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IMMUNOPHENOTYPICAL CHARACTERIZATION OF COLORECTAL CANCER LIVER METASTASIS OF PRE-TREATED AND CHEMO-NAIVE PATIENTS

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ABSTRACT (English)

Background

There is extensive evidence for molecular heterogeneity in colorectal cancer (CRC) and CRC metastasis (mCRC). At the present, there are only few information regarding the immunological characterization of mCRC and about the effect of chemotherapy and targeted drugs on the interplay between tumour and the immune system of the host. It has been proposed an integrated classification molecular and of microenvironment of mCRC, considering that no data regarding the evolution over time of these features are available so far. New immunotherapeutic strategies are currently under development and will be further studied starting from refractory settings of heavily pre-treated mCRC patients. On this basis, a specific immunological characterization of mCRC will be relevant to direct future clinical and pharmacological research. We planned an exploratory, prospective, observational study for the immune phenotypical characterization of mCRC leukocyte infiltrate from pre-treated or chemo-naive patients undergone surgical metastasis resection.

Material and Methods

Patients will be divided in two groups according to previous administration of systemic chemotherapy (pre-treated or chemotherapy vs chemo-naive or not pre-treated group). Collected metastasis need to be at least 8 cm^3 in volume to allow standard pathological and flow cytometry analysis. Flow cytometry samples were isolated in operating room selecting viable-tumor portion of metastasis, and then carried to the laboratory to be minced in small pieces and enzymatically digested. Tumor-infiltrating leukocytes have been characterized with three panels of different antibodies in terms of subset composition (CD45, CD3, CD20, CD56, CD68), maturation and activation grade (CD45, CD3, TCRγδ, CD4, CD25, CD127, CD8, CCR7, CD45-RO, PD-1, Tim-3), cytokine production, degranulation and cycling activity (IL-2, IL4, INFγ, TNFα, CD107a, Ki-67).

Results

Eleven liver metastases have been analyzed. Seven cases are in chemotherapy group and three patients were naïve. Oxaliplatin-based chemotherapy was administered in 62.5% of patients, Irinotecan-based in 25% and immunotherapy (Ipilimumab/Nivolumab) in 12.5% (one case). One case received anti-VEGF (Bevacizumab) and four cases received anti-EGFR (Cetuximab) targeted therapy. Primary tumor location was right colon in 18.2% (2 cases), left colon in 63.6% (7 cases) and rectum in 18.2% (2 cases). One B-RAF and one N-RAS mutations were mapped, the other 80% of patients had no mutations in analyzed genes. There were two patients with micro-satellite instability (MSI-H), whereas the remainder 8 were stable (MSS). Five patients (62.5%) underwent

on surgery within 6 months from the end of systemic chemotherapy administration and three (37.5%) after 6 months.

All patients are alive after a mean follow-up period of 12.19 +/- 3.3 months after operation. During follow-up, 60% of patients present a tumor recurrence with a mean progression-free survival (PFS) of 9.15 +/- 2.17 months. In particular, one case had a hepatic recurrence only, two cases pulmonary and lymph-nodal, one case peritoneal only and two cases peritoneal and pulmonary.

CT-patients have a higher percentage of infiltrating leukocytes among viable cells compared to naïve patients (p=0,048). CT-patients show a polarized response towards CD4+ T-cells (p=0.0001), whereas naïve patients towards a CD8+ T-cells response (p=0.05). CT-patients display a very consistent maturation signature independently from CT-regimen. Together T_{EM} and T_{EMRA} represent more than 95% of all T-cells in both CD4+ and CD8+, but with a different ratio. T_{EM} were more than 80% of all CD4+T-cells; within CD8+, 60% are T_{EM} and more than 35% are terminally differentiated effector. IL-7 receptor (IL-7R) is higher both in CD4+ (p=0.003) and CD8+ T-cells (p=0.045) in CT-patients compared to naïve, as IL-7R expression intensity (p=0.043 in CD4+ and p=0.035 in CD8+).

Chemotherapy regimen analysis is limited because of the small sample, but the highest percentages of infiltrating leukocytes among viable cells have been found in the patient treated with immunotherapy and in the Oxaliplatin-Irinotecan-treated one; Oxaliplatin-treated patients display variable CD45+ percentage ranging between 10 and 60%, and Irinotecan-treated patient reveals the lowest leukocyte percentages among total viable cells.

To analyze time-dependent differences of immune response in liver metastases, we divided patients in two categories considering a cutoff of 6 months from the end of chemotherapy. Patients operated later than 6 months have higher percentage of infiltrating leukocytes among viable cells (p=0.0008). and in particular of B-cells (p=0.024). On the other hand, IL-7Rexpression among CD8+ was higher near the chemotherapy (p=0.043), even though the CD4+ degranulation activity in these patients was lower (p=0.033).

Discussion and conclusion

The leading aim of our exploratory study is to describe and characterize immune infiltrate in mCRC of naive and pre-treated patients. Results can drive further studies and possibly identify the most favorable subset of patients for receiving novel immune modulating therapies.

In all samples, cytotoxic potential of infiltrating lymphocytes is a quite variable among CD4+, but high and uniform in CD8+, consistent with a T-cell effector pattern. Almost all infiltrating T-lymphocytes are CCR7-negative effector memory cells and express high levels of IL7-R that allows the maintenance of this memory pool in the metastases.

Chemotherapy reduces tumor cells leaving a higher amount of immune infiltrating cells in liver metastases ($p=0.048$). Moreover, our results demonstrate that chemotherapy treatment polarizes immune response towards CD4+ T-cells ($p=0.0001$). This effect could be partially explained by tumor microenvironment modification induced by chemotherapy, in particular because the treatment induces tumor cell death, promoting antigen presentation and CD4+ T-cells expansion. In pre-treated patients T-cells display higher level of IL7-R+ and at higher expression intensity compared to naïve patients, both in CD4+ ($p=0.003$ and $p=0.043$, respectively) and in CD8+ T-cells ($p=0.045$ and $p=0.035$, respectively). Data display that chemotherapy increases the support to memory pool maintenance inside the mCRC.

Analysis of immune infiltrate in liver metastases according to chemotherapy regimen is limited by the small sample size of patients that have been enrolled for each regimen. Nevertheless, we find some differences among regimens that can be use as working hypothesis in further works.

Analysis of immune response variation according to time from the end of chemotherapy before surgery, shows that infiltrating leukocytes in general ($p=0.0008$) and B-lymphocytes in particular ($p=0.02$) are higher after longer period from CT suspension. These patients display also a higher degranulation activity (CD107a) especially in CD4+ ($p=0.03$). On the other hand, there is a time-dependent affection of CD8+ memory pool maintenance through a lower IL7-R expression in CD8+ of late operated patients ($p=0.043$). This finding could open an interesting research field about the optimal moment for surgical resection.

The main limitation of this study is the reduced sample size. We analyzed only three naïve patients (and in some tests only two), but there were objective difficulties in enrolling such patients. Further developments of the present study will necessary include an extension of sample size, aimed to enroll more naïve, Irinotecan-treated and Oxaliplatin plus Irinotecan-treated patients.

ABSTRACT (Italian)

Introduzione

L'eterogeneità molecolare della neoplasia e delle metastasi di origine colorettale (CRC) è nota ed ampiamente dimostrata; d'altro canto, attualmente vi sono scarse informazioni circa la caratterizzazione immunologica delle metastasi epatiche di tale origine e sugli effetti della chemioterapia nell'interazione tra CRC e sistema immunitario. E' stata proposta una classificazione integrata molecolare e del microambiente tumorale di questa neoplasia in grado di descrivere la molteplicità di risposte immunitarie indotte/osservabili, considerando anche il fatto che attualmente non vi sono dati relativi all'evoluzione nel tempo di tali caratteristiche. Nuove strategie immunoterapeutiche si stanno sviluppando e saranno impiegate in pazienti pesantemente pre-trattati; pertanto la caratterizzazione immunologica delle metastasi epatiche colorettali sarà di fondamentale importanza per capire chi di essi ne potrà realmente beneficiare. Abbiamo perciò pianificato uno studio esplorativo, prospettico ed osservazionale per la caratterizzazione immunofenotipica delle metastasi da CRC in pazienti chemiotrattati o chemio-naive, che siano stati sottoposti a metastectomia.

Materiali e Metodi

I pazienti sono stati divisi in due gruppi dipendentemente dall'aver ricevuto chemioterapia sistemica pre-operatoria (gruppo CT) o meno (gruppo naive). Le metastasi resecate dovevano essere di almeno 8 cm^3 per permettere l'analisi patologica standard e quella citofluorimetrica. I campioni sono stati preparati e divisi in sala operatoria, consegnati al laboratorio per essere processati. I leucociti infiltranti il tumore sono stati isolati con digestione meccanica ed enzimatica. Sono stati allestiti tre pannelli con diversi anticorpi per analizzare i leucociti in termini di composizione (CD45, CD3, CD20, CD56, CD68), grado di maturazione ed attivazione (CD45, CD3, TCRγδ, CD4, CD25, CD127, CD8, CCR7, CD45-RO, PD-1, Tim-3), presenza intracitoplasmatica di citochine, marcatori di degranulazione o di moltiplicazione (IL-2, IL4, INFγ, TNFα, CD107a, Ki-67).

Risultati

Sono state analizzate undici metastasi. Sette casi nel gruppo pre-trattato e tre nel gruppo naive. La terapia a base di Oxaliplatino è stata somministrata nel 62.5% dei pazienti, a base di Irinotecano nel 25%, immunoterapia (Ipilimumab/Nivolumab) nel 12,5%. Un paziente è stato trattato con anti-VEGF (Bevacizumab) e quattro casi con anti-EGFR (Cetuximab). La localizzazione del tumore primario era il colon sinistro nel 63,6% (7 pazienti) dei casi, colon destro e retto nel 18,2% (2 casi)

rispettivamente. Sono state riscontrate due mutazioni nei geni mappati (un B-RAF ed un K-RAS), il restante 80% dei casi era wild-type. Sono stati riscontrati due casi di instabilità dei microsatelliti (MSI-H). Cinque pazienti (62.5%) sono stati sottoposti a chirurgia entro 6 mesi dalla fine della chemioterapia e tre (37,5%) dopo 6 mesi.

Tutti i pazienti sono vivi dopo un periodo di follow-up medio di 12.19 +/- 3.3 mesi. Durante tale periodo il 60% dei casi ha presentato una recidiva con una progressione libera da malattia di 9.15 +/- 2.17 mesi (un caso di recidiva epatica, due casi polmonare e linfonodale, un caso di peritoneale isolata e due casi di peritoneale e polmonare).

I pazienti nel gruppo chemioterapia (CT) hanno dimostrato un maggior infiltrato linfocitario nelle cellule vitali della metastasi rispetto ai naive (p=0,048). I paziente pretrattati hanno evidenziato una risposta immunitaria polarizzata verso le cellule T CD4+ (p=0.0001), mentre i naive verso i CD8+ (p=0.05). Il gruppo CT ha dimostrato un significativo pattern di maturazione dei linfociti, indipendentemente dal regime chemioterapico. Insieme i T_{EM} and T_{EMRA} rappresentano oltre il 95% delle cellule T (sia nei CD4+ and CD8+, anche se con un diverso rapporto): i T_{EM} sono oltre l'80% nei CD4+, mentre nei CD8+ il 60% sono T_{EM} e il 35% terminalmente-differenziati. Il recettore per IL-7 è espresso in più cellule nei pre-trattati rispetto ai naive, sia tra i CD4+(p=0.003) che tra i CD8+(p=0.045), e a maggiore intensità (p=0.043 nei CD4+ e p=0.035 nei CD8+).

L'analisi dell'infiltrato in base al regime chemioterapico è stata limitata dal campione ristretto, ma il maggior rapporto di leucociti infiltranti tra le cellule vitali della metastasi è stato riscontrato nei pazienti trattati con Immunoterapia o con la combinazione Oxaliplatino-Irinotecano; i pazienti trattati con Oxaliplatino mostrano una percentuale di leucociti variabile dal 10 al 60%, mentre il paziente trattato con Irinotecano ha il rapporto minore tra tutti quelli analizzati.

Per analizzare la risposta immunitaria in funzione del tempo trascorso dalla fine della chemioterapia, sono stati creati due gruppi considerando un cut-off di 6 mesi. I pazienti operati dopo più di 6 mesi dalla sospensione del trattamento hanno evidenziato una maggiore percentuale di leucociti infiltranti rispetto ai pazienti operati più precocemente (p=0.0008). In particolare ciò è stato osservato per i linfociti B (p=0.024). D'altro canto, l'espressione di IL-7R tra i CD8+ è più elevata a ridosso della terapia (p=0.043), sebbene in queste circostanze il potenziale linfocitario citotossico tra i CD4+ sia inferiore (p=0.033).

Discussione e conclusione

Lo scopo principale del nostro studio esplorativo è di descrivere e caratterizzare l'infiltrato immunitario nei pazienti chemiotrattati e naive; tali risultati potranno essere utilizzati in ulteriori studi al fine di identificare il sottogruppo di pazienti più adatto a ricevere nuove terapie.
Abbiamo evidenziato come la chemioterapia riduca il numero di cellule tumorali nelle metastasi epatiche, portando ad un maggiore infiltrato immunitario tra le cellule vitali ($p=0.048$).

Il potenziale citotossico dei linfociti è risultato essere variabile nei CD4+, ma elevato ed uniforme nei CD8+, consistente con caratteristiche effettrici. Tali linfociti effettori di memoria, esprimono alti livelli di IL7-R, che permette loro di mantenersi in vita nella metastasi.

I nostri risultati dimostrano che la chemioterapia polarizza la risposta immunitaria verso la linea CD4+ ($p=0.0001$). Questo effetto può essere parzialmente spiegato grazie al microambiente tumorale ed alle modifiche indotte dalla terapia; si può ipotizzare che il trattamento promuova la presentazione di nuovi antigeni mediante la citolisi e quindi l'espansione dei linfociti T CD4+.

I pazienti pretrattati mostrano più linfociti esprimenti IL-7R e una sua maggiore espressione rispetto ai pazienti naive sia nei CD4+ ($p=0.003$ e $p=0.043$, rispettivamente) che nei CD8+ ($p=0.045$ e $p=0.035$). Tali dati indicano che i trattamenti chemioterapici sostengono maggiormente il mantenimento dei pool di memoria infiltranti il tumore. Tale effetto è abbastanza duraturo anche dopo la sospensione della terapia: solo in CD8+ osserviamo una minore espressione di IL-7R nei pazienti operati dopo sei mesi dalla fine della CT ($p=0.043$).

L'analisi dell'infiltrato immunitario nelle metastasi epatiche in funzione del chemioterapico somministrato, è limitata dal ridotto campione di pazienti che sono stati arruolati per ciascun regime; ciò nonostante abbiamo evidenziato alcune differenze tra i diversi regimi, che potranno essere usate come ipotesi di lavoro in successivi studi.

L'analisi della risposta immunitaria in diversi momenti dalla fine della chemioterapia, ha evidenziato come i leucociti infiltranti in generale ($p=0.0008$) ed in particolare i linfociti B ($p=0.02$), siano più elevati dopo una più lunga sospensione della terapia. In questi pazienti inoltre abbiamo evidenziato una maggiore capacità citotossica apprezzabile in tutti i linfociti, ma significativa solamente nei CD4+ ($p=0.03$). Tale risultato potrà essere ulteriormente approfondito per definire il miglior momento per la resezione chirurgica.

La limitazione maggiore di questo studio è la ridotta numerosità campionaria, dovuta principalmente a grandi difficoltà oggettive nell'arruolamento dei casi non trattati. Successivi sviluppi di questo studio dovranno necessariamente prevedere un incremento del campione, mirato

ad includere più pazienti non trattati, trattati con Irinotecano o con l’associazione Oxaliplatino-Irinotecano.

BACKGROUND

Colorectal cancer and hepatic metastasis

According to the World Health Organization (WHO), colorectal cancer (CRC) is the third commonest neoplasm and the fourth cancer-related cause of death [1].

Twentyfive-30% of patients present at diagnosis a synchronous metastasis, whereas up to 50% of patients treated with curative-intent primary tumor resection, will recur at a distant site (metachronous metastasis). Of all these distant recurrence, 70% will occur in the liver, followed by peritoneum, lung and other sites. Liver metastases is the leading cause of death in CRC patients and, if not treated, average survival is 6-8 months [1].

Best therapeutic option is surgical resection, but, before actual effective chemotherapy regimens, liver metastasis were considered resectable only in 10-15% of cases. Even with liver resection, patients treated in combination with systemic chemotherapy had a poor prognosis (12-16 months of overall survival) [2].

Multidisciplinary team decisions, evolving treatments and patient care are at the present contributing in ameliorating CRC prognosis. Particularly, novel chemotherapy agents are very effective and a median overall survival of 16-22 months can be achieved at the present for not-resectable liver metastases patients. Actual systemic chemotherapy demonstrate a response rate higher than 50%, that converts in a resection rate up to 30–40% of cases. All these advances, lead to a significant improvement of survival up to 28-46 months, with a 5-years overall survival rates of 25–40% (prior <8%) [3,4].

Nevertheless, recurrence rate and mortality is still high so there is a continue effort in finding new strategies and treatments in the scientific community.

As a result of this effort, one of the most interesting ideas emerging in the last two decades, are that gross anatomical tumor characteristics are not the only fundamental features. Molecular and metabolic pathways, gene alterations, surrounding environment (namely liver “normal” cells) and interplay with patient immune system start to emerge as key-concepts to understand tumor biology [5]

It has been demonstrated that liver and lung are the most frequent metastatic site due to anatomical reasons (first and second colon and rectum drainage site), but this mechanistic hypothesis do not explain all changes that are required to CRC tumor cells to originate a metastasis [6].

The “seed and soil” hypothesis could be used to postulate that the metastatic ability of CRC is obtained through tumor cells with stemness features and migration skills (the “seed”) together with an harbouring microenvironment, modified to promote tumor cells localization, growth and escape from immune response (the “soil”) [7].

CRC cells have to gain the ability of generating metastasis by genetic rearrangement that lead epithelial to mesenchymal transition (EMT) [8]. Intrinsic and tumor-mediated immunosuppressive microenvironment of the liver could promote metastasis formation [9].

Metastasis microenvironment and immune response

Colorectal cancer (CRC) liver metastases represents a complex microenvironment in which tumor cells, stroma and immune cells interact to determine disease control or progression. Impact of immune cells in cancer control has been already reported [10]; such control depends on a multitude of different factors, ranging from type and composition of immune infiltrate, localization of infiltrate in the metastases, orientation and differentiation of immune cells and host genetics.

After these findings “immunoediting theory” has been elaborated addressing immune cells to have a major role in suppress tumor (killing tumor cells), control progression (equilibrium phase) and eventually in selecting resistant clones (tumor evasion) [11].

On the other hand, it has also been demonstrated that immune system can also play a role in promoting tumor progression, especially when chronic inflammation is predominant over adaptive immune response, stimulating proliferation of cancer cells, promoting angiogenesis and metastasis [12]

Tumor microenvironment (TME) and infiltrating cells

Many different types of cells constitute the tumor microenvironment: epithelial, endothelial and lymphatic vessels, stromal cells (e.g. cancer associated fibroblast CAF) and infiltrating immune cells. Infiltrating immune cells include T lymphocytes (effector CTLs, T-helper T-h, T-regulatory

cells T-reg), B-lymphocytes, natural killer cells (NK), dendritic cells (DCs), myeloid derived suppressor cells (MDSC), macrophages (M1 and M2) and granulocytes [13].

The interplay of such cells, their intrinsic activity and signaling products are determinant in cancer cells invasiveness and progression and host immune response.

Composition of TME is different among cancer histology types and can vary between patients with the same histology. To make the environment even more complex, TME cells composition could also vary in the same patients in different metastases according to the originating tumor sub-clone (theory of intratumoral heterogeneity, ITH) [14].

TME is crucial to promote or inhibit immune cell infiltration and finally clinical outcome. In general, high infiltration of immune cell promoting adaptive response relates to a better clinical outcome, whereas high prevalence of mesenchymal cells has a worst prognosis [15]. High CD8+ T cells relates to improved clinical outcome in many different cancer types, with the exception of kidney and glioma cancer [16]. In CRC a good prognosis seems to be related to infiltration of Th1, follicular helper T-fh, M1 macrophages, NK cells and DCs, whereas M2, MDSCs and Th17 (T helper cell subset activated by IL-17 that controls mainly the myeloid lineage of immune response) have been reported to be detrimental [17-23].

Many studies reported also that the site and patterns of infiltration relate to clinical outcome, actually cells can be at the center of the tumor or in interface with hepatic tissue and tumor (invasive margin). [24]. It has also been demonstrated an important role in tumor control of tertiary lymphoid structures (TLS), that represent lymphoid aggregates resembling lymph nodes having a T zone with mature DCs [25].

High density of CD45RO+ T cells (memory T) and CD8+ in center of tumor and invasive margin are related to better clinical outcome as presence of mature DCs, Th-follicular and B cells in TLS, probably because the role of TLS in T-cell recruitment and promotion of adaptive response [24,26].

Accumulation of CD20+ has been associated with longer disease free interval and better overall survival; actually CD20+ presenting cells are fundamental to proper CD4+ and CD8+ T cells development and this can give an advantage in tumor control (speculatively it could be possible that higher CD20+ levels reflects a better general status of the patients, capable of an effective immune response even after heavy systemic chemotherapy; further studies are required [27].

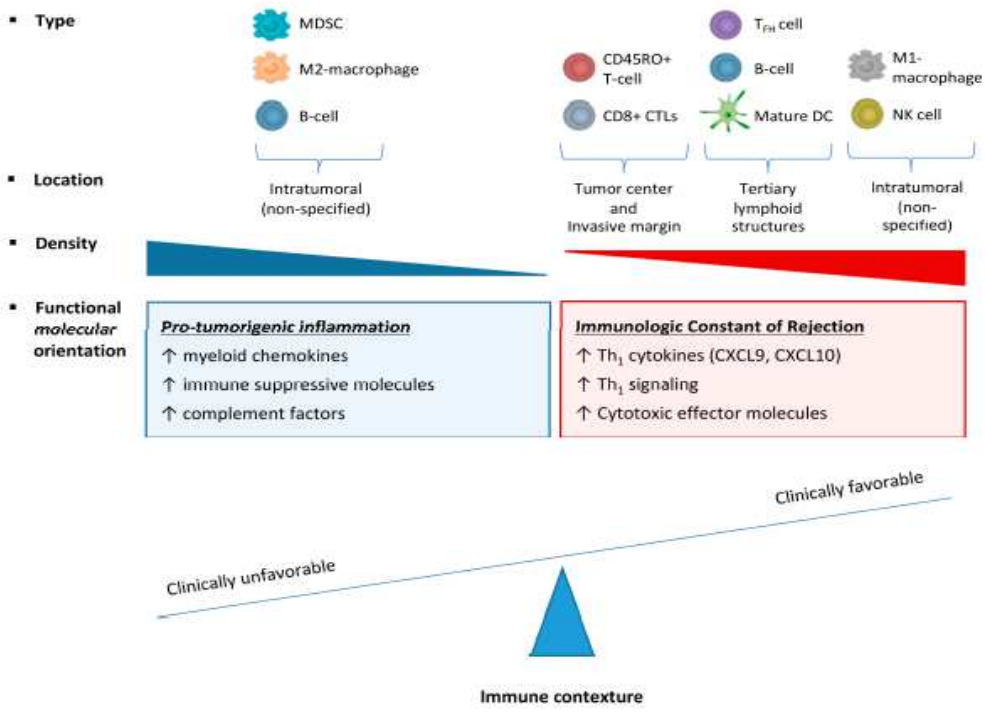
There are evidences showing some differences in immune infiltration pattern between primary tumor and metastasis. Liver metastasis have a higher infiltration of CD3+ T cells and CD45RO+ in

the invasive margin and CD8+ cells in the centre of tumor and invasive margin, compared to CD20+ B cells and FoxP3+ T-reg cells in the center of tumor in primary site (colon or rectum) [28].

Two studies showed no correlation between primary and metastatic (liver or lung) immune cell distribution pattern [29]. It is not fully understood the mechanism of such difference, it could be related to intratumoral heterogeneity (different clones originates different metastasis with preferential sites) or, more probably, the different microenvironment (colon, liver or lung) plays a fundamental role in activation of immune system.

Another recent work analyzed correlation between primary tumor Immunoscore *(see next chapter for detailed description)* and metastases' Immunoscore-like classification in CRC patients. Only Immunoscore of metastasis relates with survival at multivariate analysis, showing that prognosis depends on immune response (and consequently on TME) of metastasis rather than of primary tumor [30].

There are few evidences of relation between previous systemic treatments, TME changes and immune infiltration patterns, even though a high TILs presence at invasive margin of liver metastasis seems to give a favorable prognosis, in analogy with primary tumor TILs [31].



Adapted from Roelands et al

Immunologic and molecular predictive signatures of CRC and liver metastases

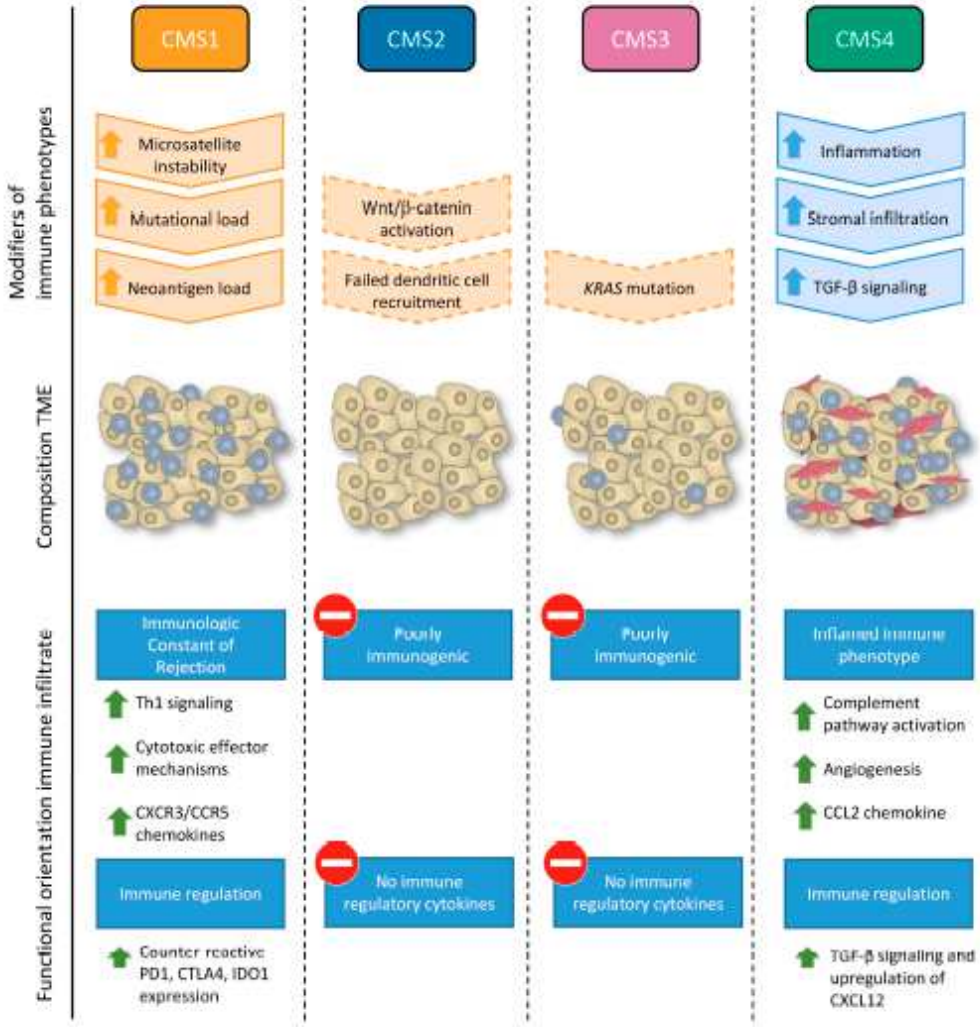
Following the previous reported results, in the last years it has been raised an important interest in histological and immunological quantification of immune cells to develop a scoring system that could possible fill the gap of classical anatomical classification (TNM) of colorectal cancer.

Such efforts lead to “Immunoscore” [32] and classification of molecular subtypes (Consensus Molecular Subtypes, CMS) [33].

Those novel scoring systems has proven to be useful in adding prognostic evaluation combined to TNM [34,35] and are even more accurate than MSI-status alone in predicting survival of CRC metastatic patients [36].

In 2012 Immunoscore (“I”) has been proposed as a novel tool to evaluate the degree of immune infiltration, considering number of CD8+ and CD45RO+ cells in the center of tumor and in invasive margin; the score variates from 1 (low infiltration) to 4 (high infiltration). When Immunoscore has been applied to a large cohort of patients (602 cases), predicts recurrence with high accuracy (in multivariate analysis only “I” score was signifcative whereas T and N stages where not), actually 5-years survival of I4 patients was 86.2% compared to 27.5% of I1 patients [37].

In 2015 an international expert consortium has proposed a classification of four CRC molecular subtypes (CMS1 to 4), based on gene-expression analysis. MicroSatellite instability (MSI) is main feature of first subtype (CMS1), whereas Chromosomal INstability (CIN) is the key of the remainder three [33].



Adapted from Roelands et al

CMS1 (“immunogenic”, 14% of early stage CRC, good prognosis) has hypermutation and hypermetilation of multiple genes, BRAF V600E mutation and it is characterized by a very high immune infiltration (mainly CTLs, CD4Th1 and NK cells).

CMS2 (“canonical”, 23% of early stage CRC) shows a marked upregulation of WNT and MYC gene products and high EGFR and HER2 expression.

CMS3 (“metabolic”, 13% of early stage CRC) is peculiar among others CIN subtypes, presenting with MSI 30% of cases, lower copy-number alterations and an high level of KRAS activating mutations leading to metabolic cell reprogramming.

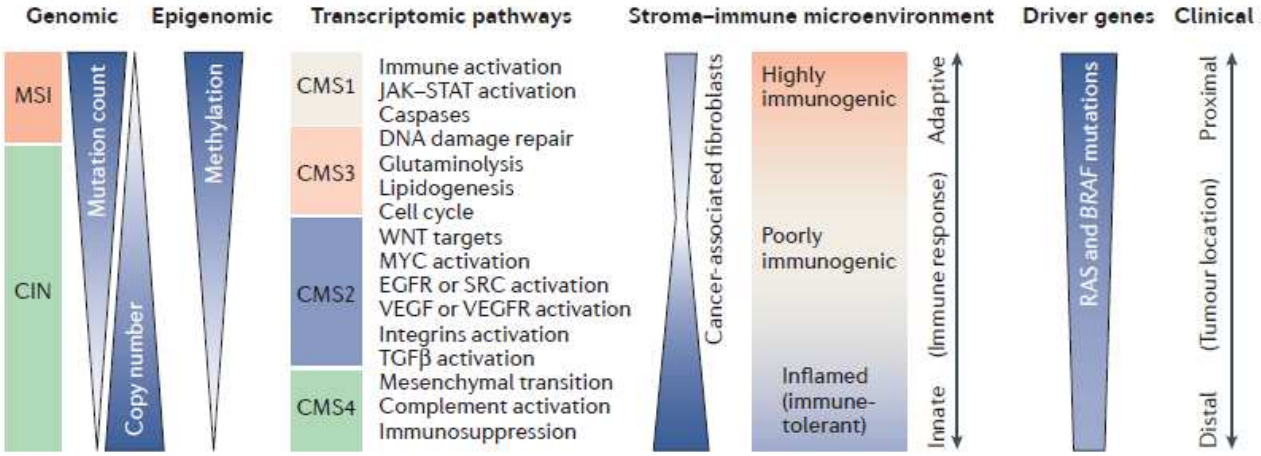
CMS4 (“mesenchymal”, 23% of early stage CRC, worst prognosis) is characterized by activation of epithelial-mesenchymal transition (EMT) pathways, extracellular matrix remodeling, TGFb, myeloid chemokines, angiogenetic factors and complement components.

TME composition varies between CMSs and clinical outcomes are different according to subtypes.

In CMS1, microsatellite instability (MSI), due to deficient DNA repair systems, produces a large amount of neo-antigens that recruit an effective immune response polarized in Th1 direction, leading to high cytotoxic activity (CTLs) thorough CXC3 and CCR5 chemokines.

CMS4 prognosis is significantly worse than CMS1, probably because of different pattern of immune response: CMS4 has higher expression of myeloid chemokines, angiogenetic factors, complement components, whereas CMS1 has a very prominent T-mediated immune response (high Th1 and T-cell attracting chemokines) [38].

It has been demonstrated, through cell-specific gene expression profiling, that transcripts associated with poor prognosis (e.g. TGFb signaling) in CMS4 are produced by TME cells (mainly by cancer associated fibroblasts CAFs and endothelial cells), rather than immune cells themselves [39].



Adapted from Dientsmann et al

Therefore, prognosis of patients in CMS4 derives more than interaction between tumor and microenvironment rather than the activity of immune system. A similar mechanism of resistance has been shown in breast cancer: in presence of high levels of TGFb, tumors show a “immune benefit disable (IBD)” subtype leading to a worst prognosis [40].

According to molecular subtypes, also mechanisms of tumor evasion are different.

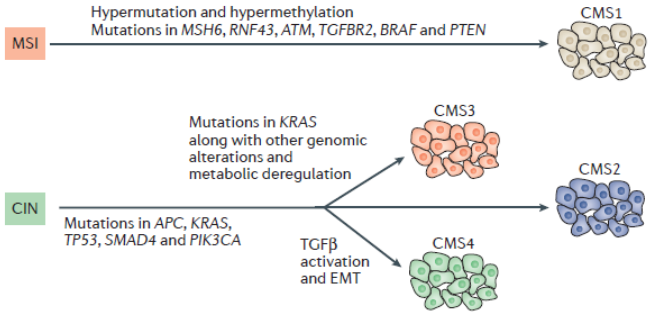
In CMS1 upregulation of CTLA4, PD-L1 and PDCD1 (in normal immune homeostasis those are surface receptors used to promote lymphocytes programmed death (PD) to decrease immune

response or destroy auto-reacting lymphatic clones), leads to reduction of activity of antigen-specific immune cells and finally to tumor progression [38].

In addition, upregulation of non-canonical human leukocyte antigen G (HLA-G) and E (HLA-E) by colorectal tumor cells inhibits NK activity. Notably this pathway has also been described in CMS4 and some sub-types of CMS2 and CMS3 (HLA-G is expressed by plancetar cells and may play a role in immune tolerance during pregancy; HLA-E is a cofactor for NK activity that is usually low expressed in normal cells) [41,42].

In CMS4 overexpression by stromal cells in TME of TGFb and CXCL12 (a chemokine that regulates CD20 expression on B cells and that is chemotactic for mesenchymal stem cells), reduces adaptive response and promotes angiogenesis and tumor diffusion [38].

TGFß and pro-tumoral genes are not overexpressed in CMS2, since this subtype avoid immune control preventing tumor infiltration by immune cells [43]. In CMS2 a possible escape mechanism could be derived from evidences in other tumors type (such as melanoma and lung cancer), in which upregulation of WNT/b-catenin leads to reduction or abolition of dendritic cells (DCs) recruitment through suppression of CCL4 gene transcription (a chemokine expressed by immune cells, mainly macrophages, and it is chemotactic for NK cells) [44]



KRAS mutation, that is main CMS3 characteristic, in addition with LKB1 mutation (a tumor suppressor gene that controls cell growth and proliferation when energy and nutrient levels are scarce [45] demonstrated in lung cancer an active recruitment of neutrophils and production of cytokines suppressing T-cells activity [46].

Another mechanism of evasion in CMS2 and CMS3 is downregulation of genes related to Major Histocompatibility Complex of class I (MHC-I); MHC-I is fundamental in antigen presentation and MHC-I loss or downregulation prevents or reduces tumor antigen recognition by immune cells [47]

Aim of the study

There is extensive evidence for molecular heterogeneity in CRC. Studies have revealed that intra-tumor heterogeneity can be highly variable within primary tumors or between primary and metastatic sites [48].

Moreover, tumor heterogeneity can be linked to targeted therapies in terms of acquired resistance mutations. In the era of precision medicine, specific molecular characterization of primary tumor and metastasis taking into account the dynamism of the disease and the actual therapeutic target should be considered.

Nowadays there is not enough information regarding the immunological characterization of CRC metastasis. Especially, few data are available about the effect of chemotherapy and targeted drugs on the interplay between tumour and the immune system of the host [49].

Immune system takes part to different phases of tumour growth. It is a dynamic balance that can be differentially modulated by several agents, resulting in immunosuppression or immunostimulation [50].

No data regarding the evolution over time of these features are available so far. Immune therapy represents a promising option for the treatment of an increasing number of malignancies. New immunotherapeutic strategies are currently under development and will be further studied starting from refractory settings of heavily pre-treated mCRC patients.

On this basis, a specific immunological characterization of CRC metastasis will be relevant to direct future clinical and pharmacological research.

As surgery is a therapeutic option in the treatment of mCRC, a percentage of mCRC patients undergo to resection of metastasis before or after medical treatment. These tumour samples could be useful to define the immune signature of colorectal metastatic disease.

Based on the above reported considerations, it has been planned an exploratory, prospective, observational study for the immunophenotypical characterization of colorectal cancer metastasis from pre-treated *vs* chemo-naive patients.

Primary objective:

- To describe patterns of tumor-infiltrating leukocytes in colorectal cancer metastasis.

Secondary objectives:

- to correlate immunological features of metastasis with previous treatment received;
- to explore the impact of specific immunological features of metastasis with patients' outcome.

MATERIALS AND METHODS

Patients' selection

Patients could have been treated for primary tumor in other institutions (in case of metachronous liver metastases) and then been referred to a Clinical Oncology unit for chemotherapy or to a Surgical Unit for evaluation of liver resection (and eventually primary tumor resection in case of synchronous liver metastases). See results section for a detailed list of participating Units.

Patients enrolled in the study have to meet the following inclusion criteria:

- metastatic liver disease from colorectal cancer (pathological confirmation required to perform cytofluorimetric analysis)
- good functional status (ECOG PS0 and PS1) to be fit for surgery for metastatic disease
- availability of clinical data (pre-operative and follow-up)
- written consent for the study
- age >18 years

After selection and fulfilling of all the previous requirements, patients are divided in two groups according to previous administration of systemic chemotherapy: pre-treated or chemotherapy group vs naive or not pre-treated group.

All clinical variables related to patient status, tumor type (localization, mutational status, TNM classification), treatment received (type of chemotherapy, number of cycles and duration of treatment, use of targeted therapy), surgical procedure and follow-up (overall survival OS, disease free survival DFS or progression free survival PFS) have been collected.

Sample collection

Collected metastasis need to be at least 8 cm^3 in volume to allow standard pathological and flow cytometry analysis. The surgeon in operating room has dissected samples for the study and contextually the anatomopathologist selected a small portion of 1cm^3 from a viable-tumor interface zone (peripheral zone of metastasis) and designated it to flow cytometry.

Molecular and traditional pathological assessment

The liver specimen was routinely assessed after 24 hours formalin fixation and then routine hematoxylin-eosin slide evaluation.

IHC (immunohistochemistry) staining was performed on 4 µm-thick formalin-fixed/paraffin-embedded (FFPE) tissue sections collected from primary tumor, with antibodies to hMLH1 (Dako ES05; ready to use), hMSH2 (Dako FE11; Ready to use) hMSH6 (Dako EP49; diluition 1:50) and hPMS2 (Dako EP51; diluition 1:50).

DNA was extracted from primary tumor FFPE 10-micron thick sections after deparaffinnization. KRAS/NRAS and BRAF mutation analysis was performed by mass spectrometry (Kit Myriapod Colon status, Diatech Pharmacogenetics – MassArray analyzer) for KRAS/NRAS exons 12,13,59,61,117 and 146 and for BRAF exon 600.

The determination of microsatellite instability was also evaluated by multiplex amplification with fluorescent primers and subsequent DNA fragment analysis on automated sequencer (Titano MSI kit CE-IVD Diatech Pharmacogenetics).

Flow cytometry analysis

The liver metastases surgical sample was sliced and stored O.N. at 4°C in RPMI medium (Roswell Park Memorial Institute medium or RPMI 1640) with 10% of FBS (fetal bovine serum). Slices were then chopped and digested with Tumor Dissociation Kit (MiltenyiBiotec, 130-095-929) in a GentleMACS Octo Dissociator (MiltenyiBiotec, 130-096-427), with Heaters running program 37°C_h_TDK_2 or 37°C_h_TDK_3. Erythrocytes were lysed.

Analysis of tumor infiltrating leukocytes was performed by multicolor flow cytometry. For cell surface staining, FcR Blocking was performed (FcR Blocking Reagent, Miltenyi Biotec, 130-059-901).Thereafter, the Fixable Viability Stain 780 (BD Horizon, 565388) and the following anti-human antibodies were used: CD45-BV786 (clone HI30), CD3-BV510 (clone SK7), CD20-PE-Cy7 (clone 2H7), CD56-APC-R700 (clone NCAM16.2), CD11 CD4-PerCP-Cy 5.5 (clone SK3), CD25-PE-Cy7 (clone 2A3), CD127-APC-R700 (clone HIL-7R-M21), CD8-BV605 or CD8-APC-R700 (clone SK1), CD45RO-BV650 (clone UCHL1), CD197(CCR7)-Alexa-647 (clone 150503), CD279(PD-1)-PE-CF594 (clone EH12.1), CD366(TIM-3)-PE (clone 7D3), all from BD Bioscience.

Cells were then processed using the Cytofix/Cytoperm kit (BD Biosciences, 554715) or Transcription Factor Buffer set (BD Biosciences, 562725), according to the manufacturer's instructions.

Finally, for the intracellular staining the following anti-human antibodies were used: CD68-FITC (clone Y1/82A), CD107a-PE-Cy7 (clone H4A3), Ki67-BV421 (clone B56), IL-2-PE (clone MQ1-17H12), IL-4-APC (clone 8D4-8), INF-γ-Alexa488 (clone B27) and TNF-α-BV650 (clone MAb11) all from BD Bioscience. Cells were acquired on a LSR II flow cytometer (BD Bioscience), and data were analyzed using FlowJo software (TreeStar Inc, v10).

Antibody panel 1 characterized leukocytes subset composition, panel 2 described lymphocytes maturation and activation grade, panel 3 monitored cytotoxic granules and cytokine production, and cycling/proliferative activity.

Leucocyte count is an estimation of white blood cells present in the observed sample and it has been obtained by optical microscopy after dying with Trypan blue.

Tumor infiltrating leukocytes composition (panel 1)

First panel described immune infiltrating cells, in particular T- and B-lymphocytes, Natural Killer (NK) cells and Macrophages. To identify these immune-subsets the following markers (listed with essential biological meaning) have been used.

CD45

Called also Protein Tyrosine Phosphatase receptor Type C (PTPRC) or leukocyte common antigen (LCA) is a transmembrane receptor expressed in different isoforms in all hematopoietic cells with the exception of red blood, platelets and plasma cells. CD45 function is essential for cell growth and mitotic cycle regulation. It is used to identify white blood cells among tumoral and stromal cell types [51].

CD3

A T-cell specific co-receptor expressed in T lymphocytes membrane (and at lower level in Purkinje cells). After antigen recognition, association of CD3 with T-cell receptor (TCR) leads to activation of CD4 (T-helper) and CD8 (cytotoxic) lymphocytes. It is used to differentiate lymphocytes among

other immune cells [52].

CD20

A specific B-cell membrane protein, necessary for development and differentiation of B-cells into plasma cells. It probably acts as a calcium channel following ligation of the B cell receptor with antigen. CD20 is used to identify B-lineage lymphocytes [53].

CD56

also called Neural Cell Adhesion Molecule (NCAM), is expressed in membrane surface of neurons and skeletal muscle cells playing a role in cell-to-cell adhesion, neuronal growth and synaptic plasticity. In hematopoietic lineage it is highly expressed in natural killer cells. NK cells are a type of cytotoxic lymphocyte essential in innate (but also in acquired) immune response, able to destroy cells that are not expressing MHC class I (such as tumor cells that use the downregulation of MHC to mask tumor neo-antigens that could be recognized by adaptive immunity) [54].

CD68

a protein highly expressed in circulating monocytes, tissue macrophages and osteoclasts. CD68 is part of the family of scavenger receptors (CD68 type D) with a role in clear cellular debris, promotes phagocytosis and mediates recruitment and activation of macrophages. CD 68 acts as a protein binder to tissue -specific lectins or selectins, allowing targeting and homing of macrophages [55].

<u>Lymphocytes activation and maturation (panel 2)</u>

Panel 2 described T-cells sub-types, identifying CD4+ and CD8+ T-cells, TCR-γδ Lymphocytes (TCRγδ), regulatory T-cells (Treg), their maturation grade as Naïve, central memory (T_{CM}), effector memory (T_{EM}) and terminally differentiated effector memory RA-expressing (T_{EMRA}). In addition, two markers had been used to determine the presence of immune-checkpoints molecules (PD-1and TIM-3).

CD4

This antigen is involved in the recognition of MHC class II molecules and is a co-receptor for HIV. CD4 is primarily expressed in a subset of T-lymphocytes, also referred to as T helper cells, but may also be expressed by other cells in the immune system, such as monocytes, macrophages, and

dendritic cells. CD4 functions to initiate or augment the early phase of T-cell activation through its association with the T-cell receptor complex and protein tyrosine kinase, Lck.

CD8

Cluster of Differentiation 8 is a cell surface glycoprotein found on most cytotoxic T lymphocytes that mediate efficient cell-cell interactions within the immune system. The CD8 antigen acts as a co-receptor with the T-cell receptor on the T lymphocyte to recognize antigens displayed by an antigen presenting cell (APC) in the context of class I MHC molecules. CD8 is found on a T cell subset of normal cytotoxic/suppressor cells which make up approximately 20-35 % of human peripheral blood lymphocytes. The CD8 antigen is also detected on natural killer (NK) cells, subpopulations of peripheral blood null cells, thymocytes and bone marrow cells. The CD8 co-receptor functions as either a homodimer composed of two alpha chains, or as a disulfide-linked heterodimer composed of one alpha and one beta chain. Both alpha and beta chains share significant homology to immunoglobulin variable light chains. The majority of CD8+ T cells express CD8 as a alpha/beta heterodimer.

TCR-γδ

"Normal" T-cell receptor (TCR) is composed by one alpha and one beta subunit; TCR-γδ cells (Tγδ or TCRgd or Tgd) is a less frequent subtype of T lymphocytes with an invariant TCR composed by one gamma and one delta subunit, able to recognize a broad range of antigens (mainly lipidic) not presented by MHC molecules. TCR-γδ cells can have also direct cytotoxic activity or indirectly through activation of other immune cells and cytokine production [57]. TCR-γδ cells are a subsets of CD3+ cells. TCR-γδ cell function and behaviour in tumoral tissue are not completely understood. In many cancers they act as direct antitumor cells (leukemia, neuroblastoma and some carcinomas) [58], but it has been reported a pro-tumor role in colorectal cancer (CRC), in which a subtype of TCR-γδ (γδT17) are responsible of the chronic inflammation through IL-17 secretion, leading to expansion of myeloid-derived suppressor cells (MDSC) [59].

Regulatory T cells (Tregs)

Treg cells are a subset of CD4+-Tcells with a suppressive role in normal immunity and in self-tolerance, necessary to prevent autoimmune lethal diseases (e.g. in thymus and enteric mucosa) [60]. Treg cells activation in periphery is promoted when a naive CD4+ T-cell recognizes an antigen in absence of optimal co-stimulation (e.g. instestinal commensal bacteria) or in presence of TGFb produced by tumor microenviroment [61]. Proliferation of Treg is promoted by FOXP3

(forkhead box P3) a transcriptional regulator that converts naïve T-cells into Tregs, with suppressive activity [62]. Treg express high levels of CD25[63] and a combination of staining for CD127 along with staining for CD25 can be used to discriminate Treg cells from activated T cells. CD4+,CD25+,CD127−/low T cells show high FoxP3 expression and potent suppressive activity [64].

IL7-Receptor CD127

IL-7 is a protein with differentiation and homeostatic role in lymphoid lineage cells (T, B and NK). IL-7 promotes differentiation of hematopoietic stem cell in lymphoid progenitor cell, from which will derive lymphocytes, as opposed to IL-3 stem cell stimulation towards myeloid progenitor.
As many other IL receptors, IL-7 receptor is a heterodimer composed of a gamma chain (common to many other IL-R) and a specific sub-unit (IL-R alpha, CD127). Dosing IL-7R on lymphocytes surface can estimate the proliferation and lymphoid cells pool maintenance during immune response [65].

Naive, central memory, effector memory and TEMRA T-lymphocytes

The study of lymphocyte behavior and biology has recently overcome, mainly thanks to cytofluorimetry, the original concept of a dual division of T lymphocytes (CD3+) in helper (CD4+) and effector (CD8+) categories.

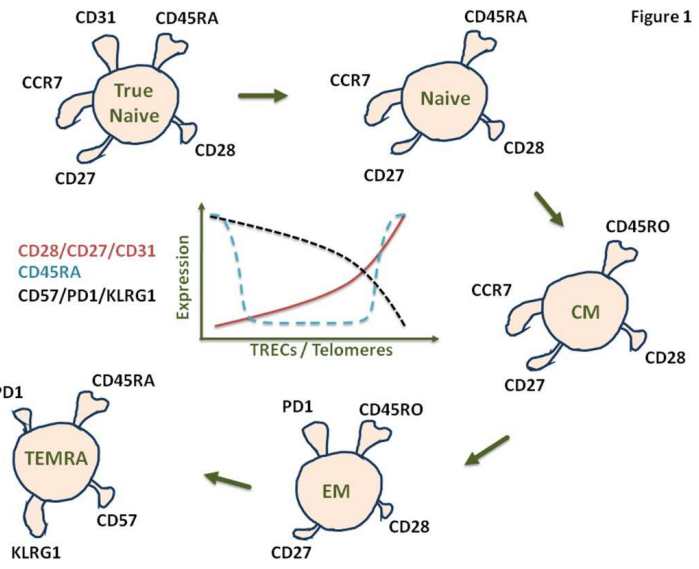


Figure 1

Biological behavior of T cells has been successfully explained describing a pool of lymphocytes with different phenotypes, homing and functions. T cell populations have been divided into four cell types using surface markers as C-C motif chemokine receptor (CCR)−7 and CD45RA (an isoform of CD45). Such pool of lymphocytes includes naive T cells (TN) and antigen-experienced memory T cells (Tm) divided into central memory (Tcm) and effector memory (Tem). Naive T cells express both CCR7 and CD45RA (Tn CCR7+/CD45RA+), whereas primed T cells have three different subsets that can be differentiated by CCR7 and CD45RA expression patterns: T central memory (Tcm CCR7+/CD45RA-), T effector memory (Tem CCR7-/CD45RA-) and terminally differentiated effector memory cells re-expressing CD45RA (Temra CCR7-/CD45RA+) [66].

Tcm cells express CD62L and CCR7, that promote lymph-nodes migration and prompt proliferation in case of antigen re-exposition. Tem cells can migrate in periphery and infiltrate tissue because lack CD62L and have immediate effector functions. T memory cells derive from central memory (Tcm) and persists for life because their of ability to self-renew. A short-lived subtype with an extremely high cytolytic phenotype (granzymes and perforin) are Te (T effector) cells that can derive from Tcm and Tem after repeated antigen contact [67].

PD-1

PD-1 is a cell surface receptor member of the immunoglobulin superfamily expressed on T and pro-B cells. PD-1 has a regulatory (suppressive) role in immune response (immune check-point) protecting from autoimmune diseases; PD-1 binds to its ligands (PD-L1 and L2) in the context of MCH TCR-antigen interaction acting as a negative co-stimulator to promote apoptosis in activated T-cells and inhibiting apoptosis in regulatory T-cells (T-Regs).
In addition to immunosuppressive microenvironment mechanisms (as secretion of inhibitory cytokines), many tumor types (including gastric, ovarian, lung and renal carcinomas) over-express PD-L1, down-regulating effector anti-tumor T-cell activity and facilitating immune evasion (tumor imunoediting).
After these findings, several clinical trials used monoclonal antibodies against PD-1 (and anti-CTLA-4 antibody with analogous physiologic mechanism) to prevent immune evasion, with promising clinical outcomes for tumor “immunotherapy” [68].

TIM-3

T cell immunoglobulin and mucin domain 3 (TIM-3) is a member of the TIM gene family that are surface receptors expressed in T and myeloid lineages cells. TIM-3 has an inhibitory role in T cell-mediated immune responses, leading to reduced proliferation and cytokines production.
TIM-3 plays a role in control of autoimmunity and is overexpressed in chronic viral infections, many types of cancers (in CD8+ in melanoma and CD4+/CD8+ in lung cancer) and in exhausted effector lymphocytes [70].

Cytokine secretion (panel 3)

Panel 3 has been performed to describe intracytoplasmic presence of cytokines (IL-2, IL-4, INF-γ, TNF-α) in CD4+ and CD8+ T-cells, and their cellular activity in terms of proliferation (Ki-67) and presence of cytotoxic granules (CD107a).

IL-4

The interleukin 4 is a cytokine with many physiological roles, produced by Th2 lymphocytes after differentiation from naive helper T cells (Th0), even though is not fully understood which is the first cell type producing IL-4 (could be basophils). IL-4 stimulates proliferation of activated B and T cell, M2 class macrophages (leading to fibrosis), differentiation of B-cell in plasma cell and IgE class switch [72].

Ki-67

Ki-67 (Kiel (German city) - 67 (number of clone of Hodgkin lymphoma line from which it was isolated)) is a nuclear protein associated with cellular replication and ribosomal RNA transcription. Ki-67 is present in all replicating phases (G1, S, G2 and M) and it is absent in G0 (cell quiescence), so it is used clinically and experimentally to determine the replication grade of observed cells [73].

CD107a

Lysosomal-associated membrane protein 1 (LAMP-1 or Cluster of Differentiation 107a) is a transmembrane protein, mainly associated with lysosome, expressed in many cellular types.
LAMP-1 is primary located across lysosomal membrane with homeostatic functions (pH, lysosomal integrity and catabolism control), but can be expressed in cellular membrane after lysosomal fusion acting as selectins-ligand in cell to cell adhesion (as in cytotoxic effector phase of T-lymphocytes and NK cells). CD107a is studied as a degranulation and cytotoxic activity indicator on effector cells [74].

Statistical analysis

Since the study is exploratory, no formal hypothesis for sample sizing have been postulated. Nevertheless, the inclusion of 5 chemo-naive patients and 5 patients pre-treated with systemic chemotherapy plus or minus a targeted drug is considered reasonable for the purpose of the study.
Cytofluorimetric data, collected clinical variables, overall survival (OS), disease free survival (DFS) or progression free survival (PFS) of the two groups (chemotherapy vs naïve patients and grouped chemotherapy regimens), have been analyzed using ANOVA test, Student T-test, Chi Square or Fisher exact test and Log-Rank (Mantel-Cox) when appropriate, with SPSS software v. 20.0 (SPSS Inc. Chicago IL).
A cut-off p value of 0.05 has been considered for approving or rejecting working hypothesis (H_0) indicative for relation between analyzed variable and testing feature(s). A p value of 0.05-0.07 has been separately reported in results.
Data have been tabulated and depicted with appropriate tables and graphs to help results reading.

Institutions collaborating to the study

Since management of stage IV colo-rectal cancers is multidisciplinary, many different Units have collaborated to the present study. Here we report laboratory, pathological, surgical and oncological Units, with their respective affiliations, that collaborate in patients' treatment, follow-up or in sample collection and analysis:

- Tumor Immunology laboratory; Dept. of Surgery, Oncology and Gastroenterology, Immunology and Oncology section; University of Padova
- Pathology unit; Dept. of Medicine; Pathology and Cytology unit; University of Padova
- 1st Oncology unit, Veneto Institute of Oncology IRCSS, Padova
- 1st Surgical Clinic; Dept. of Surgery, Oncology and Gastroenterology; University of Padova
- Hepatobiliary and Liver transplant unit, Dept. of Surgery, Oncology and Gastroenterology; University of Padova
- Oncological surgery of the esophagus and digestive tract unit, Veneto Institute of Oncology IRCSS, Padova

RESULTS

Clinical characteristics

Eleven liver metastases have been collected and analyzed coming from 10 patients of metastatic colorectal cancer (one patient had resection of two lesions and both have been included in the study and considered as two different cases). Seven patients have been pre-treated with systemic chemotherapy (CT group) and three patients have been only surgically treated with colic resection and/or palliation (e.g. oostomy or intestinal by-pass) and liver metastases biopsy or excision (Tab I).

Gender was male in 45.5% and female in 54.5 % of cases. Mean age at operation was 55.7 +/- 15.6 years. The main part (87.5%) of CT-patients received one line of chemotherapy; the other 12.5% received two lines before surgery. Oxaliplatin-based chemotherapy was administered in 62.5% of patients, Irinotecan-based in 25% and immunotherapy (Ipilimumab/Nivolumab) in 12.5% (one case). One case received anti-VEGF (Bevacizumab) and four cases received anti-EGFR (Cetuximab) targeted therapy in addition to cytotoxic chemotherapy.

Primary tumor location was right colon in 18.2% (2 cases), left colon in 63.6% (7 cases) and rectum in 18.2% (2 cases). One B-RAF and one N-RAS mutations were mapped, the other 80% of patients had no mutations in analyzed genes. There were two patients with micro-satellite instability (MSI-H), whereas the remainder 8 were stable (MSS).

Five patients (62.5%) underwent on surgery within 6 months from the end of systemic chemotherapy administration and 3 (37.5%) after 6 months. Metastatic samples used for flowcytometry analysis had a mean weight of 1.74 +/- 0.91 grams.

All patients were alive after a mean follow-up period of 12.19 +/- 3.3 months after operation. During follow-up, 60% of patients presented a tumor recurrence with a mean progression-free survival (PFS) of 9.15 +/- 2.17 months (one case had a hepatic recurrence only, two cases pulmonary and lymph-nodal, one case peritoneal only and two cases peritoneal and pulmonary).

Table I. Patients characteristics

Met ID	Patient age	Primary tumor site	CT regimen	Mutational status	Satellite status	Flowcytometry sample Weight (g)	Total leukocytes in flowcytometry sample	Leukocytes/gram in flowcytometry sample
1	72,1	Right colon	OX	NRAS mut	MSI-H	1,7	1,5E+07	8,8E+06
2	50,8	Left colon	OX	All WT	MSS	1	1,0E+07	1,0E+07
3	39,1	Left colon	IRI/cet	All WT	MSS	4,3	5,6E+06	1,3E+06
4	67,8	Rectum	OX-IRI/bev	All WT	MSS	1,3	2,0E+06	1,5E+06
5	66,6	Rectum	OX/pani	All WT	MSS	1,5	6,5E+06	4,3E+06
6	22,8	Right colon	ipili/nivol	All WT	MSI-H	1,6	7,5E+06	4,7E+06
7	62,5	Left colon	OX/pani	All WT	MSS	1,8	5,0E+06	2,8E+06
8		Left colon	OX/pani	All WT	MSS	2	3,0E+06	1,5E+06
9	72,1	Left colon	Naive	All WT	MSS	1,1	6,0E+06	5,5E+06
10	51,7	Left colon	Naive	BRAF mut	MSS	1,63	0,6E+06	0,4E+06
11	44,6	Left colon	Naive	All WT	MSS	1,14	7,5E+06	6,6E+06

Tumor infiltrating leukocytes composition

Among all leukocytes infiltrating the 11 samples (detected with CD45), the vast majority were T-lymphocytes, followed by macrophages, B-lymphocytes and NK cells. Moreover, an estimation of absolute number of cell sub-types revealed that T-cells were $8.0x10^6 \pm 1.6x10^6$/gram, macrophages $1.3x10^6 \pm 2{,}5x10^6$/gram, B-cells $0.2x10^6 \pm 4.9x10^6$/gram and NK cells $0.2x10^6 \pm 0.6x10^6$/gram (Tab II, Fig. 1A).

Table II. Leukocytes sub-populations

	% intra CD45+		Leukocytes count (absolute number) in flowcytometry samples	
	Mean (n=11)	SD	Mean (n=11)	SD
LT (CD3+)	62,7	25,6	8,0E+06	1,6E+06
LB (CD20+)	1,2	1,6	1,9E+05	5,0E+06
NK (CD56+)	1,0	1,1	2,5E+05	6,7E+05
Mϕ (CD68+, CD3neg,CD20neg,CD56neg)	6,7	3,0	1,3E+06	2,5E+06

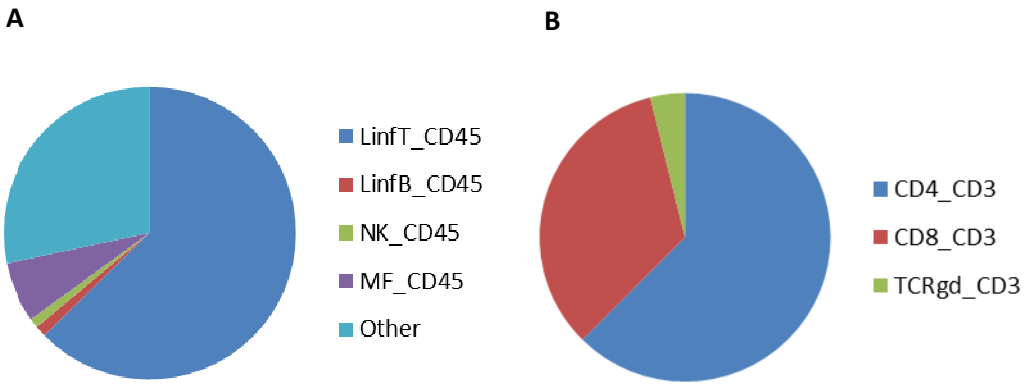
One metastases (ID Met 10) had a very low level of infiltrating leukocytes, making possible to execute only this first leukocytes sub-populations analysis. It was possible to characterize T-cells subpopulations with further analysis only on the other ten metastases.

Analyzing T-lymphocyte sub-population, CD4+ cells were the most represented among CD3+ cells, followed by CD8+ cells and TCRγδ cells. Among CD4+, T-regulatory cells (Treg) were 7.1%. Absolute number estimation of such cell populations showed that CD4+ T-cells are $5.2x10^6 \pm 1.1x10^7$/gram, CD8+ T-cells are $2.3x10^6 \pm 4.1x10^6$/gram, TCRγδ cells $2.7x10^5 \pm 5.2x10^5$/gram and Tregs $5.0x10^5 \pm 1.2x10^6$/gram (Tab. III and Fig. 1B).

Table III. T-cell subpopulations

	% intra CD3+ or CD4+		Lymphocyte count (absolute number)	
	Mean (n=10)	SD	Mean (n=10)	SD
CD4+intra CD3+	57,9	15,5	5,2E+06	1,1E+07
CD8+ intra CD3+	31,2	11,4	2,3E+06	4,1E+06
TCRγδ+intra CD3+	3,6	1,1	2,7E+05	5,2E+05
Tregs (CD25+/CD127low) intra CD4+	7,1	4,5	5,0E+05	1,2E+06

Figure 1. Leukocyte (A) and Lymphocyte (B) sub-populations



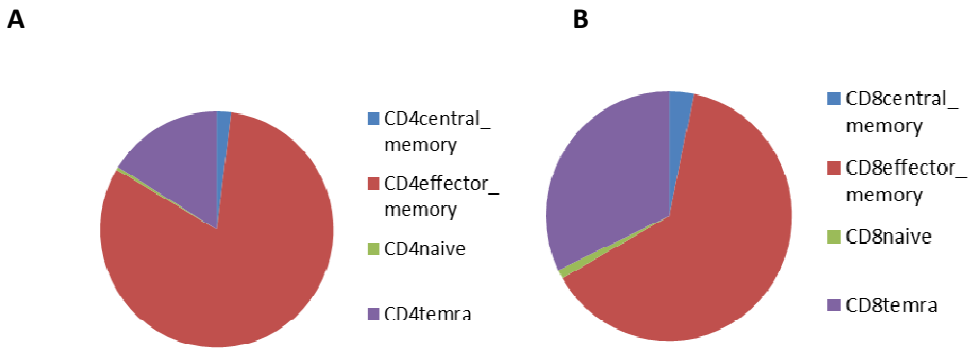
Analysis of CD4+ and CD8+ T-cells with the two maturation markers CCR7 and CD45RO, showed that the vast majority of these cells were effector cells and in particular effector memory (T_{EM}, CCR7neg/CD45RO+) and terminally differentiated effector memory (T_{EMRA}, CCR7neg/CD45ROneg), with a very low level of naïve T-cells (CCR7+/CD45ROneg) or central memory (T_{CM}, CCR7+, CD45RO+). Our results show that naïve and T_{CM} together accounted less than 4% in both T-cells sub-types (Tab. IV and Fig. 2).

Table IV. Differentiation of CD4+ and CD8+ T-cells

	% of CD4+		Lymphocyte count (absolute number)	
	Mean (n=10)	SD	Mean (n=10)	SD
CD4+ naive	0,4	0,9	7,9E+04	1,4E+05
CD4+ T_{CM}	2,0	1,9	6,7E+03	7,9E+03
CD4+ T_{EM}	81,7	14,7	4,4E+06	9,6E+06
CD4+ T_{EMRA}	16,1	14,2	8,0E+05	1,8E+06

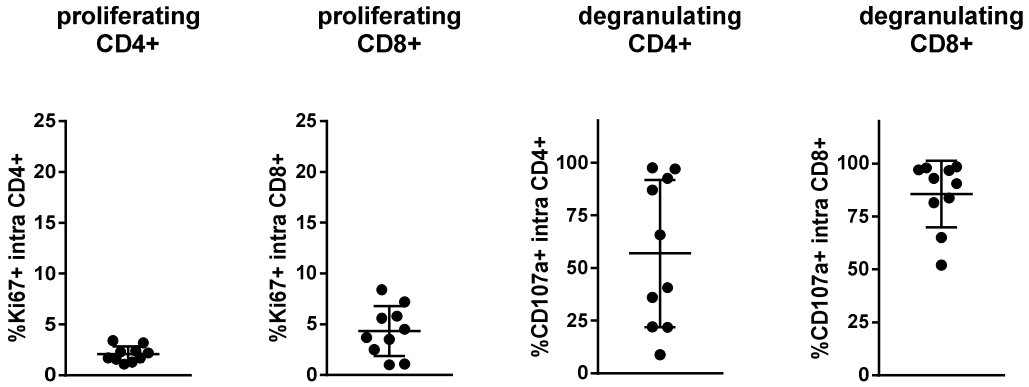
	% of CD8+		Lymphocyte count (absolute number)	
	Mean (n=10)	SD	Mean(n=10)	SD
CD8+ naive	1,3	1,3	3,7E+04	5,5E+04
CD8+ T_{CM}	3,3	5,5	2,6E+04	4,8E+04
CD8+ T_{EM}	57,2	16,1	1,3E+06	2,6E+06
CD8+ T_{EMRA}	38,5	16,1	9,1E+05	1,6E+06

Figure 2. Differentiation of CD4+ (A) and CD8+ (B) T-cells



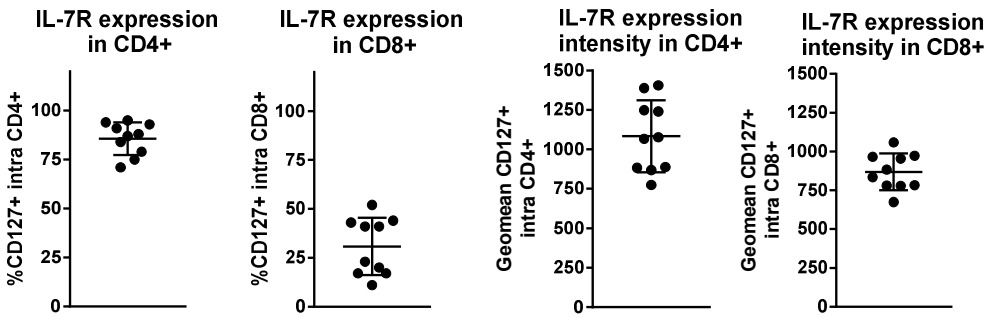
Evaluation of a proliferation marker (Ki67) described a limited proliferative capability of both CD4+ and CD8+ infiltrating T-cells (less than 10% were cycling cells). On the other hand, cytotoxic activity analysis (measured as presence of intracytoplasmic cytotoxic granules CD107a+) displayed a high activity for CD8+ T-cells in all samples and a more variable (but present) activity for CD4+ T-cells (Fig 3).

Figure 3. Proliferation and cytotoxic activity of CD4+ and CD8+ T-cells.



IL-7 is a crucial survival factor for memory T-cells and in particular, its presence supports maintenance of the memory-cells pool. To evaluate CD4+ and CD8+ T-cells capability to maintain memory pools, IL-7 receptor (IL-7R or CD127) was tested as an instructive marker. CD127 was expressed on the membrane in a higher percentage of CD4+ (p=0.0001) if compared to CD8+ T-cells and with a more pronounced intensity expression (p=0,001) (Fig. 4).

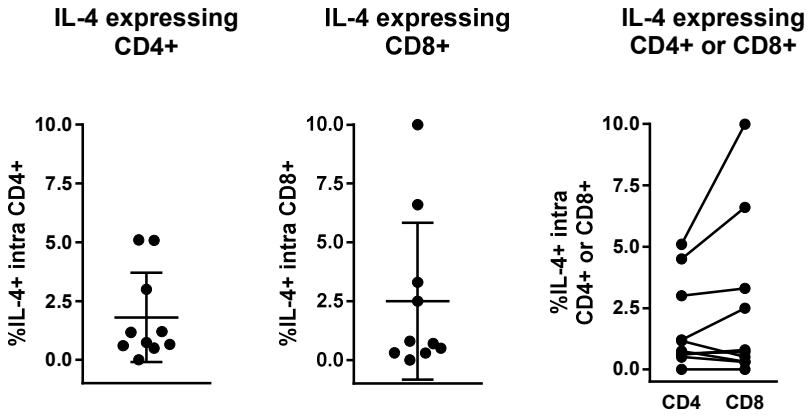
Figure 4. IL7-R expression on CD4+ and CD8+ T-cells



	Mean	SD	p
CD4_IL7R	85,7	8,3	0,0001
CD8_IL7R	31,0	14,6	
Intens_IL7R_CD4	1084,0	228,2	0,001
Intens_IL7R_CD8	868,3	118,2	

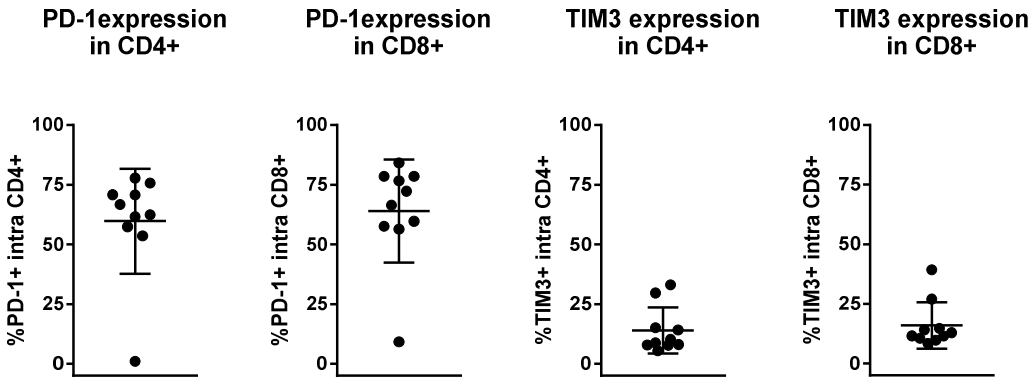
Despite the complex processing procedure of sample disgregation and the long interval between surgical excision and cytofluorimetric labelling, it has been tried an analysis of intracytoplasmic cytokine production. IL-4 was the only detectable cytokine among that evaluates in panel 3 (see Materials and Methods). IL-4 expression was not detectable in all samples. The IL-4 positive samples showed both CD4+ and CD8+ cytokine expressing T-cells, even though IL-4 expressing T-cells displayed low expression intensity (Fig. 5 and data not shown).

Figure 5. IL-4 expression in CD4+ and CD8+ T-cells. In the right panel, CD4+ and CD8+ connected came from the same sample.



Finally, immuno-checkpoint markers have been analyzed. PD-1 was expressed in both CD4+ and CD8+ in more than 50% of T-cells. The outlier (ID Met 6) that appeared with a very lower percentage of PD-1 expressing T-cells is the patient who received anti-PD-1 therapy. On the other hand, TIM-3 has a low expression in both sub-populations (Fig. 6).

Figure 6. Immuno-checkpoint markers expression in CD4+ and CD8+ T-cells



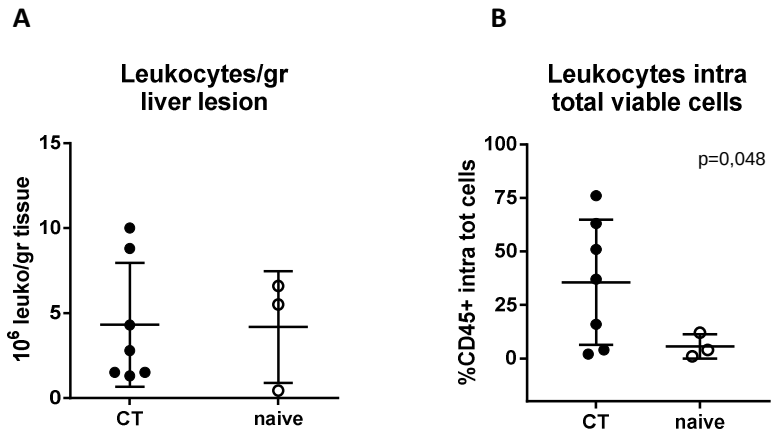
Pre-treated and naïve patients

To determine the effect of chemotherapy in liver metastases infiltrating leukocytes, it has been performed a comparative analysis between patients that received systemic chemotherapy before operation (Chemotherapy group) and patients that have been treated with surgical resection first (naïve group).

Since immunotherapy regimen has the main target of activating and stimulating patient's immune system against tumor cells, to avoid confounding factor, patient treated with immunotherapy has been excluded in this comparative analysis of cytofluorimetric results.

Even though optic microscopy cell count with viable dye Trypan-blue did not demonstrate difference in absolute number of infiltrating leukocytes per gr of tissue (Fig. 7A), cytofluorimetric data of leukocytes (%CD45+) among viable cells displayed a different distribution in pre-treated (CT-group) and naïve patients (Fig. 7B). In particular CT-patients had a higher percentage of infiltrating leukocytes among viable cells compared to naïve patients (p=0,048). Not all viable cells have been precisely characterized with specific markers. Otherwise, flowcytometry data described their size (FSC, Forward Scatter) and cellular complexity (SSC, Side Scatter cytometric parameter), and it is plausible to consider them mainly tumor cells also because normal liver tissue have been mainly eliminated from biopsies by the pathologist dissection in operating room before standard coloration and eventually by the biologist before cytofluorimetric procedures.

Figure 7. Leukocytes in liver lesions



Analyzing leukocyte sub-populations, some differences between CT- and naïve patients emerged, but none of these reached statistical significance. In particular, B-cells were more represented in CT-patients and NK cells have a higher ratio in naïve patients. On the other hands, T-cells and Macrophages were present in similar percentages in the two groups of patients (Fig. 8 and Tab. V).

Figure 8. Leukocytes sub-populations in CT- and naïve patients

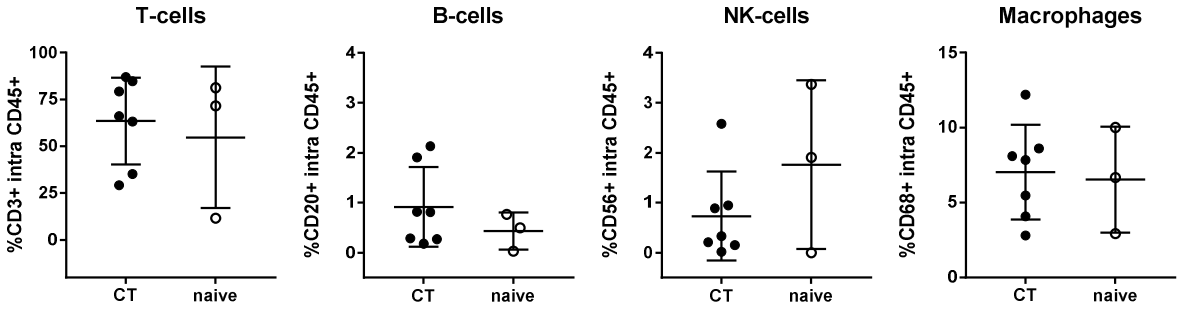


Table V. Leukocyte sub-populations in CT- and naïve patients

	% intra CD45+				
	CT-patients		Naive		P
	Mean (n=10)	SD	Mean (n=10)	SD	
LT (CD3+)	63,5	23,1	54,8	37,7	n.s.
LB (CD20+)	0,9	0,8	0,4	0,4	n.s.
NK (CD56+)	0,7	0,9	1,8	1,7	n.s.
Mϕ (CD68+, CD3neg,CD20neg,CD56neg)	7,0	3,2	6,5	3,5	n.s.

On the other hand, T-lymphocytes analysis revealed that CT-patients showed a polarized response towards CD4+ T-cells, strongly more represented among CD3+ if compared to naïve patients and with a very small dispersion around the mean value (p=0,0001). In contrast, naïve patients displayed a polarization towards a CD8+ T-cells response if compared to CT-group (p=0,005). In addition, TCRγδ and T-regs were more represented in pre-treated patients, even if these differences did not reach statistical significance (Fig. 9 and Table VI).

Figure 9. T-cells subsets distribution between CT-patients and naive

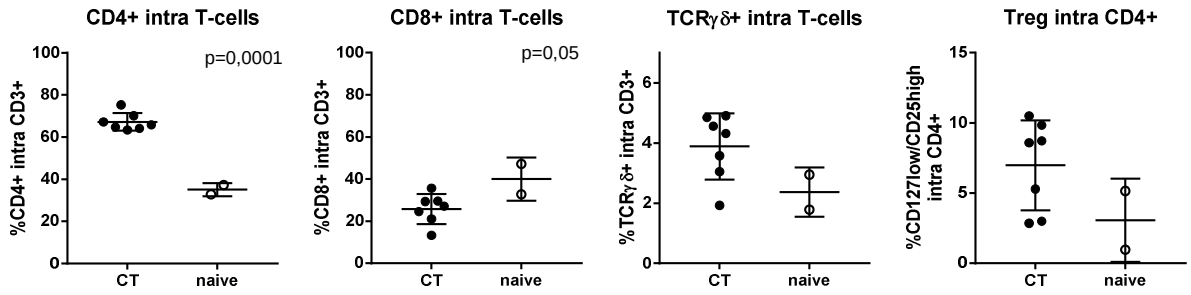


Table VI. Lymphocyte subsets in CT-patients and naive

	% intra CD3+ or CD4+				
	CT		Naive		P
	Mean (n=9)	SD	Mean (n=9)	SD	
CD4+ (intra CD3+)	67,2	4,2	35,1	3,1	0,0001
CD8+ (intra CD3+)	25,8	7,1	40,0	10,2	0,05
TCRgd+ (intra CD3+)	3,9	1,1	2,4	0,8	n.s.
Tregs (intra CD4+)	7,0	3,2	3,1	3,0	n.s.

Absolute estimated number of T-cells per gram of tissue were higher in pre-treated patients, as B-cells and NK cells; macrophages are, on the opposite, higher in naive patients (see Fig. 10 and Tab. VII).

Figure 10. Absolute estimated number of Leukocyte subsets per gram of tissue

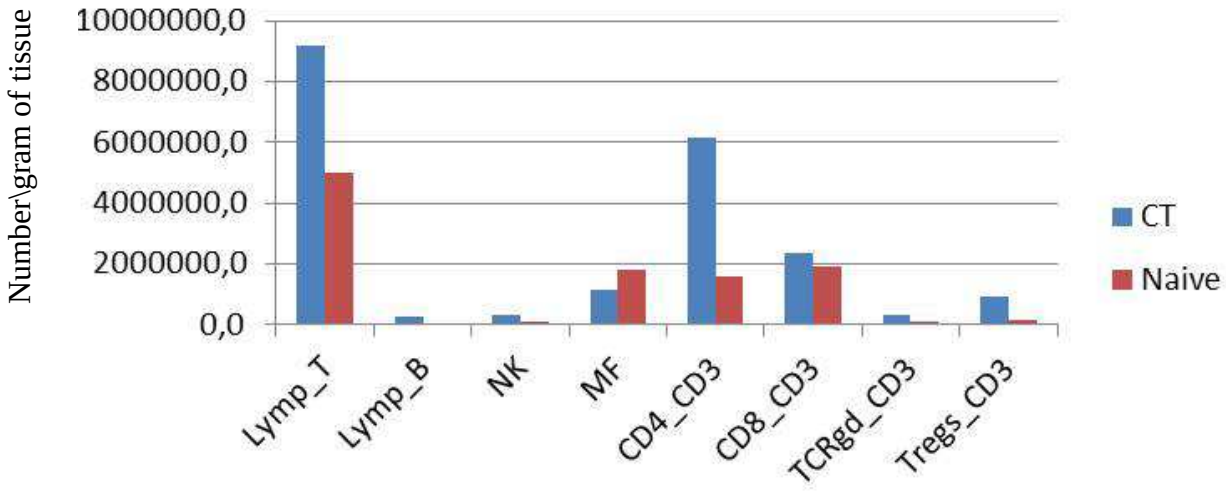


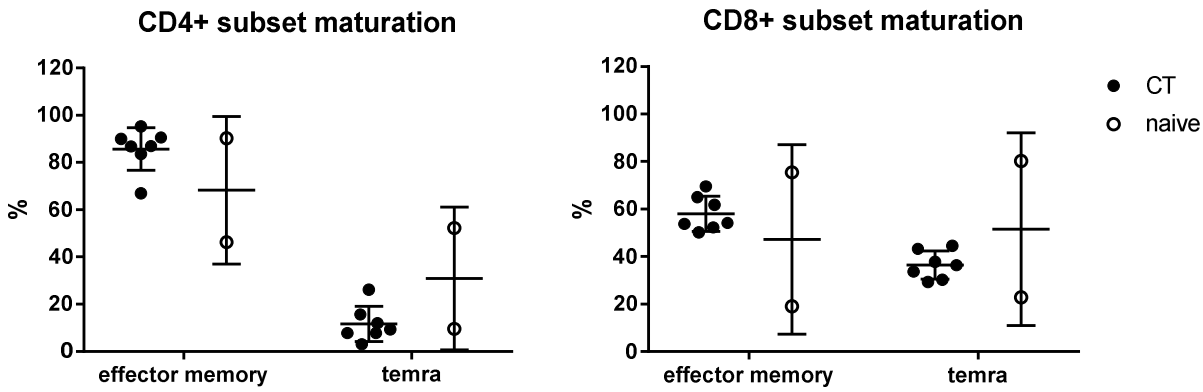
Table VII. Absolute estimated number of Leukocytes per gram of tissue

		LT (n°CD3+/gr)	LB (CD20+/gr)	NK (CD56+/gr)	Mϕ (CD68+/gr)
CT	Mean (n=9)	9,2E+06	2,6E+05	3,1E+05	1,2E+06
	SD	1,9E+07	5,8E+05	8,0E+05	2,6E+06
Naive	Mean (n=9)	5,0E+06	3,0E+04	1,1E+05	1,8E+06
	SD	9,5E+05	1,4E+04	1,1E+05	2,7E+06

		CD4+ (n°/gr)	CD8+ (n°/gr)	TCRgd+ (n°/gr)	Tregs (n°CD25+/CD127low /gr)
CT	Mean (n=9)	6,1E+06	2,3E+06	3,1E+05	6,1E+05
	SD	1,3E+07	4,7E+06	5,8E+05	1,3E+06
Naive	Mean (n=9)	1,6E+06	1,9E+06	1,1E+05	5,2E+04
	SD	2,1E+05	8,8E+05	1,4E+04	5,4E+04

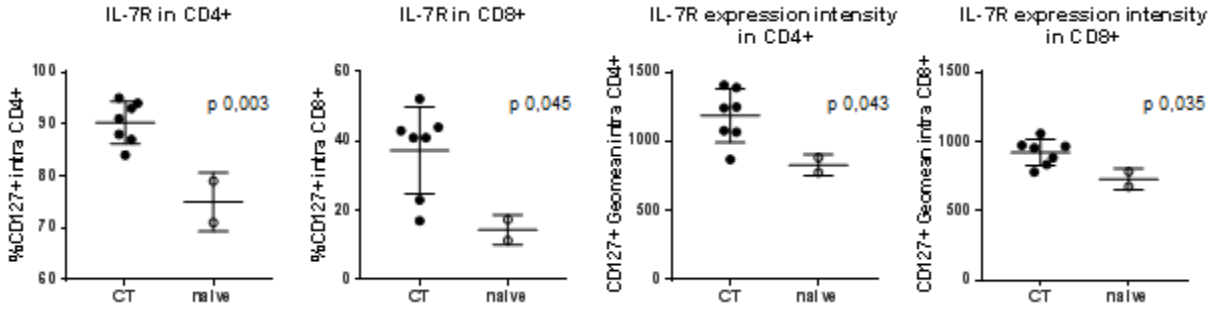
A comparative analysis of T-cells maturation between CT and naïve group was not able to display any difference because of the great dispersion of naïve patients in this characterization. On the contrary, CT-patients displayed a very consistent maturation signature with a minimal dispersion around the mean and independently from CT-regimen. Together T_{EM} and T_{EMRA} represented more than 95% of all T-cells in both CD4+ and CD8+, but with a different ratio. T_{EM} were more than 80% of all CD4+T-cells. On the other hand, within CD8+, 60% were T_{EM} and more than 35% were terminally differentiated effector (Fig. 11).

Figure 11. T-lymphocytes maturation in CT and naive patients



To dissect differences in the capacity of generating and supporting memory pools, expression of IL-7R was determined. The percentage of cells positive for this cytokine receptor was higher both in CD4+ (p=0,003) and CD8+ T-cells (p=0,045) in CT-patients if compared to naïve patients. Moreover, also IL-7R expression intensity was higher in CT-group if compared to naïve patients (p=0,043 in CD4+ and p=0,035 in CD8+; Fig. 12 and Tab. IX). This data support the idea that CT-regimen encourages and sustains T-cell memory pools more efficiently than absence of treatment.

Figure 12. IL-7R expression and intensity in CT and naïve patients

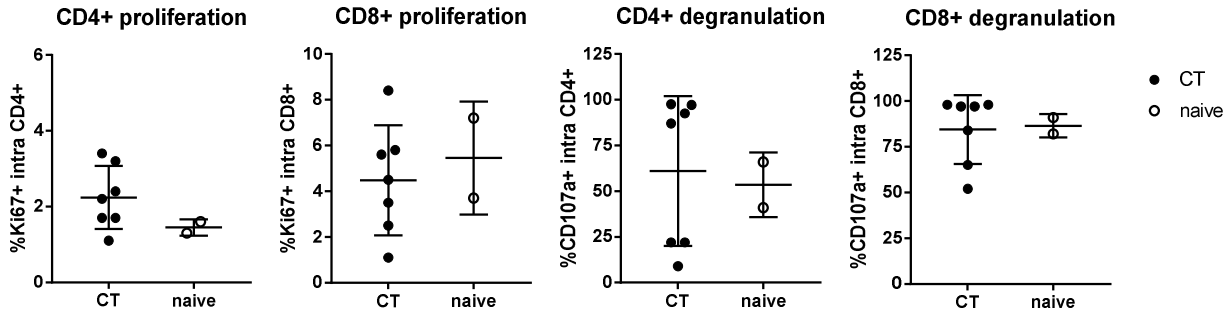


Tab IX. IL7-R expression and intensity in CT and naïve patients

	CT		Naive		P
	Mean	SD	Mean	SD	
% cells IL7R+ intra CD4+	90,29	4,1	75,0	5,7	0,003
% cells IL7R+ intra CD8+	37,3	12,5	14,3	4,3	0,045
Intens_IL7R+_CD4+	1185,0	192,6	828,5	77,1	0,043
Intens_IL7R+_CD8+	921,0	94,3	728,5	77,1	0,035

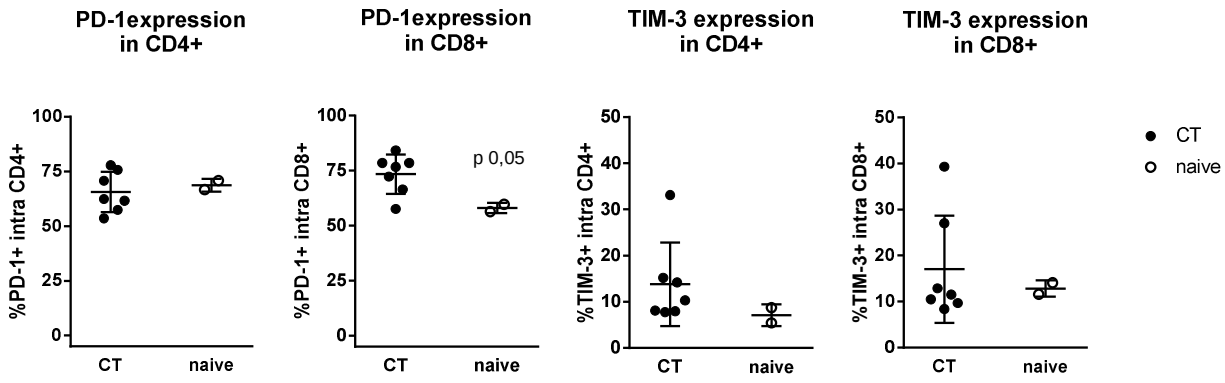
Flow cytometry analysis of proliferative capacity and degranulation activity did not reveal differences between CT and naïve patients rather highlighted a great spreading especially in CT-group (Fig. 12bis and Tab. X).

Figure 12bis. Proliferation and activity of CD4+ and CD8+ T-cells.



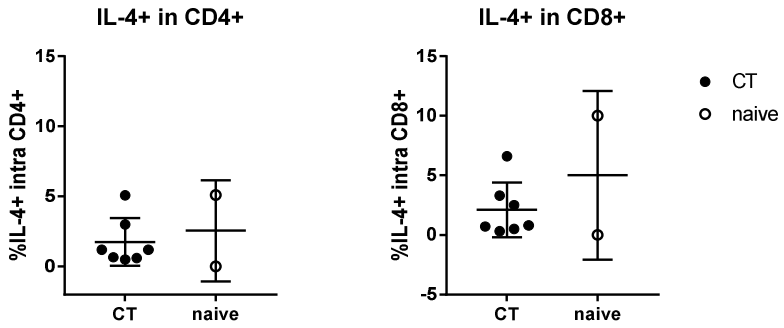
The percentage of PD-1 expressing cells was elevated in both T-cells of CT and naïve group. Only among CD8+, PD-1+cells from CT-patients were more represented than in naïve group (p=0.05). The other immune-checkpoint marker analyzed TIM-3 did not allow to discriminate CT- and naïve patients (Fig. 13 and Tab X).

Figure 13. PD-1 and TIM-3 expression in CT and naïve patients



Neither the analysis of the presence of IL-4 granules in CD4+ and CD8+ T-cells revealed to be instructive to distinguish pretreated from untreated group (Fig.14 and Tab X).

Figure 14. IL-4 levels in T-cells from CT and naive patients



Tab X. Proliferation, checkpoint, IL-4 in CT and naïve patients

		CD4_KI67	CD8_KI67	CD4_PD1	CD8_PD1	CD4_TIM3	CD8_TIM3	CD4_IL4	CD8_IL4
CT	Mean	2,2	4,5	65,6	73,5	13,8	17,0	2,5	3,5
	SD	0,8	2,4	9,3	8,9	9,0	11,6	1,6	3,3
Naive	Mean	1,5	5,5	68,8	58,1	4,7	12,9	2,6	5,0
	SD	0,2	2,5	2,9	2,3	4,4	1,8	3,6	7,1
p		ns	ns	ns	**0,05**	ns	ns	ns	ns

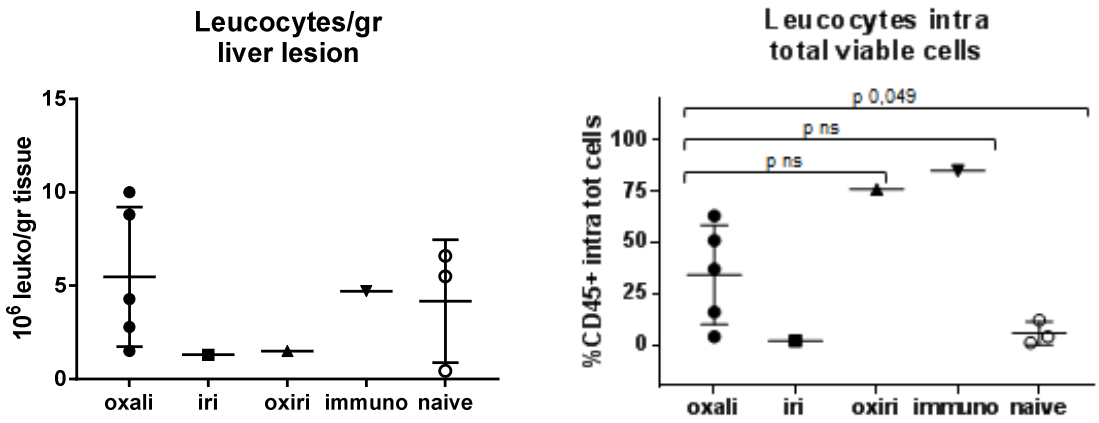
Chemotherapy regimens

To determine if different regimens of chemotherapy (Oxaliplatin, Irinotecan, Oxaliplatin plus Irinotecan and Immunotherapy) could affect infiltrating leukocytes, a comparative analysis of all collected variables has been performed using CT regimen as comparison factor.

Percentage of infiltrating leukocytes among viable cells was the highest in the patient treated with immunotherapy (PT_{IMM}; at pathological report there was a complete response with no viable tumor cells), and similar in oxaliplatin-plus-irinotecan-treated one ($PT_{OX\text{-}IRI}$). Oxaliplatin-treated patients (PTS_{OX}) displayed variable CD45+ percentage ranging between 10 and 60%. Irinotecan-treated (PT_{IRI}) and naïve patients revealed low leukocyte percentages among total viable cells (Fig. 15).

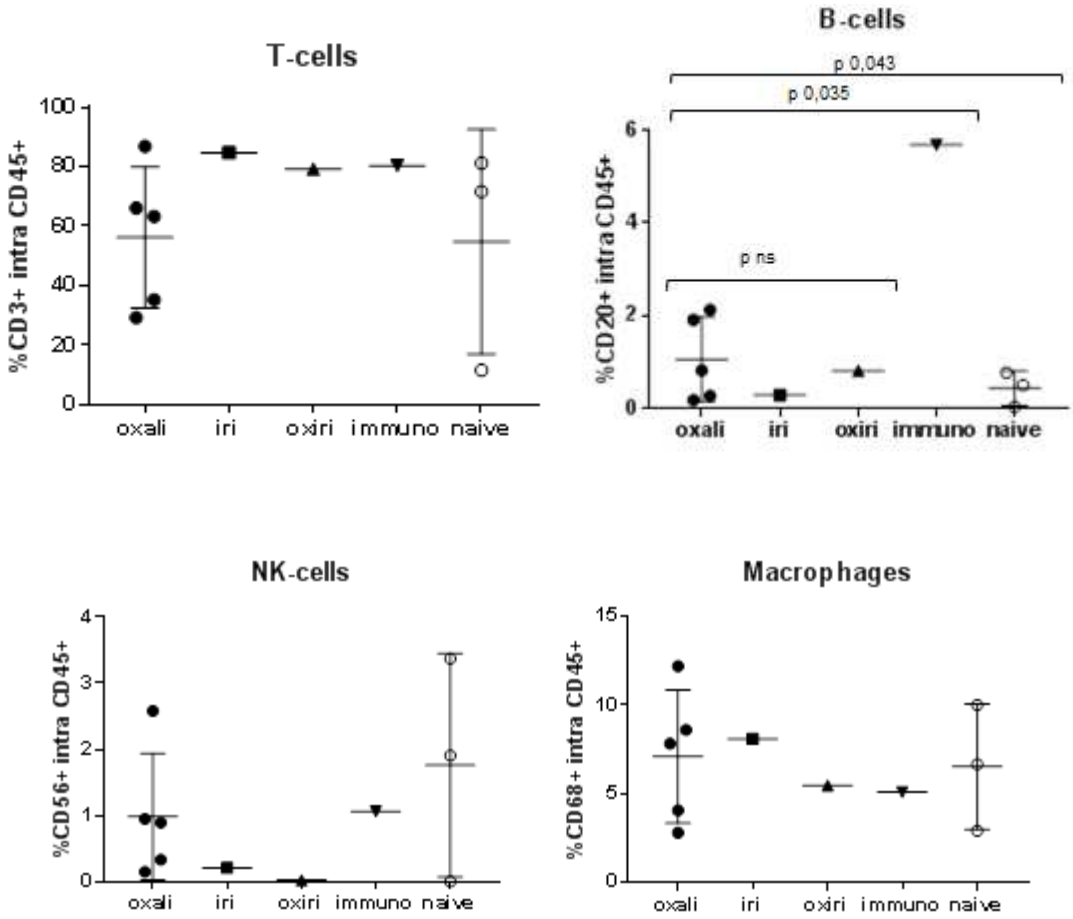
The estimated number of leukocytes obtained with optical microscopy are lower in patients treated with Irinotecan (alone or in association with Oxaliplatin).

Figure 15. Infiltrating leukocytes in different CT-regimen treated patients



Even though the majority of infiltrating leukocytes were T-cells independently from chemotherapy regimen, in PTS_{OX} a certain spreading was observed. B-cells were significantly higher in PT_{IMM}, although percentages were smaller than 10% of all leukocytes (p=0.0035). NK cells and macrophages did not differentiate CT regimens (Fig. 16, Tab XI).

Figure 16. Leukocytes sub-populations in different CT-regimen patients

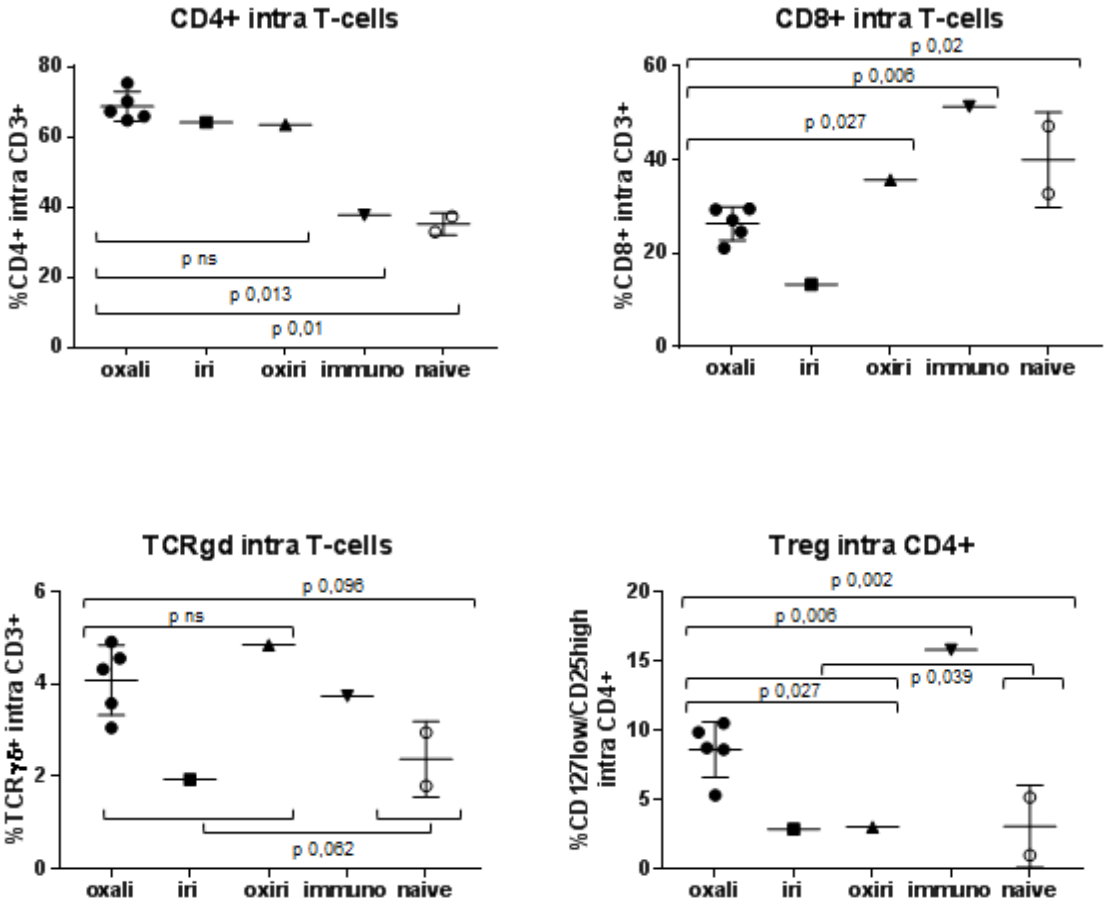


Among T-lymphocytes, CD4+ percentages were great and comparable in PTS_{OX}, PT_{IRI} and $PT_{OX\text{-}IRI}$ and low in PT_{IMM}, generating a statistically significant difference (p=0.013; Fig. 17, Tab XI).

On the other hand, CD8+ T-cells were higher in PT_{IMM} if compared to all other treated patients (p=0,006). Among the "classic" cytotoxic chemotherapy regimens, $PT_{OX\text{-}IRI}$ displayed the highest level of CD8+ T-cells and PT_{IRI} the lowest one (p=0.027).

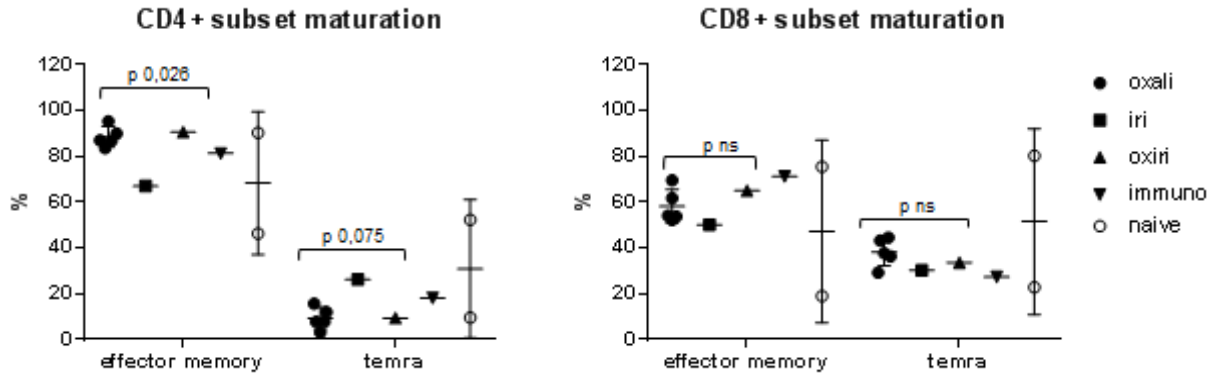
TCRgd in the patient treated with Irinotecan are lower that other cytotoxic regimens (p ns). Treg are very high in immuno-treated patient (p=0.006) and in Oxaliplatin-treated patient among cytotoxic regimens (p=0.027) (Fig. 17).

Figure 17. T-cell subsets in different CT-regimen patients



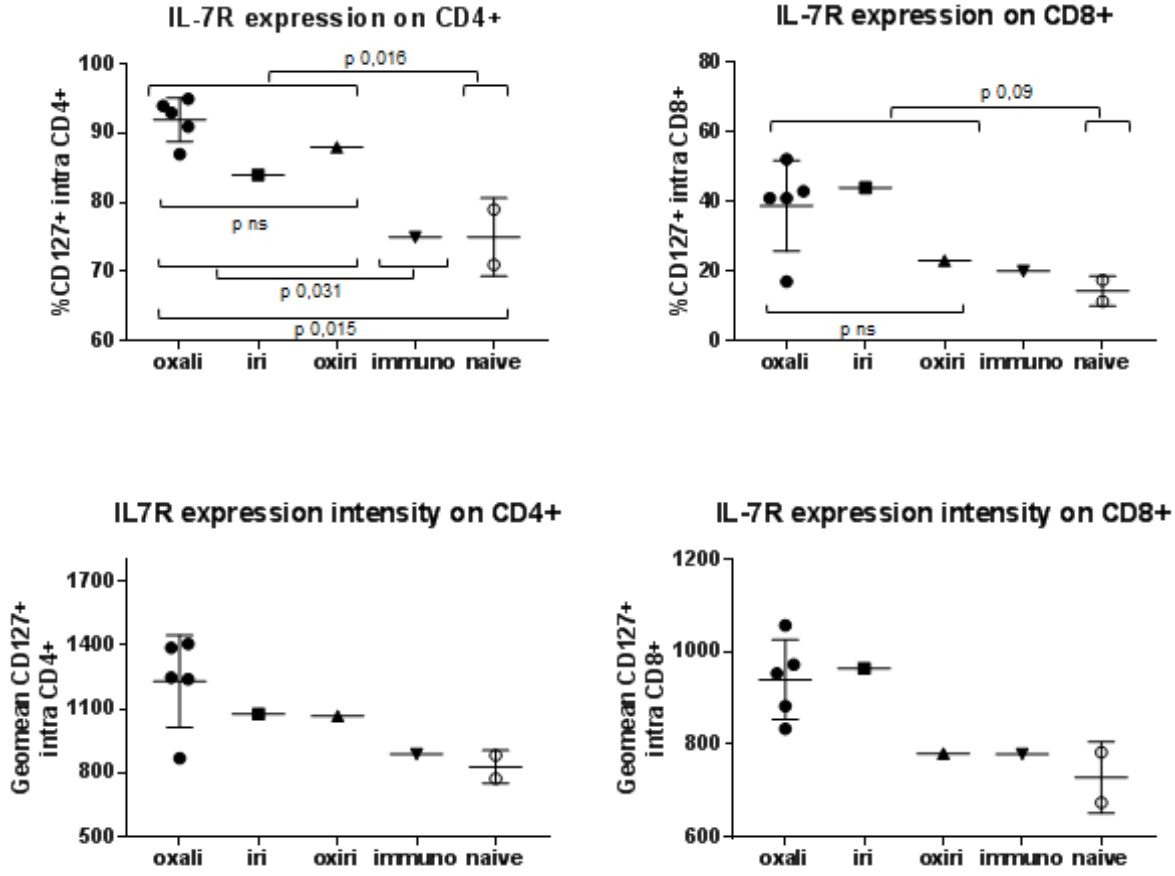
Lymphocytes maturation analysis in classical CT regimens, showed a high proportion of CD4+ T_{EM} and low CD4+T_{EMRA} in PT_{OX}, $PT_{OX\text{-}IRI}$ and PT_{IMM} treated patients, whereas PT_{IRI} has a significantly lower level of CD4-T_{EM} (p=0,026) and a higher presence of CD4+T_{EMRA}. Data from CD8+ lineage displayed similar tendency, even though they did not reach statistical significance (Fig. 18).

Figure 18. T-cell subsets maturation in different CT-regimen patients



Considering IL7 receptor expression, all treated patients show high level, whereas immuno-treated patient has low level of IL7-R (p=0,031). PT_{OX-IRI} and PT_{IMM} displayed a low level of IL-7R expression in CD8+ T-cells, but the differences did not allow discriminating them from the other patients. IL-7R expression intensity disclosed concordant results (Fig.19, Table XI).

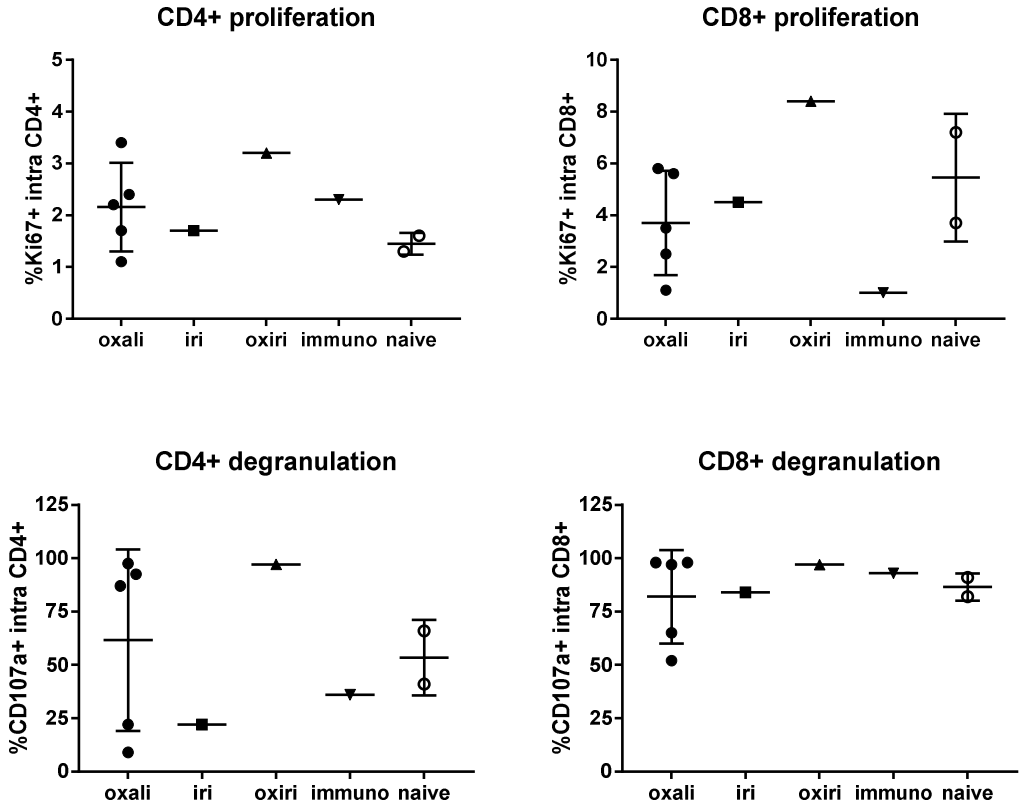
Figure 19. IL-7R expression in infiltrating T-cells



CD4+ and CD8+ T-cells proliferation did not statistically differ among analyzed groups, even though $PT_{OX\text{-}IRI}$ displayed the highest level of Ki-67+ T-cells if compared to other CT regimens.

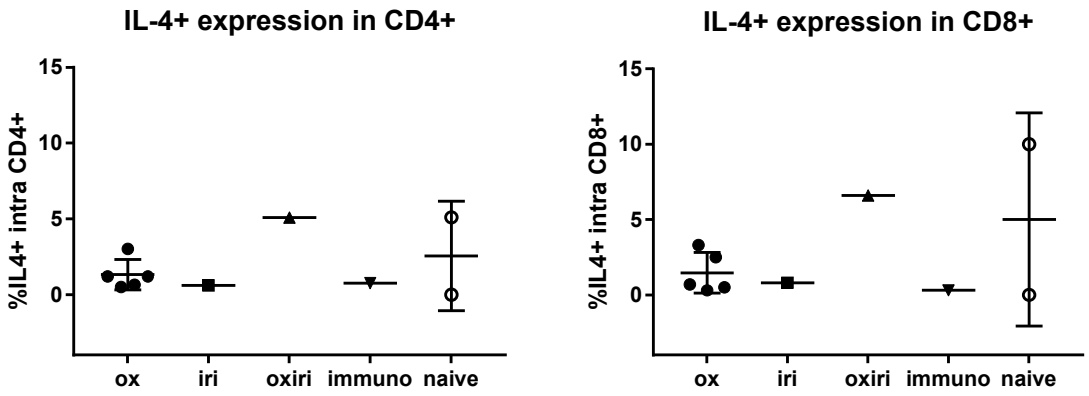
CD4 degranulation (CD107a) show dispersed results among different regimens and in Oxaliplatin treated patients. CD8 cells degranulation (CD107a) is more similar among groups (Fig. 20, table XI).

Figure 20. T-cells proliferation and degranulation capacity in different CT-regimen patients



About cytokines, IL-4 production was higher in $PT_{OX\text{-}IRI}$ if compared with other regimens, among CD4+ and CD8+ T-cells (Fig. 21).

Figure 21. IL-4 expression in T-cells of different CT-regimen patients



PD-1 expression in CD4+ and CD8+ T-cells was significantly lower in PT_{IMM} (p=0.012 and p=0.003, respectively). Analyzing all the other regimens, PD-1+ cell portion was high in pre-treated patients, without any difference between CT-regimens. On the other hand, TIM-3 expression was highly variable in both CD4+ and CD8+ T-cells from PTS_{OX} (Fig. 22 and Tab XI).

Figure 22. Immuno-checkpoint molecules expression in T-cells of different CT-regimen patients

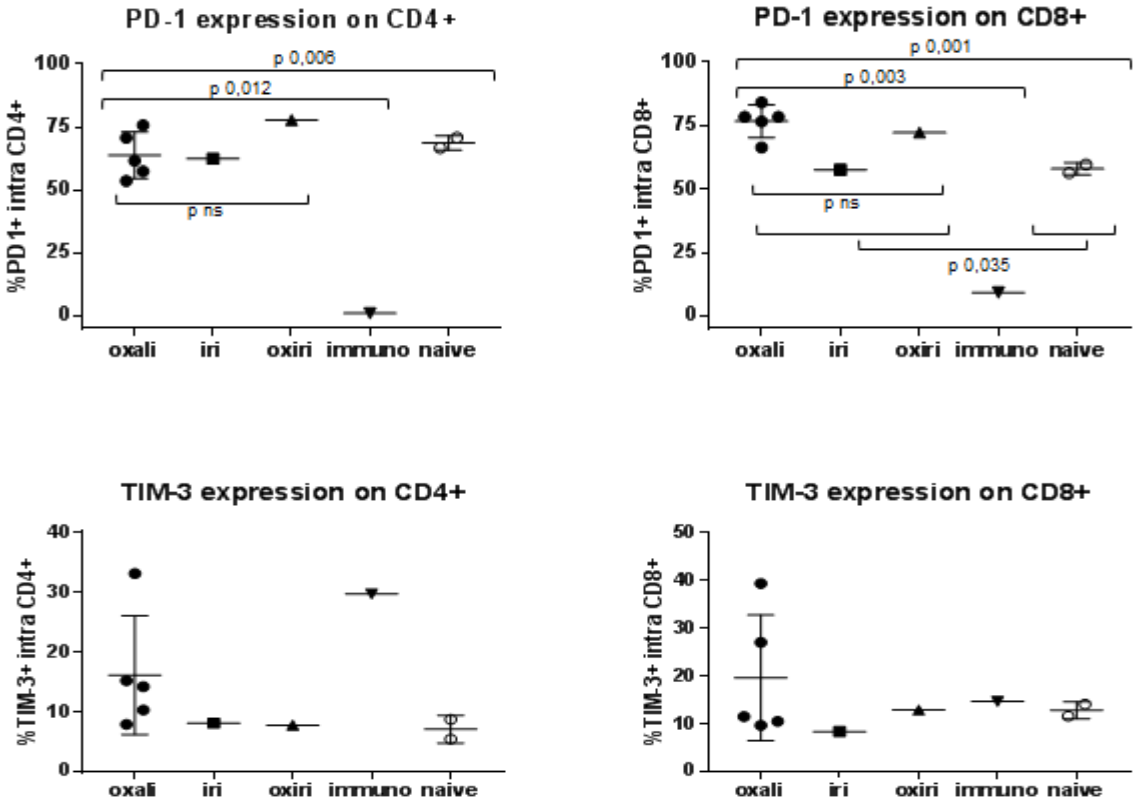


Table XI. Results of comparison of CT regimens and Immunotherapy

	Oxaliplatin		Irinotecan	Immuno	Ox-iri	**p (excluding naive)**		Naive		p (including naive)	
	Mean (n=5)	SD	Mean (n=1)	Mean (n=1)	Mean (n=1)	**ox vs iri vs oxiri**	**ox vs iri vs oxiri vs imm**	Mean (n=3)	SD	all CT and naive	oxali vs iri vs oxiri vs naïve (excl imm)
Linf_T	56,2	23,7	84,7	80,4	79,2	**ns**	**ns**	54,8	37,7	ns	ns
Linf_B	1,1	0,9	0,3	5,7	0,8	**ns**	**0,035**	0,4	0,4	0,009	ns
NK	1,0	1,0	0,2	1,1	0,0	**ns**	**ns**	1,8	1,7	ns	ns
MF	7,1	3,8	8,1	5,1	5,5	**ns**	**ns**	6,5	3,5	ns	ns
CD4	68,6	4,2	64,1	37,7	63,4		**0,013**	35,1	3,1	0,010	0,001
CD8	26,3	3,6	13,3	51,4	35,7	**0,027**	**0,006**	40,0	10,2	0,020	0,039
TCRgd	4,1	0,8	1,9	3,7	4,9	**ns**	**ns**	2,4	0,8	0,096	0,062
Tregs	8,6	2,0	2,9	15,8	3,0	**0,067**	**0,027**	3,1	3,0	0,023	0,065
CD4central_memory	2,7	1,8	4,5	1,0	0,4	**ns**	**ns**	0,3	0,3	ns	ns
CD4effector_memory	88,5	4,4	66,9	81,2	90,5	**0,026**	**ns**	68,3	31,2	ns	ns
CD4naive	0,1	0,1	3,0	0,3	0,0	**0,0001**	**0,0001**	0,2	0,4	0,0001	0,0001
CD4temra	9,2	4,8	26,1	18,1	9,4	**0,075**	**ns**	30,9	30,2		
CD8central_memory	2,1	1,1	18,8	1,0	1,1	**0,0001**	**0,001**	0,8	0,5	0,0001	0,0001
CD8effector_memory	58,3	7,3	50,1	71,2	65,1	**ns**	**ns**	47,2	39,9	ns	ns
CD8naive	1,8	1,7	1,0	0,9	0,5	**ns**	**ns**	0,8	0,8	ns	ns
CD8temra	38,2	6,1	30,2	27,3	33,6	**ns**	**ns**	51,5	40,6	ns	ns
CD4_PD1	63,8	9,2	62,4	1,1	77,8	**ns**	**0,012**	68,8	2,9	0,006	ns
CD8_PD1	76,9	6,5	57,6	9,2	72,3	**ns**	**0,003**	58,1	2,3	0,001	0,035
CD4_TIM3	16,1	9,9	8,1	29,7	7,8	**ns**	**ns**	4,7	4,4	ns	ns
CD8_TIM3	19,6	13,1	8,4	14,7	12,9	**ns**	**ns**	12,9	1,8	ns	ns
CD4_IL7R	92,0	3,2	84,0	75,0	88,0	**ns**	**0,031**	75,0	5,7	0,015	0,016
CD8_IL7R	38,8	13,1	44,0	20,0	23,0	**ns**	**ns**	14,4	4,3	ns	0,092
Intens_IL7R_CD4	1230,2	216,0	1077,0	888,0	1067,0	**ns**	**ns**	828,5	77,1	ns	ns

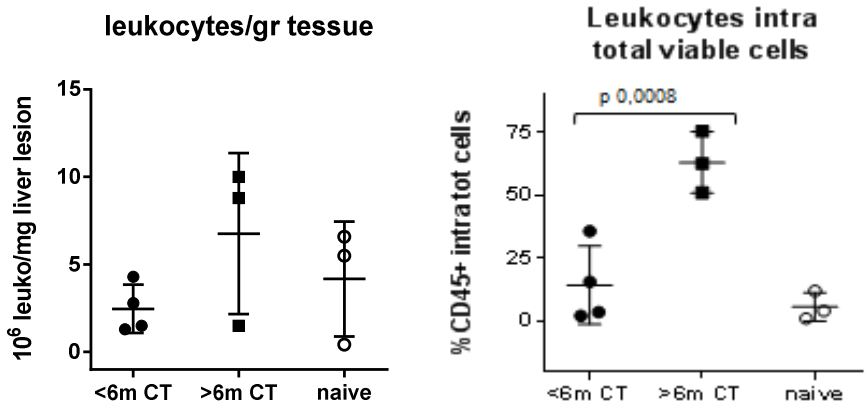
Intens_IL7R_CD8	940,4	86,2	965,0	779,0	780,0	**ns**	**ns**	728,5	77,1	ns	ns
CD4_KI67	2,2	0,9	1,7	2,3	3,2	**ns**	**ns**	1,5	0,2	ns	ns
CD8_KI67	3,7	2,0	4,5	1,0	8,4	**ns**	**ns**	5,5	2,5	ns	ns
CD4_CD107a	61,6	42,5	22,0	36,0	97,1	**ns**	**ns**	53,5	17,7	ns	ns
CD8_CD107a	82,0	21,9	84,0	93,0	97,0	**ns**	**ns**	86,5	6,4	ns	ns
CD4_IL4	1,8	1,2	3,0	0,7	5,1	**ns**	**ns**	2,6	3,6	ns	ns
CD8_IL4	3,1	3,5	1,6	0,3	7,0	**ns**	**ns**	5,0	7,1	ns	ns

Interval between chemotherapy and surgery

To analyze time-dependent evolution of immune response in liver metastases, a two categories comparison scheme was created, according to the interval free from systemic chemotherapy and surgery (less than 6 months and more than 6 months from the treatment). Patients treated within 6 months from the end of CT received Oxaliplatin-based regimen in 3 cases and Irinotecan in one case; patients in group > 6 months received Oxaliplatin in 2 cases and Oxaliplatin plus Irinotecan in one case. As seen in previous section, Immunotherapy-treated patient had particular biological features, so it has been excluded to reduce confounding factors.

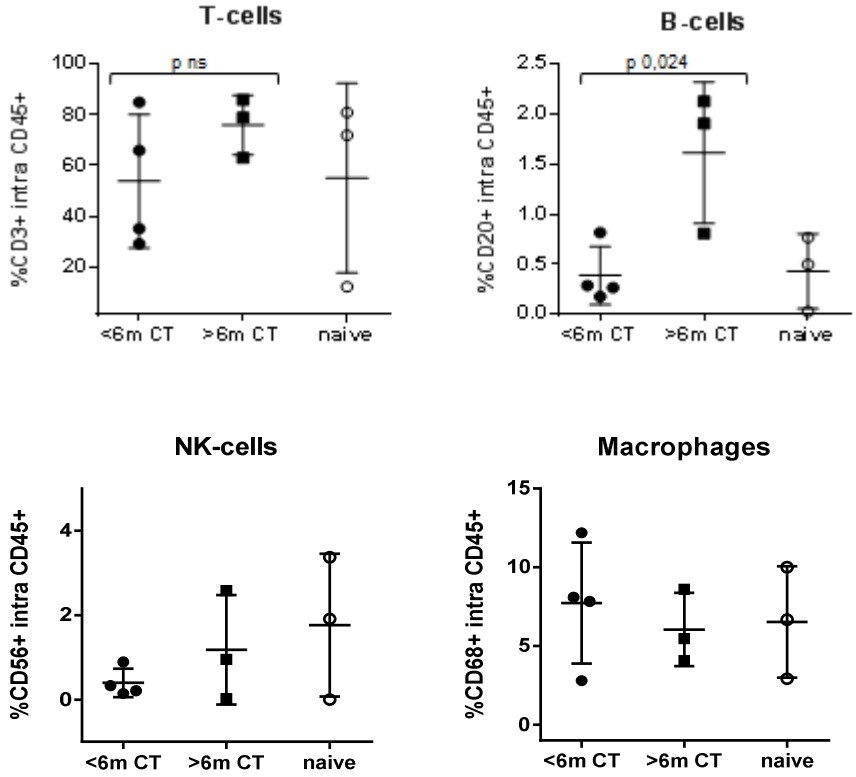
Estimated number of infiltrating leukocytes obtained with optical microscopy, revealed a tendency to a higher number of these cells per gr of tissue in patients with the longer interval free from CT. The same group of patients had a higher percentage of infiltrating leukocytes among viable cells (p=0.0008; Fig. 23).

Figure 23. Leukocytes in liver lesions of patients resected less than 6m (<6m) and more than 6m (<6m) from systemic CT



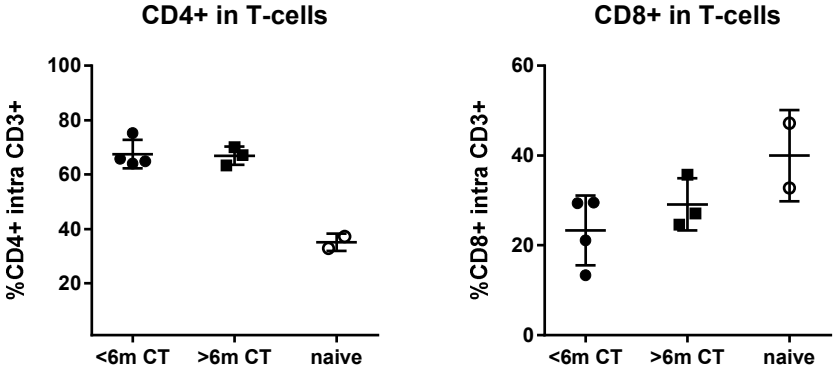
Analyzing flow cytometry data, T- and B-cells percentages appeared higher after 6 months of therapy, even though only in the second case the difference reached significance (p=0,024). NK cells had a tendency to be higher in long interval from CT (>6 months), macrophages were present in the same proportion in all groups (Fig. 24 and Tab. XII).

Figure 24. Leukocytes subsets in patients resected less than 6m (<6m) and more than 6m (<6m) from systemic CT



There was no difference among T-lymphocyte subsets comparing the two time-dependent groups. Only CD8+ T-cells percentages displayed a tendency to be higher in long interval from CT (Fig. 25 and Tab XII).

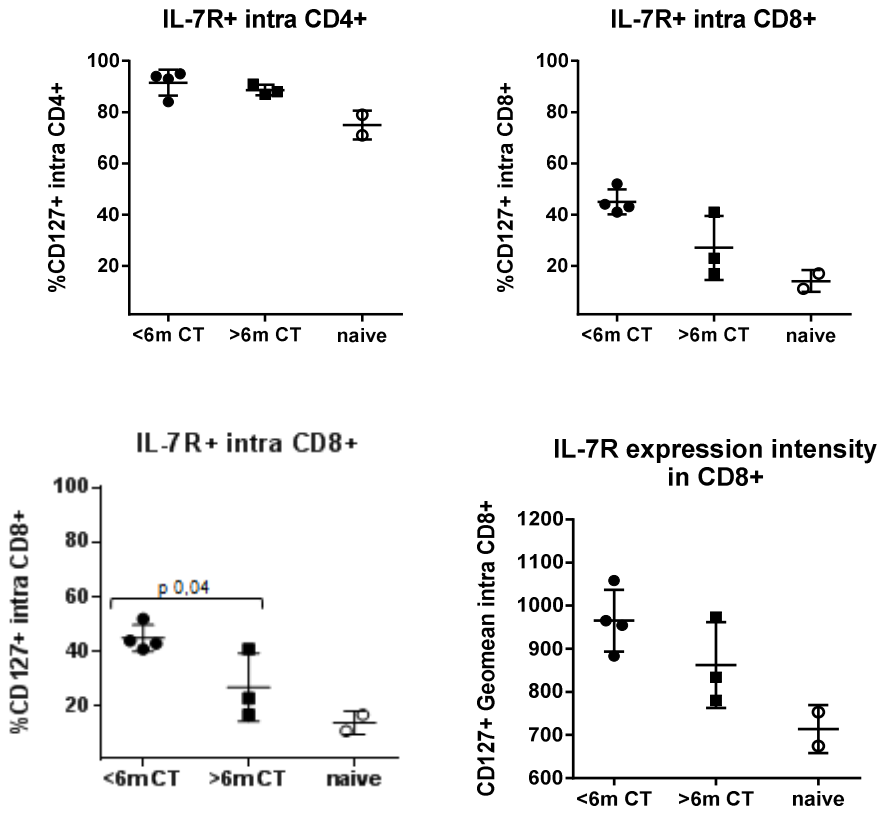
Figure 25. T-cell subsets in patients resected less than 6m (<6m) and more than 6m (<6m) from systemic CT



Time after the end of chemotherapy does not affect maturation of T-lymphocytes (data not shown and Tab XII).

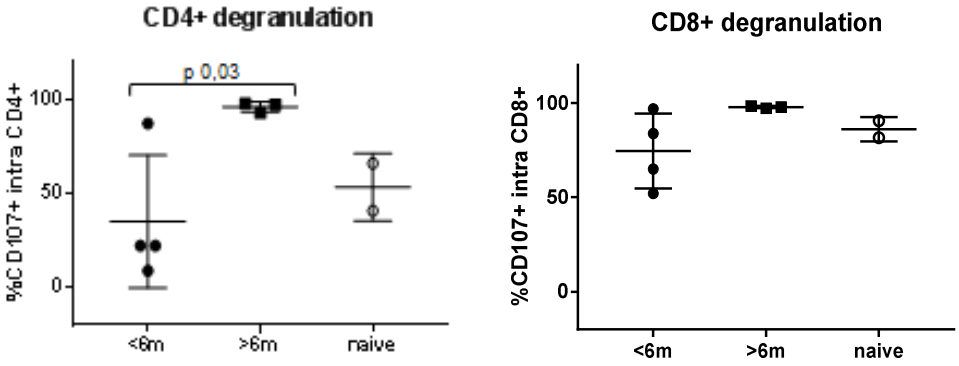
On the other hand, the expression of IL-7R appeared to be related to time from CT suspension only in CD8+ T-cells, being lower for longer suspension period (p=0,043). Intensity of IL7-R expression shows the same trend, even though it did not reach significance (Fig. 26 and Tab XII).

Figure 26. IL-7R expression in T-cells from patients resected less than 6m (<6m) and more than 6m (<6m) from systemic CT



There is no time-dependent differences in proliferation capacity among these infiltrating T-lymphocytes, which principally had effector functions (*data not shown*). On the other hand, cytometry data displayed a negative CT-impact on degranulation activity in patients with a short interval between surgery and CT. This phenomenon affected both CD4+ and CD8+ T-cells, but it reached statistical relevance only in CD4+ (p=0.033; Fig. 27 and Tab XII).

Figure 27. Degranulation activity of T-cells from patients resected less or more than 6 months from systemic CT suspension.



PD-1 expression did not differ in CD4+ and CD8+ T-cells according to the time of CT suspension (data not shown). On the other hand, patients that had been operated after 6 months from CT end, displayed a tendency to have higher percentages of TIM-3-expressing CD4+ and CD8+ T-cells, despite a notable dispersion (Fig. 28 and Tab XII).

IL-4 analysis showed a tendency towards higher levels in CD4+ and CD8+ T-cells of patients with long interval from CT, even though it didn't reach statistical significance (Fig. 29 and Tab XII).

Figure 28. Immuno-checkpoint TIM-3 expression in T-cells from patients resected less or more than 6 months from systemic CT suspension.

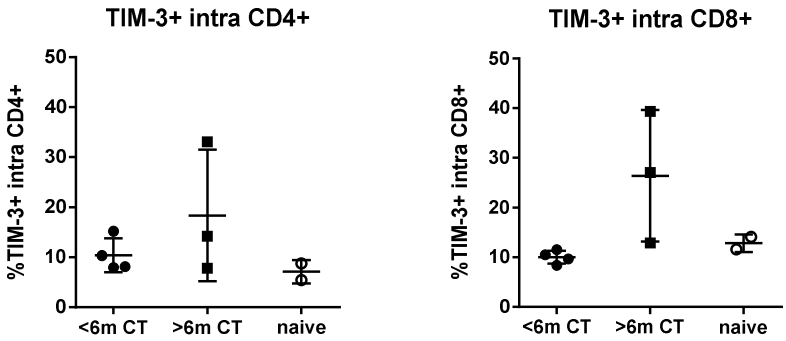
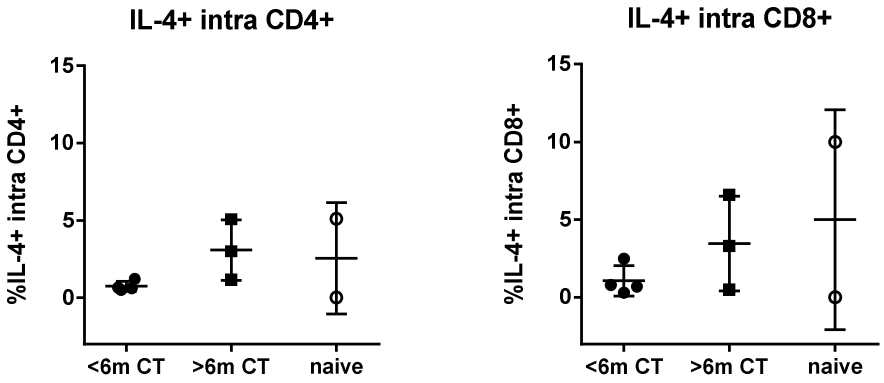


Figure 29. IL-4 expression in T-cells from patients resected less or more than 6 months from systemic CT suspension.



Tab XII. Analysis of time from CT to surgery

	<6m		>6m		p
	Mean (n=5)	SD	Mean (n=3)	SD	<6m vs >6m
Linf_T	53,9	26,1	76,5	12,0	ns
Linf_B	0,4	0,3	1,6	0,7	**0,024**
NK	0,4	0,3	1,2	1,3	ns
MF	7,7	3,8	6,1	2,3	ns
CD4	67,5	5,3	66,9	3,4	ns
CD8	23,3	7,8	29,1	5,8	ns
TCRgd	3,7	1,3	4,1	0,9	ns
Tregs	6,7	3,2	7,4	3,9	ns
CD4central_memory	3,5	2,0	1,4	1,2	ns
CD4effector_memory	84,0	12,1	88,0	3,9	ns
CD4naive	0,9	1,4	0,1	0,1	ns
CD4temra	12,2	10,0	10,9	4,2	ns
CD8central_memory	6,0	8,5	2,0	1,5	ns
CD8effector_memory	56,5	8,8	60,2	5,9	ns
CD8naive	1,1	0,5	2,0	2,3	ns
CD8temra	36,8	8,2	35,9	2,1	ns
CD4_PD1	67,6	6,8	62,9	13,0	ns
CD8_PD1	71,2	11,7	76,4	3,6	ns
CD4_TIM3	10,4	3,4	18,4	13,2	ns
CD8_TIM3	10,0	1,3	26,4	13,2	ns
CD4_IL7R	91,5	5,1	88,7	2,1	ns
CD8_IL7R	45,1	4,9	27,0	12,5	**0,043**
Intens_IL7R_CD4	1277,8	153,1	1061,3	189,6	ns
Intens_IL7R_CD8	965,0	71,9	862,3	99,6	ns
CD4_CD107a	35,0	35,2	95,7	2,7	**0,033**
CD8_CD107a	6,4	97,7	19,9	0,6	ns
CD4_IL4	1,8	1,1	3,3	2,0	ns

CD8_IL4	2,0	2,1	5,4	4,0	ns

DISCUSSION AND CONCLUSION

In the present study, we describe infiltrating leukocytes of 11 hepatic lesions from 10 patients affected by colo-rectal metastatic tumors. Seven patients have been pre-treated with systemic chemotherapy whereas three patients did not receive any treatment (naive). Different chemotherapy regimens have been used: Oxaliplatin-based, Irinotecan-based or a combination of them; one case has been treated with check-point inhibitors (Ipilimumab/Nivolumab).

The main aim of our exploratory study is to describe and characterize immune infiltrate in naive and pre-treated patients. Results can drive further studies and possibly identify the most favorable subset of patients for receiving novel therapies (such as immuno-modulating drugs or vaccines).

Flowcytometry data give a first indirect demonstration of chemotherapy effect, that reduces tumor cells in liver metastases from treated patients, showing a higher amount of immune-infiltrate among total viable cells ($p=0.048$; Fig. 7).

Coherently with other published data, the majority of infiltrating leukocytes are T-lymphocytes, followed by macrophages, B-lymphocytes and NK cells [75].

Lymphocyte cytotoxic potential is variable among CD4+, but high and uniform in CD8+ T-cells, consistent with an effector pattern (Fig. 3). Moreover, maturation patterns confirmed that almost all infiltrating T-lymphocytes are T effector memory (T_{em}) and RA-expressing effector memory (T_{EMRA}) (Fig. 10).
On the other hand, the infiltrating lymphocytes are barely proliferating (Fig. 3), but the major part of them displayed an important expression of IL7-R, that allows the maintenance of memory pool in the metastases (Fig. 4) [76].

Finally, those effector lymphocytes express extensively PD-1 and barely TIM3, showing an active population susceptible to immunomodulation, but not yet “exhausted” (Fig. 6) [77,78].
Even though presence of infiltrating T-cells is not higher in treated patients compared to untreated ones, our results demonstrate that chemotherapy treatment polarizes immune response towards CD4+ T-cells ($p=0.0001$; Fig. 9). This effect could be partially explained by tumor microenvironment modification induced by chemotherapy, in particular because the treatment induces tumor cell death, promoting antigen presentation and CD4+ T-cells expansion [79,80].

It could be possible that macrophages recruited in the metastases by chemotherapy-induced cellular death, play a major role in presenting new antigens causing a shift in the effector cytotoxic response towards CD4+ T-cells lineages. Alternatively, antigens could be presented in lymphoid organs, as

in a “classical” immune response, or in tertiary lymphoid structures (TLS) associated to metastases [81].

Independently by the CT-regimen, pre-treated patients display T-cells presenting higher level of IL7-R compared to naïve patients (Fig. 12). In particular the therapy impacts on the portion of cells that present the receptor and improves its expression intensity both in CD4+ ($p=0.003$ and $p=0.043$, respectively) and in CD8+ T-cells ($p=0.045$ and $p=0.035$). These data demonstrate that chemotherapy treatment indirectly support memory pools inside the metastatic site.

Combining these results with previously reported ones, it can be affirmed that pre-treated patients have an “immune-activated pattern”, reasonably pointed towards new and/or different tumor antigens induced by chemotherapy cytotoxic effect [79-81].

These findings could be relevant to answer the main question of the study about the best group of patients (pre-treated or naïve) to be submitted to immuno-modulating therapies, such as check-point inhibitors or tumor-antigens based vaccines. Since pre-treated patients show a more effective and polarized immune response, it seems attractive and reasonable the idea of using chemotherapy as a “starter” for immune response that can be later “fueled” with subsequent immune-modulating therapies. On the other hand, also other elements have to be considered. Not all tumors have the same capability of elicit immune response, as demonstrated in studies of molecular subtype classification of colorectal cancer [33]. Moreover, patients with tumors with a low infiltration can benefit from chemotherapy as “immunogenic stimulator”. Whereas patients with microsatellite instability, that in general have a vigorous immune response against tumor, could have a detrimental effect following a “standard” cytotoxic systemic chemotherapy because of a depression of actively replicating immune cells [82,83].

Analysis of immune infiltrate in liver metastases according to chemotherapy regimen is limited by the small sample size of patients that have been enrolled for each regimen. Nevertheless, we find some differences among regimens that can be use as working hypothesis in further works.

It immediately appears clear that patient treated with **Ipilimumab/Nivolumab regimen** strongly differs from other treated patients. She has the highest presence of infiltrating leukocytes among viable cells ($p= 0.0049$; Fig.15), the highest level of B-lymphocytes ($p=0.035$; Fig. 16) and T-reg ($p=0.006$; Fig.17), finally displays a prominent CD8+ T-cells response ($p=0.006$; Fig 17). Probably these results depends not only on the immunotherapy regimen, but also on the particular

immunological status of the patient in “ending/closing phase” of the immune response, since she reached the pathological complete response with no more tumor cells in the liver lesion analyzed. Accordingly, this patient has the lowest expression of IL7-R in both CD4+ (p=0.031) and CD8+ lymphocytes (Fig. 19) [27, 84].

Oxaliplatin-treated patients compose the largest group of treated patients. They display a great dispersion about the presence of infiltrating leukocytes among viable cells (Fig. 15), T-cells and Macrophages (Fig. 16), suggesting that not only CT-regimen controls and determines these parameters. Other elements that could influence these features are primary tumor location, interval from CT end, molecular and genetic profile of the tumor.

On the other hand, in this group data support a minimal dispersion in the percentage of CD4+ (70%) and CD8+ (28%) and the consequently CD4+/CD8+ ratio around 2.5. Moreover, all samples display quite all CD4+ with an effector memory phenotype (Fig. 18) and expressing IL7-R at high intensity (Fig. 19). On the other hand, it can also be observed a higher immunosuppressive population, represented by higher infiltrating Treg in this group compared to other regimens (p=0.027, Fig. 17).

This findings show that Oxaliplatin has a direct effect on CD4+ T-cells phenotype and differentiation and promotes memory pool maintenance, suggesting that this regimen has an intrinsic higher effectiveness as cytotoxic agent, but also elicits a partial immunosuppression at tumor site.

Interestingly, the **Irinotecan-treated patient** has a low level of leukocytes intra total viable cells (Fig. 15), the lowest level of CD8+ T-cells (p= 0.027; Fig. 17), TCRγδ (p= n.s.; Fig. 17) and effector memory CD4+ T-cells (p=0.0026; Fig. 18) compared to other CT-regimens. This CT-treatment does not seem to support cytotoxic immune response, but it is important to underline that those results have to be carefully considered since they are referred to a single patient and they need to be confirmed before to be related to a “less immunogenic” chemotherapy.

The patient treated with **Oxaliplatin-Irinotecan combined regimen** shows a very high level of immune infiltration (almost the same level of immuno-treated regimen; p=0.0049; Fig.15), probably because of effectiveness of this regimen in killing tumor cells (that are still well represented at pathological report after surgery, in contrast with immuno-treated patient). Moreover, this regimen displays the highest level of CD8+ T-cells among all CT-regimens (p=0.027; Fig. 17). The high level of effector CD8+ T-cells could suggest that combination of Oxaliplatin and Irinotecan is the most immunogenic among cytotoxic regimens.

Our data show that different treatments can promote CD4+ or CD8+ T-cells lineages, but data are not sufficient to determine if infiltration rate is induced by tumor cytotoxic activity and intrinsic efficacy of the specific drug only, or also by different tumor-host-chemotherapy interactions. Probably all together these factors cause different response patterns. [85].

Literature reports a slightly higher response rate for Oxaliplatin compared to Irinotecan (7 to 51% vs 9 to 35%), both in association with continuous administration of 5-FU/LV [86]. These CT-regimens are used as "conversion" therapy because they induce metastases regression and make eligible for radical surgery, an un-resectable patient at diagnosis, even though there are no evidence in terms of overall and disease-free survival increasing.

Analysis of immune response variation according to time from the end of chemotherapy before surgery, shows that infiltrating leukocytes in general ($p=0.0008$; Fig. 23), and B-lymphocytes in particular ($p=0.02$; Fig. 24) are higher after 6 months from CT suspension.

Time does not determine any difference among CD4+ and CD8+ T-cells distribution and maturation pattern of both lymphocytes lineages. On the other hand, it is possible to demonstrate a time-dependent affection of CD8+ memory pool maintenance through a lower IL7-R expression in late operated patients ($p=0.043$; Fig. 26). [87]. Analogous trend can not be observed in CD4+ lymphocytes, perhaps because IL-7R expression among CD4+ requires more than 6 months to decrease.

Conversely, we observed a lower degranulation activity (CD107a) in patients operated close to chemotherapy administration. This effect is valuable in both CD4+ and CD8+, but reaches statistical significance only among CD4+ T-lymphocytes ($p=0.03$; Fig. 27). These data are consistent with a cytotoxic effect of CT-treatment charged to lymphocytes in patients with short-term interval after therapy suspension.

From a clinical point of view, to identify the more advantageous interval between surgery and chemotherapy suspension is paramount. It would be better to operate when CD8+ memory pool maintenance is at its best (less than 6 months) or it would be better to wait for cytotoxic capability recovery? Certainly, a larger group of patients is needed to shed light on this complex phenomenon.

On the other hand, these results have to be cautiously interpreted because of a possible selection bias. Naïve, short and long interval patients are all different patients and so results across time do not express variations of immune response of the same patient. Since long interval patients are only

“good responders” with no disease progression after a long chemotherapy suspension period, and that patients with progression have not been enrolled, it could be possible that long interval patients have been positively selected for surgery. For this reason is it possible that they do not represent a realistic evolution of immune response of short time patients after CT-suspension.

The main limitation of this study is the reduced sample size. We analyzed only three naïve patients (and in some tests only two), but there were objective difficulties in enrolling such patients.

Firstly, naïve patients are referred to surgeon because of primary tumor symptoms that require immediate treatment (unstoppable and life-threatening bleeding or mechanical bowel obstruction). Considering this, there are ethical issues (patients in deteriorated general conditions in which adding a liver biopsy for the study could be harmful or impossibility to obtain pre-operative informed consent) and practical aspects (coordinate urgent operations and laboratory availability for processing and analyzing samples) that reduced collection of these patients.

Another point is the increasing effectiveness of screening programs (reduction of advanced stages patients at diagnosis) that luckily make critical presentation of naïve patients everyday less frequent.

There is also a different distribution of patients among chemotherapy regimens, which reflects the actual drugs clinical use. The vast majority of patients receive Oxaliplatin-based chemotherapy as first line, according to international guidelines and Oncologists preferences [88].

Immunotherapy is quite new in clinical practice and not widespread. Moreover, only a minority of patients (namely MSI-H) with CRC can be eligible for this therapy [82,84].

Irinotecan, even though could be used as first line therapy according to guidelines as alternative to Oxaliplatin, is usually proposed as second-line option in case of progression to Oxaliplatin regimen, reducing the number of patients receiving Irinotecan and surgery (a progressing patient is usually considered not eligible for surgery) [89].

Oxaliplatin plus Irinotecan is a very effective combination therapy that is usually proposed as “conversion” therapy to reduce metastatic lesion size and number, to become operable (at presentation lesions could be too disseminated or technically impossible to resect). Use of Oxaliplatin/Irinotecan combination is limited by high grade of CT-related side effects (it can be proposed only to a patients with in good clinical conditions) and because after that regimen there are virtually no other effective lines of therapy [88,89].

Further developments of the present study will necessary include an extension of sample size, aimed to enroll more naïve, Irinotecan-treated and Oxaliplatin plus Irinotecan-treated patients (at least three more patients in each group), to limit data dispersion and empower statistical analysis.

Enlarging sample size could also permit analysis of mutational status (RAS, RAF genes) and targeted therapy (e.g. anti-EGFR antibodies) effect on immune response in liver metastases.

Another interesting aspect could be characterization of patients in terms of Consensus Molecular Subtypes (CMS), eventually to demonstrate a different pattern of immune response according to CMS profile.

Variation of immune response across time and interplay between chemotherapy, tumor adaptation and immunity of the patient are extremely difficult to analyze due to biological complexity that required a wide specimen availability. To this aim, we could propose an exploratory study, collecting from the same patient biopsies and peripheral blood samples, before and after chemotherapy, if patient is eligible for surgical procedure of metastases excision. In particular, repeated PBMC collection display immune response modifications through monitoring of circulating T_{CM} as precursors of infiltrating effector memory cells. This approach will clarify if selection bias affects our present data and provide easily collectable specimens.

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