016/j.ejso.2021.05.016.
38. Desiderio, J., Chao, J., Melstrom, L., Warner, S., Tozzi, F., Fong, Y., Parisi, A., & Woo, Y. (2017). The 30-year experience-A meta-analysis of randomised and high-quality non-randomised studies of hyperthermic intraperitoneal chemotherapy in the treatment of gastric cancer. *European journal of cancer (Oxford, England : 1990)*, *79*, 1–14. https://doi.org/10.1016/j.ejca.2017.03.030.
39. Kusamura, S., Barretta, F., Yonemura, Y., Sugarbaker, P. H., Moran, B. J., Levine, E. A., Goere, D., Baratti, D., Nizri, E., Morris, D. L., Glehen, O., Sardi, A., Barrios, P., Quénet, F., Villeneuve, L., Gómez-Portilla, A., de Hingh, I., Ceelen, W., Pelz, J. O. W., Piso, P., … Peritoneal Surface Oncology Group International (PSOGI) and the French

National Registry of Rare Peritoneal Surface Malignancies (RENAPE) (2021). The Role of Hyperthermic Intraperitoneal Chemotherapy in Pseudomyxoma Peritonei After Cytoreductive Surgery. *JAMA surgery*, *156*(3), e206363. https://doi.org/10.1001/jamasurg.2020.6363.

40. Papantoni, E., Ntatsis, K., Kyziridis, D., Kalakonas, A., Hristakis, C., & Tentes, A. A. (2021). Twenty-years' experience with cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) for pseudomyxoma peritonei (PMP). *Journal of B.U.ON. : official journal of the Balkan Union of Oncology*, *26*(4), 1647–1652.
41. Graham, I., Boston, A., Hayward, R., & Berri, R. (2024). Outcomes following cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) for peritoneal malignancies: 10 Year experience. *American journal of surgery*, *230*, 78–81. https://doi.org/10.1016/j.amjsurg.2024.01.031.
42. Baghban, R., Roshangar, L., Jahanban-Esfahlan, R., Seidi, K., Ebrahimi-Kalan, A., Jaymand, M., Kolahian, S., Javaheri, T., & Zare, P. (2020). Tumor microenvironment complexity and therapeutic implications at a glance. *Cell communication and signaling : CCS*, *18*(1), 59. https://doi.org/10.1186/s12964-020-0530-4.
43. Wang, L., Geng, H., Liu, Y., Liu, L., Chen, Y., Wu, F., Liu, Z., Ling, S., Wang, Y., & Zhou, L. (2023). Hot and cold tumors: Immunological features and the therapeutic strategies. *MedComm*, *4*(5), e343. https://doi.org/10.1002/mco2.343
44. Lasek W. (2022). Cancer immunoediting hypothesis: history, clinical implications and controversies. *Central-European journal of immunology*, *47*(2), 168–174. https://doi.org/10.5114/ceji.2022.117376.
45. Farhood, B., Najafi, M., & Mortezaee, K. (2019). $CD8^+$cytotoxic T lymphocytes in cancer immunotherapy: A review. *Journal of cellular physiology*, *234*(6), 8509–8521. https://doi.org/10.1002/jcp.27782
46. Ohue, Y., & Nishikawa, H. (2019). Regulatory T (Treg) cells in cancer: Can Treg cells be a new therapeutic target?. *Cancer science*, *110*(7), 2080–2089. https://doi.org/10.1111/cas.14069
47. Downs-Canner, S. M., Meier, J., Vincent, B. G., & Serody, J. S. (2022). B Cell Function in the Tumor Microenvironment. *Annual review of immunology*, *40*, 169–193. https://doi.org/10.1146/annurev-immunol-101220-015603.
48. Khanal, S., Wieland, A., & Gunderson, A. J. (2023). Mechanisms of tertiary lymphoid structure formation: cooperation between inflammation and antigenicity. *Frontiers in immunology*, *14*, 1267654. https://doi.org/10.3389/fimmu.2023.1267654

49. Sautès-Fridman, C., Petitprez, F., Calderaro, J., & Fridman, W. H. (2019). Tertiary lymphoid structures in the era of cancer immunotherapy. *Nature reviews. Cancer*, *19*(6), 307–325. https://doi.org/10.1038/s41568-019-0144-6
50. Habif, G., Crinier, A., André, P., Vivier, E., & Narni-Mancinelli, E. (2019). Targeting natural killer cells in solid tumors. *Cellular & molecular immunology*, *16*(5), 415–422. https://doi.org/10.1038/s41423-019-0224-2.
51. Wculek, S. K., Cueto, F. J., Mujal, A. M., Melero, I., Krummel, M. F., & Sancho, D. (2020). Dendritic cells in cancer immunology and immunotherapy. *Nature reviews. Immunology*, *20*(1), 7–24. https://doi.org/10.1038/s41577-019-0210-z
52. Wang, X., Qiu, L., Li, Z., Wang, X. Y., & Yi, H. (2018). Understanding the Multifaceted Role of Neutrophils in Cancer and Autoimmune Diseases. *Frontiers in immunology*, *9*, 2456. https://doi.org/10.3389/fimmu.2018.02456.
53. Aras, S., & Zaidi, M. R. (2017). TAMeless traitors: macrophages in cancer progression and metastasis. *British journal of cancer*, *117*(11), 1583–1591. https://doi.org/10.1038/bjc.2017.356.
54. Chesney, J. A., Mitchell, R. A., & Yaddanapudi, K. (2017). Myeloid-derived suppressor cells-a new therapeutic target to overcome resistance to cancer immunotherapy. *Journal of leukocyte biology*, *102*(3), 727–740. https://doi.org/10.1189/jlb.5VMR1116-458RRR.
55. Zunino, B., & Ricci, J. E. (2015). Hyperthermic intra-peritoneal chemotherapy and anticancer immune response. *Oncoimmunology*, *5*(1), e1060392. https://doi.org/10.1080/2162402X.2015.1060392.
56. Nevo, N., Lee Goldstein, A., Bar-David, S., Abu-Abeid, A., Dayan, D., Lahat, G., & Nizri, E. (2023). Immunological effects of heated intraperitoneal chemotherapy can be augmented by thymosin α1. *International immunopharmacology*, *116*, 109829. https://doi.org/10.1016/j.intimp.2023.109829.
57. Geva, R., Alon, G., Nathanson, M., Bar-David, S., Nevo, N., Aizic, A., Peles-Avraham, S., Lahat, G., & Nizri, E. (2023). PD-1 Blockade Combined with Heated Intraperitoneal Chemotherapy Improves Outcome in Experimental Peritoneal Metastases from Colonic Origin in a Murine Model. *Annals of surgical oncology*, *30*(5), 2657–2663. https://doi.org/10.1245/s10434-022-13025-7.
58. Scutigliani, E. M., Liang, Y., Crezee, H., Kanaar, R., & Krawczyk, P. M. (2021). Modulating the Heat Stress Response to Improve Hyperthermia-Based Anticancer Treatments. *Cancers*, *13*(6), 1243. https://doi.org/10.3390/cancers13061243.
59. Dellinger, T. H., Han, E. S., Raoof, M., Lee, B., Wu, X., Cho, H., He, T. F., Lee, P., Razavi, M., Liang, W. S., Schmolze, D., Priceman, S. J., Lee, S., Lin, W. C., Lin, J. F.,

Kebria, M., Hakim, A., Ruel, N., Stewart, D. B., Wang, E. W., … Rodriguez-Rodriguez, L. (2022). Hyperthermic Intraperitoneal Chemotherapy-Induced Molecular Changes in Humans Validate Preclinical Data in Ovarian Cancer. *JCO precision oncology*, *6*, e2100239. https://doi.org/10.1200/PO.21.00239

60. Moukarzel, L. A., Ferrando, L., Dopeso, H., Stylianou, A., Basili, T., Pareja, F., Da Cruz Paula, A., Zoppoli, G., Abu-Rustum, N. R., Reis-Filho, J. S., Long Roche, K., Tew, W. P., Chi, D. S., Sonoda, Y., Zamarin, D., Aghajanian, C., O'Cearbhaill, R. E., Zivanovic, O., & Weigelt, B. (2022). Hyperthermic intraperitoneal chemotherapy (HIPEC) with carboplatin induces distinct transcriptomic changes in ovarian tumor and normal tissues. *Gynecologic oncology*, *165*(2), 239–247. https://doi.org/10.1016/j.ygyno.2022.02.022.
61. Takahashi, A., Torigoe, T., Tamura, Y., Kanaseki, T., Tsukahara, T., Sasaki, Y., Kameshima, H., Tsuruma, T., Hirata, K., Tokino, T., Hirohashi, Y., & Sato, N. (2012). Heat shock enhances the expression of cytotoxic granule proteins and augments the activities of tumor-associated antigen-specific cytotoxic T lymphocytes. *Cell stress & chaperones*, *17*(6), 757–763. https://doi.org/10.1007/s12192-012-0348-0.
62. Franko, J., Brahmbhatt, R., Tee, M., Raman, S., Ferrel, B., Gorvet, M., & Andres, M. (2020). Cellular Immunoprofile of Peritoneal Environment During a HIPEC Procedure. *Annals of surgical oncology*, *27*(13), 5005–5013. https://doi.org/10.1245/s10434-020-08870-3.

9. PUBLICATIONS

- Corrao F, Zizzo MG, Tutone M, Melfi R, Fiduccia I, Carollo PS, Leonardo AD, Caldara G, Perriera R, Pace A, Belmonte B, **Sammataro S**, Pibiri I, Lentini L. *Nonsense codons suppression. An acute toxicity study of three optimized TRIDs in murine model, safety and tolerability evaluation.* Biomed Pharmacother. 2022 Dec;156:113886. doi: 10.1016/j.biopha.2022.113886. Epub 2022 Oct 18. PMID: 36265311.
- Belmonte B, Di Lorenzo G, Mangogna A, Bortot B, Bertolazzi G, **Sammataro S**, Merighi S, Martorana A, Zito G, Giorgiutti A, Bottin C, Zanconati F, Romano A, Ricci G, Biffi S. *PARP-1, EpCAM and FRα as potential targets for intraoperative detection and delineation of endometriosis: a quantitative tissue expression analysis.* (Minor revision)

10. ACKNOWLEDGMENTS

- Tumor Immunology Unit, University of Palermo: Prof. Claudio Tripodo, Prof. Beatrice Belmonte, Dott. Valeria Cancila.
- Ginecologic Oncology Unit, Hospital Civico, Di Cristina, Benfratelli, Palermo: Prof. Vito Chiantera, Dott. Mariano Catello Di Donna.
- Oncologic Surgery Unit, Hospital Civico, Di Cristina, Benfratelli, Palermo: Prof. Pierenrico Marchesa, Dott. Antonino Picciurro.
- A.C.T.A Group Srl, Napoli.