General Index

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Title: The predictive role of the immune system for response to therapy and survival in patients with solid tumors.

Synopsis

1.   Breast Cancer (BC), Pancreatic Adenocarcinoma (PDAC) and Malignant Mesothelioma (MMe) are aggressive forms of solid tumors. The immune system behaves toward cancer cells as for infections, but insight into tumor cell-autonomous molecular pathways affecting these features are lacking, especially in correlation with response to targeted therapies targeting immune-related molecular pathways such as the mTOR inhibitor everolimus in HR+/HER2- BC. A better understanding of the correlation between the molecular biology of the role of the immune system and patients' survival and response to therapy is the object of my research.

2.   First of all I used an *in silico* approach (cBioPortal) to investigate possible correlations between survivals of patients with solid tumors and genomic alterations, using the gene list from the 730 immune-related (with additional 40 housekeeping genes) PanCancer IO 360[TM] panel. The genetic alterations that were considered from the panel were mutations, amplifications, deep deletions and multiple alterations.

3.   Secondly through an *in vitro* analysis, 62 Paraffin-embedded formalin fixed (PEFF) samples of MMe were analysed for Tumour Infiltrating Lymphocytes (TILs) and Programmed death-ligand 1 (PD-L1) expression[1]. Patients were divided in 4 groups according to a cut-off of the percentage of TILs found per sample as measured by means of immunohistichemistry: "0" or absent (between 0%

and 5%), "1" or low (between 6% and 25%), "2" or moderate (between 26% and 50%) and "3" or high (between 51% and 75%). Overall Survival (OS) was then correlated with different TILs' expression patterns. Moreover, PD-L1 expression was assessed within the tumour as well as in the adjacent stroma on the same samples. Higher expression of peritumoral TILs (Group 2 + 3) versus Group 0 and 1 correlated with improved OS (p-value = 0,02). On the contrary, PD-L1 expression seemed to be inversely correlated with clinical outcomes, even in the absence of statistical significance (Hazard Ratio, HR: 1.76; p=0.083 95% IC: 0.92-3.36 in areas within the tumour; HR: 1.60; p= 0.176 95%; Confidence Interval, CI: 0.80-3.19 in areas within the stroma). No relationship between TILs and PD-L1 expression was identified.

4.   Thirdly we investigated the correlation between the prognosis of 12 PDAC patients and the presence of tumour infiltrating lymphocytes through IHC and the expression of 521 immune system genes using Nanostring nCounter Vantage and related bioinformatics[2]. Twenty immune system genes were significantly differentially expressed in patients with a good prognosis relative to patients with a worse prognosis: Toll-like Receptor 2 (*TLR2*) and Toll-like Receptor 7 (*TLR7*) (Toll-like receptor superfamily); *CD4, CD37,* Forkhead box P3 *(FOXP3), Protein Tyrosine Phosphatase Receptor Type C (PTPRC)* (B cell and T cell signalling); Interferon Regulator Factor 5 (*IRF5)*, Interferon Regulator Factor 8 (IRF8), Signal Transducer and Activator of Transcription 1 (STAT1), Transcription Factor E3 (TFE3) (transcription factors); Acid Nuclear Phosphoprotein 32 Family Member B (*ANP32B*), Cyclin D3 (*CCND3*) (cell cycle); Bruton's Tyrosine Kinase BTK (B cell development); Tumor Necrosis Factor (*TNF*), Tumor Necrosis Factor Receptor Superfamily Member 1A (*TNFRSF1A*) (*TNF* superfamily); Hematopoietic Cell Kinase (*HCK*) (leukocyte function); Complement C1q Subcomponent Subunit A (*C1QA*) (complement system); BCL2 Associated X (*BAX*), PNMA Family Member 1 (*PNMA1*) (apoptosis); Inhibitor of Nuclear Factor Kappa B Kinase Subunit Epsilon (*IKBKE*) (NFκB pathway). Differential expression was more than twice log 2 for *TLR7, TNF, C1QA, FOXP3,* and Cell Differentiation Antigen 37 (*CD37*).

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5.   Fourthly, to find a biomarker that could be a good prognostic factor and a predictor of response to anti-cancer treatments for predicting survival of breast cancer patients I have reviewed the literature and implemented a meta-analysis on the role of Phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit alpha (*PIK3CA)* mutational status in randomized clinical trials. Overall 1929 Breast Cancer (BC) cases were included[3]. The pooled analysis confirmed that the presence of a *PIK3CA* mutational status represents an independent negative prognostic factor (HR = 1.67, 95% CI: 1.15-2.43; p = 0.007) in BC, as previously reported. To investigate immune-related biomarkers for predicting response to everolimus therapy in Hormone-Receptor Positive and Human Epidermal Growth Factor Receptor 2 negative (HR+/HER2-) *in vitro*, neutrophil and platelet to-lymphocyte ratios (NLR and PLR), immune pathways, and TILs were characterized in correlation with the therapeutic strategy targeting mammalian target of rapamycin (mTOR) using everolimus in 2131 metastatic patients from the BALLET study, in 23 patients receiving neoadjuvant everolimus and in 15 metastatic patients at the local institution, respectively. In the 27 HR+/HER2- patients of the MREC study receiving neoadjuvant everolimus of most of the immune system pathways were activated in everolimus responders vs. non-responders. In the BALLET study quartiles of patients with lower NLR levels in the blood had higher survivals compared to patients with higher NLR: NLR ≤ 2.3 vs. NLR >2.3; NLR ≤ 3.2 vs. NLR > 3.2; and NLR ≤ 4.4 vs. NLR >4.4 (p=0.19, p=0.12 and p=0.01). In the smaller population of 15 metastatic patients, Fluorescence-Activated Cell Sorting (FACs) analyses showed that everolimus responders vs. non-responders had higher levels of CD3+ T-lymphocytes at baseline (p=0.0343) and during treatment (p=0.0233), higher levels CD8+ and CD4+ T-lymphocytes at baseline (p=0.0172, p=0.0005, respectively) and during treatment (p=0.0102, p=0.0032, respectively); while they had slightly lower levels of regulatory T-lymphocytes and Natural Killers (NKs) (p=0.0588 and p=0.0411, respectively).

6.   The knowledge produced helps to define key immune-related pathways in solid tumors. Further refining a panel of immune-system markers could help guiding clinicians in the decision-making step for giving mTOR inhibitor everolimus in HR+/HER2- BC.

Chapter 1: Immune-gene signature: a new tool for patients' selection for checkpoint inhibitors?

Rationale

1)   During their growth, tumor cells develop an evasion mechanism by manipulating immune cells to their benefit[4–6]. Immune genes are therefore important in the context of solid tumors and there is a lack of understanding on how frequencies of alterations in immune genes correlate with survival in large datasets of patients with solid tumors.

2)   In the first chapter of this thesis, I present my meta-analysis interrogating cBioPortal's multiple large-scale cancer genomic datasets with an immune-related gene list made of 770 genes (PanCancer IO 360[TM] panel).

3)   The results section of this first chapter proved that alterations in large groups of patients with 9 different types of solid tumors significantly correlated with worse survival outcomes. Tumor cells achieve immune evasion by interfering with immune signaling by hijacking immunosuppressive cells to control immune infiltration, allowing tumor cell proliferation[7,8].

4)   Our data from the first chapter suggests that genetic alterations in the immune system are a potentially new diagnostic tool indicating worse survival outcomes in solid tumors.

Materials and Methods

1)   By analyzing gene expression profiles (GEPs) on baseline tumor samples in patients with solid tumors treated with pembrolizumab, Ayers *et al* identified an immune gene signature able to score for clinical outcome. The assay was performed using a learn-and-confirm paradigm based on data from different clinical studies on pembrolizumab[9]. The authors employed a custom panel of 680 tumor- and immune-related genes using the NanoString nCounter platform (NanoString Technologies Inc.). Starting from a small pilot study, including only 19 patients affected by metastatic melanoma. Ayers *et al* were able to define an immune gene panel, the "pan-tumor T cell-inflamed GEP". The assay was then tested in 220 patients with 9 different cancers.[9] The panel contains IFN-γ-responsive genes related to antigen presentation, chemokine expression, cytotoxic activity and adaptive immune resistance. Based on the encouraging results of the study, the T cell-inflamed GEP was developed into a clinical-grade assay and is under evaluation in ongoing trials on pembrolizumab[9]. The Gene Expression Panel "PanCancer IO 360[TM]", comprising 730 genes plus 40 internal reference genes, was derived from this study.

2)   The cBioPortal is an open-access platform permitting analysis of multidimensional cancer genomics datasets of patient cohorts[10,11], including The Cancer Genome Atlas (TCGA) and Memorial Sloan Kettering (MSK) datasets. This platform was interrogated using the gene list from the PanCancer IO 360[TM] panel to search for subgroups with a different immune signature. In this analysis, online databases with gene expression data of clinical patients were exploited to investigate the alteration frequency of the abovementioned 730-gene panel list. The solid tumors included in my *in silico* analysis were bone cancer, poorly differentiated and anaplastic thyroid cancer, low grade glioblastoma (LLG), merged LLG and glioblastoma multiforme (GBM), head and neck cancer, renal cell carcinoma, pancreatic neuroendocrine tumors (NETs), soft tissue cancer, ampullary cancer, cholangiocarcinoma, oligodendroglioma, miscellaneous neuroepithelial tumor, kidney chromophobe, melanoma, hepatocellular carcinoma, pediatric Wilms, lung squamous cell

carcinoma, and thymic epithelial tumor. My analysis generated two subgroups of patients based on the presence or absence of genomic alterations within the list of included genes.

Results

1)   Alterations in signature genes for immune surveillance could indicate disrupted function of these genes, permitting tumors to go through a process of immune escape, resulting in lower patient survival. On this note, patients with genetic alterations had a tendency to worse Disease-Free Survival (DFS) and OS compared to the group of patients without genetic alterations.   The difference in DFS and OS between patients with or without genetic alterations reached statistical significance in LLG (DFS, p=0.000009997; n=1053), bone cancer (DFS, p=0.00008766; OS, p=0.0002616; n=218), poorly differentiated and anaplastic thyroid cancer (OS, p=0.0009864; n=117), merged LLG and GBM (OS, p= 0.0005608; n=1122), head and neck cancer (OS, p=0.0156; n=1515), renal cell carcinoma (DFS, p= 0.0364; OS, p= 0.0205; n=1765), breast cancer (OS, p=0.0210;n=1756), pancreatic NETs (OS, p=0.0312; n=108), soft tissue cancer (OS, p= 0.0325; n=1116) (**Figures 1.1-1.9**). The complete report of the results is included in the supplementary data.

Overall Survival Kaplan-Meier Estimate

a

Logrank Test P-Value: 0.0799

■ Cases with Alteration(s) in Query Gene(s)
■ Cases without Alteration(s) in Query Gene(s)

● Mutation ● Fusion ● Amplificati

Disease/Progression-free Kaplan-Meier Estimate

b

Logrank Test P-Value: 9.997e-5

■ Cases with Alteration(s) in Query Gene(s)
■ Cases without Alteration(s) in Query Gene(s)

Figure 1.1 Correlation between immune-gene alterations and survival of lower grade glioblastoma patients. Two subgroups of patients were generated on the basis of the presence or absence of genetic alterations within 730 immune-gene list from PanCancer IO360[TM] panel to investigate differences between Overall Survival **(a)** and Disease/Progression-Free Survival **(b).** The column graphs on the left depict the overall genetic alteration frequency of the immune panel between the two subgroups of patients. Different columns' colours show the frequency of different types of genetic alterations (mutation, fusion, amplification, deep deletion or multiple alterations).

Figure 1.2 Correlation between immune-gene alterations and survival of bone cancer patients. Two subgroups of patients were generated on the basis of the presence or absence of genetic alterations within 730 immune-gene list from PanCancer IO360^{TM} panel to investigate differences between Overall Survival **(a)** and Disease/Progression-Free Survival **(b).** The column graphs on the left depict the overall genetic alteration frequency of the immune panel between the two subgroups of patients. Different columns' colours show the frequency of different types of genetic alterations (mutation, fusion, amplification, deep deletion or multiple alterations).

Figure 1.3 Correlation between immune-gene alterations and survival of Neuroendocrine tumors. Two subgroups of patients were generated on the basis of the presence or absence of genetic alterations within 730 immune-gene list from PanCancer IO360[TM] panel to investigate differences between Overall Survival. The column graphs on the left depict the overall genetic alteration frequency of the immune panel between the two subgroups of patients. Different columns' colours show the frequency of different types of genetic alterations (mutation, fusion, amplification, deep deletion or multiple alterations).

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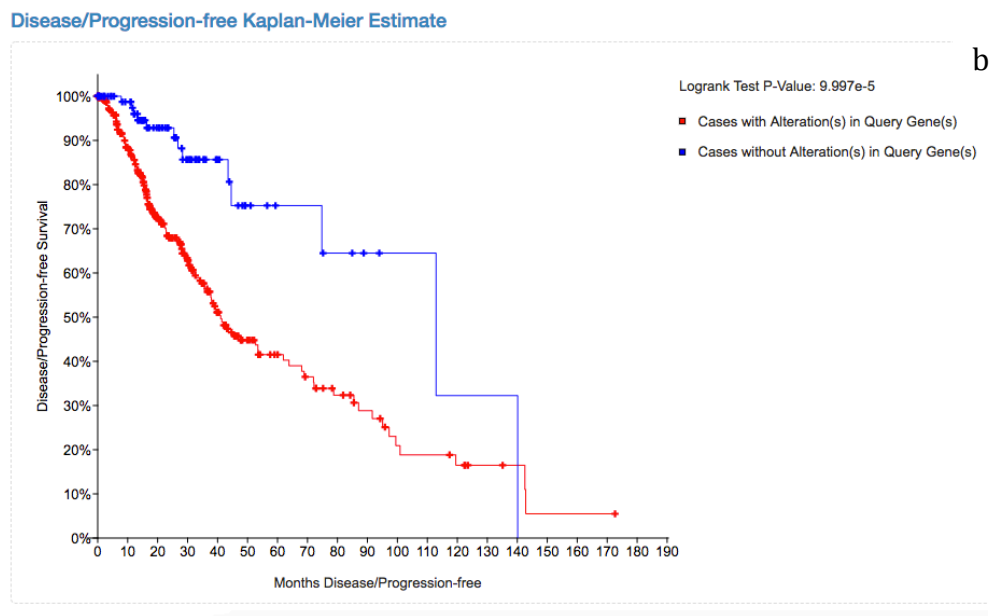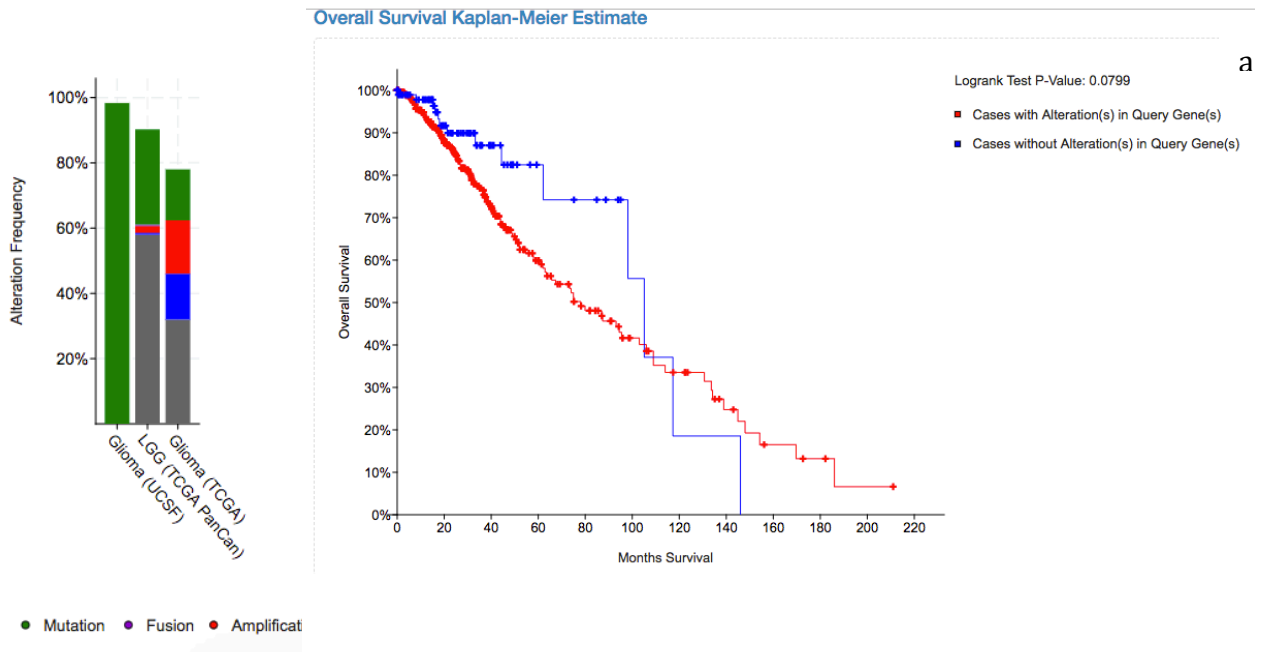**Figure 1.4 Correlation between immune-gene alterations and survival of Anaplastic Thyroid Cancers.** Two subgroups of patients were generated on the basis of the presence or absence of genetic alterations within 730 immune-gene list from PanCancer IO360^{TM} panel to investigate differences between Overall Survival. The column graphs on the left depict the overall genetic alteration frequency of the immune panel between the two subgroups of patients. Different columns' colours show the frequency of different types of genetic alterations (mutation, fusion, amplification, deep deletion or multiple alterations).

Figure 1.5 Correlation between immune-gene alterations and survival of Diffused Glioma. Two subgroups of patients were generated on the basis of the presence or absence of genetic alterations within 730 immune-gene list from PanCancer IO360™ panel to investigate differences between Overall Survival. The column graphs on the left depict the overall genetic alteration frequency of the immune panel between the two subgroups of patients. Different columns' colours show the frequency of different types of genetic alterations (mutation, fusion, amplification, deep deletion or multiple alterations).
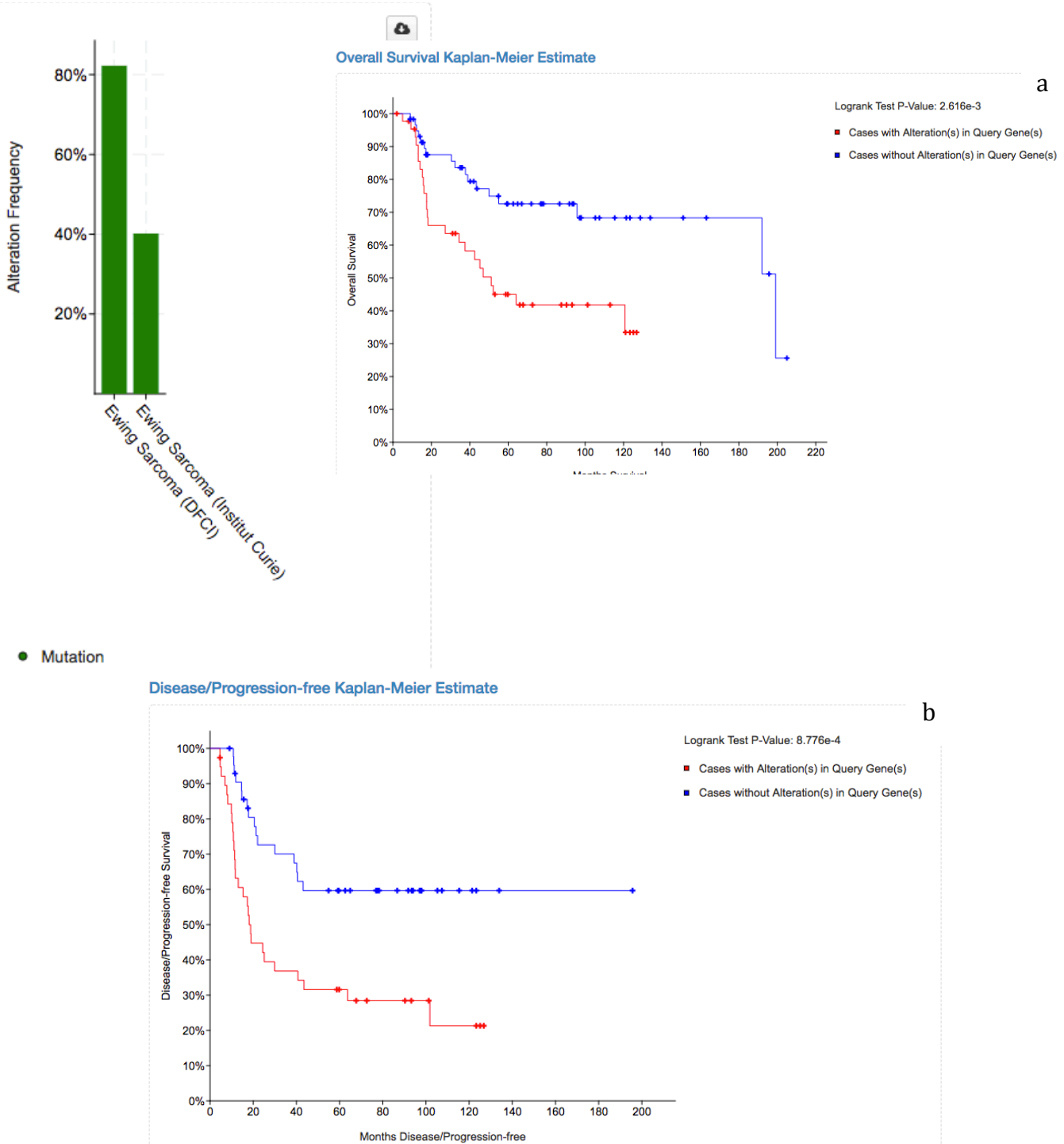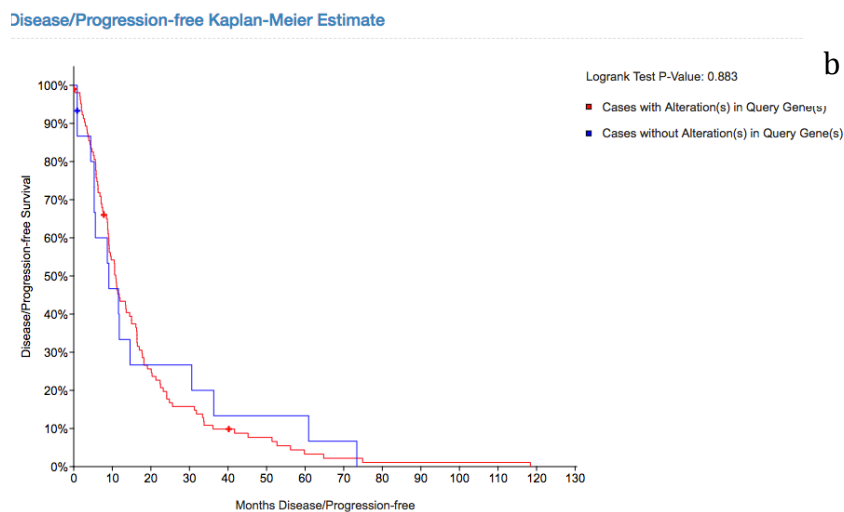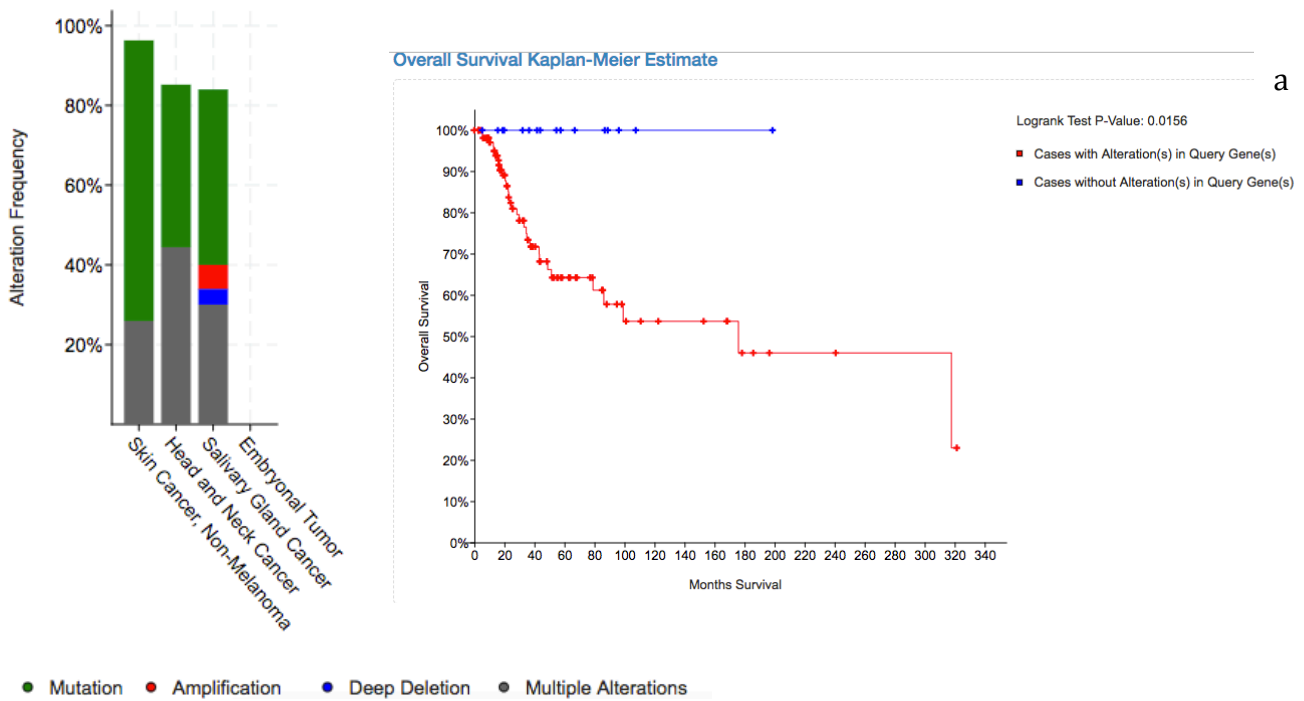
Figure 1.6 Correlation between immune-gene alterations and survival of Head and Neck Cancers. Two subgroups of patients were generated on the basis of the presence or absence of genetic alterations within 730 immune-gene list from PanCancer IO360[TM] panel to investigate differences between Overall Survival **(a)** and Disease/Progression-Free Survival **(b)**. The column graphs on the left depict the overall genetic alteration frequency of the immune panel between the two subgroups of patients. Different columns' colours show the frequency of different types of genetic alterations (mutation, fusion, amplification, deep deletion or multiple alterations).
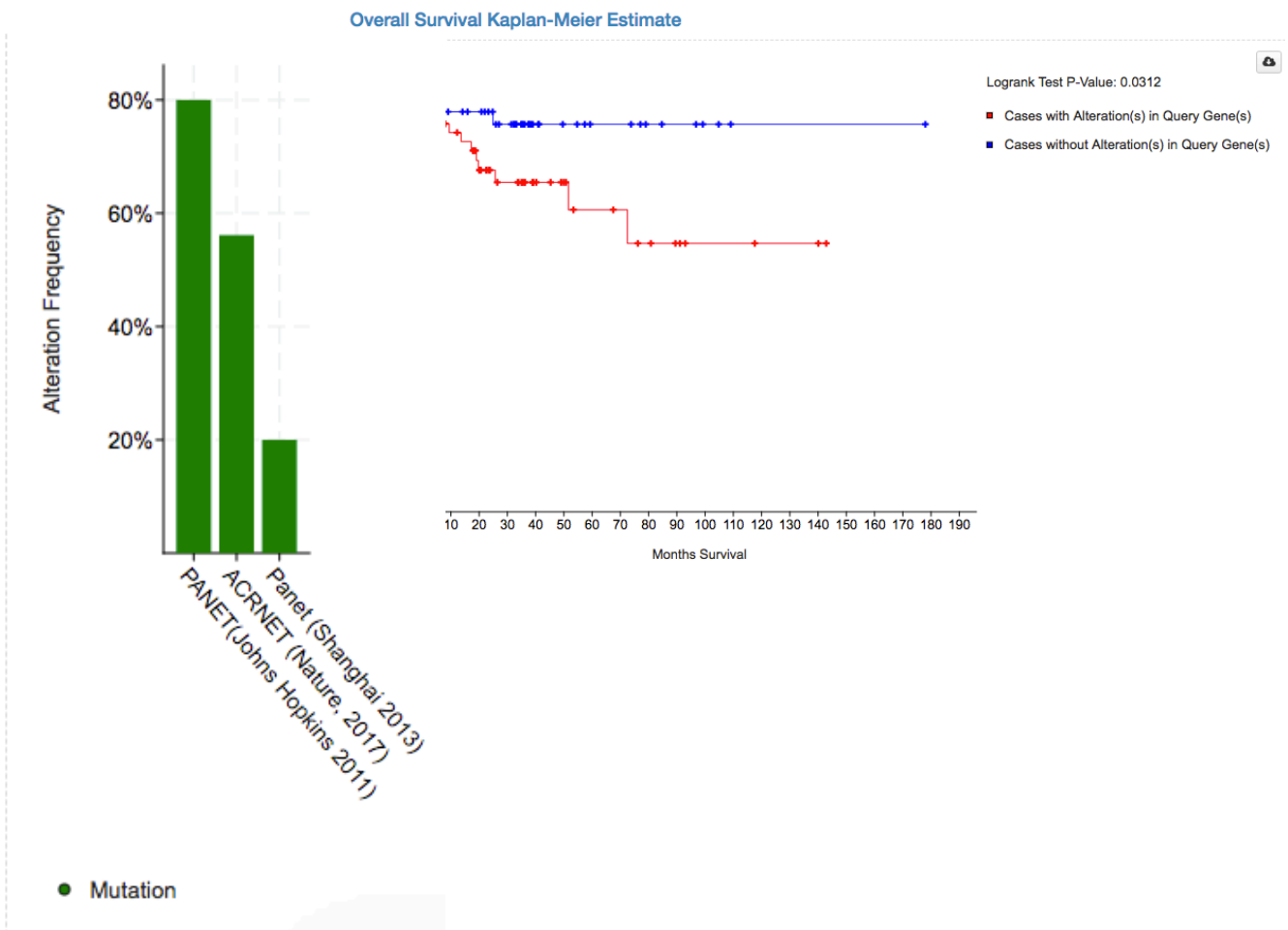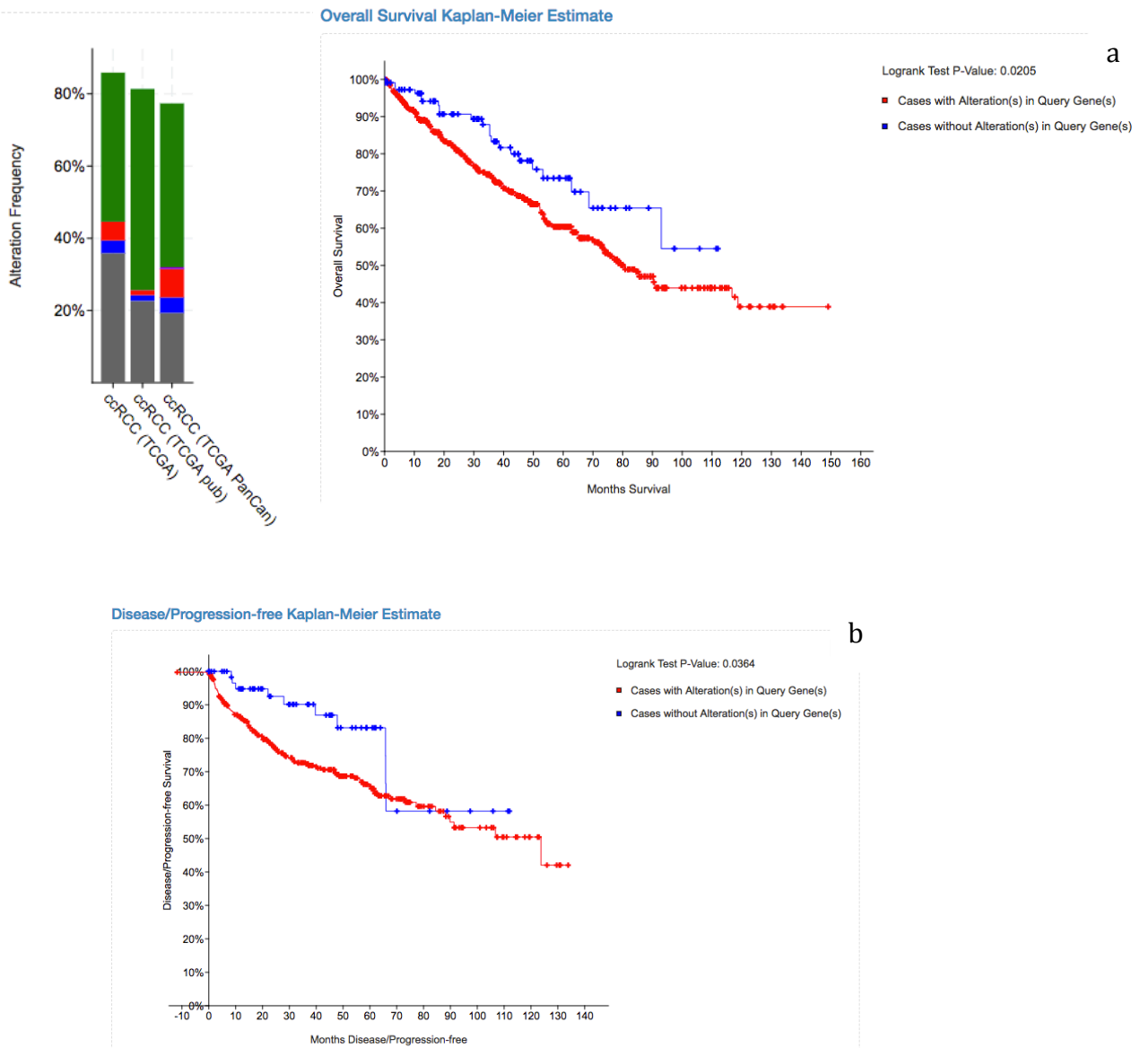
Figure 1.7 Correlation between immune-gene alterations and survival of Renal Cell Carcinoma. Two subgroups of patients were generated on the basis of the presence or absence of genetic alterations within 730 immune-gene list from PanCancer IO360^{TM} panel to investigate differences between Overall Survival **(a)** and Disease/Progression-Free Survival **(b)**. The column graphs on the left depict the overall genetic alteration frequency of the immune panel between the two subgroups of patients. Different columns' colours show the frequency of different types of genetic alterations (mutation, fusion, amplification, deep deletion or multiple alterations).
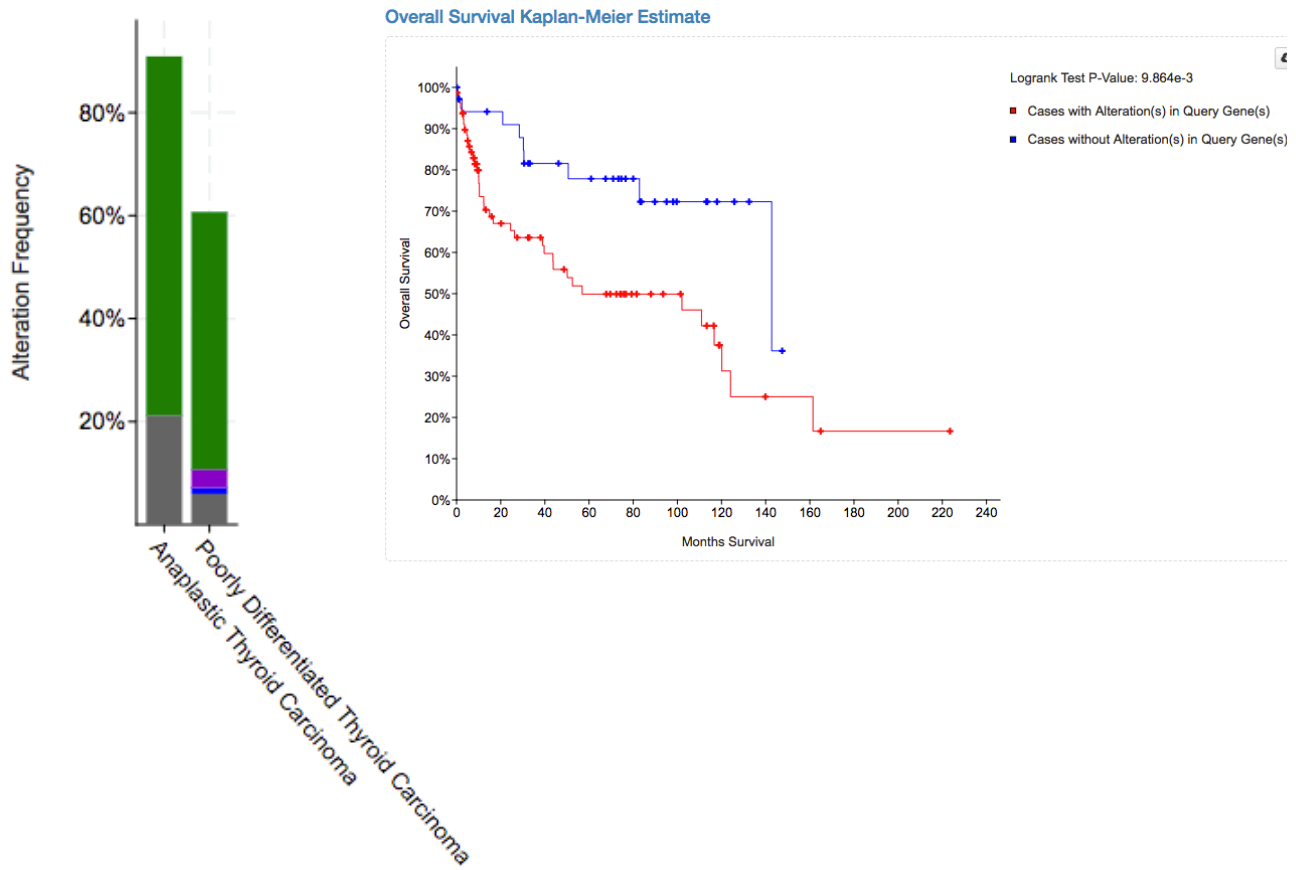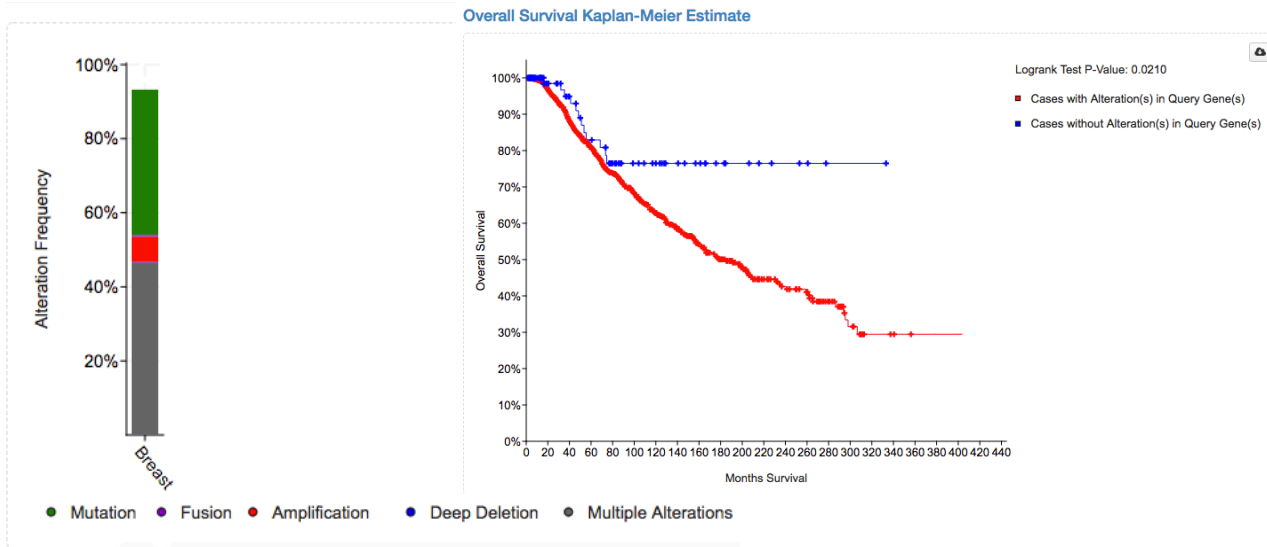
Figure 1.8 Correlation between immune-gene alterations and survival of Breast Cancer Patients of Memorial Sloan Kettering. Two subgroups of patients were generated on the basis of the presence or absence of genetic alterations within 730 immune-gene list from PanCancer IO360^{TM} panel to investigate differences between Overall Survival. The column graphs on the left depict the overall genetic alteration frequency of the immune panel between the two subgroups of patients. Different columns' colours show the frequency of different types of genetic alterations (mutation, fusion, amplification, deep deletion or multiple alterations).
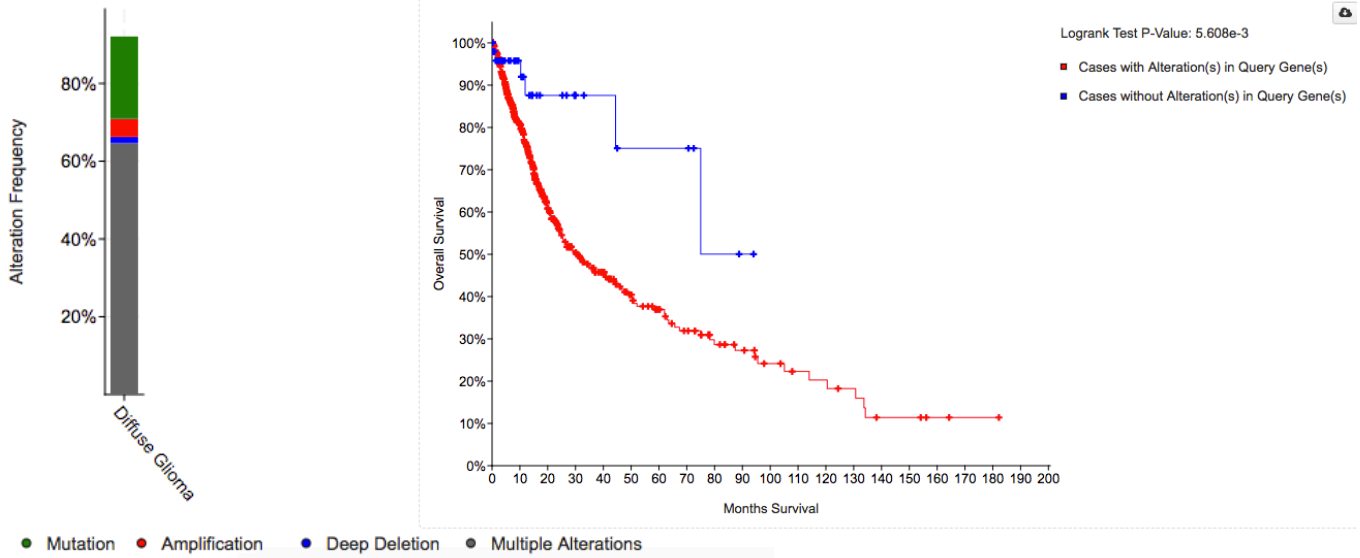
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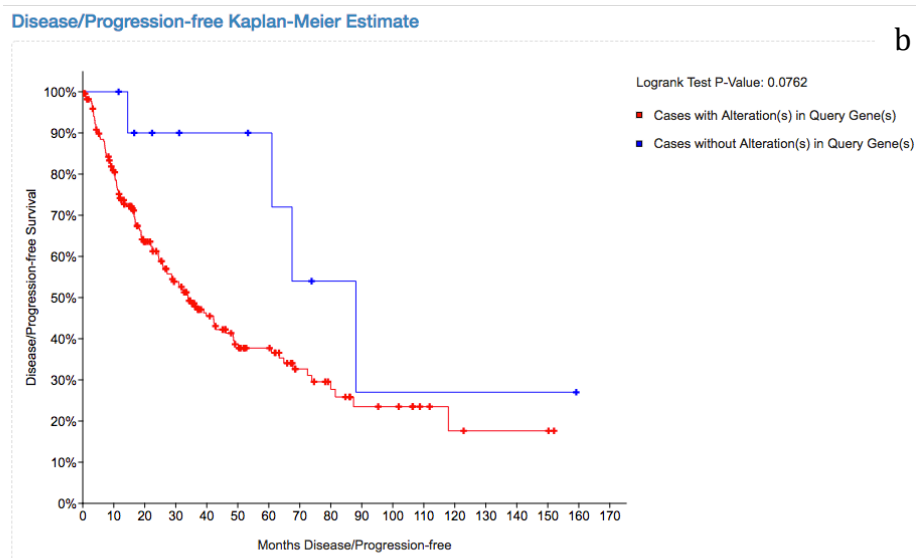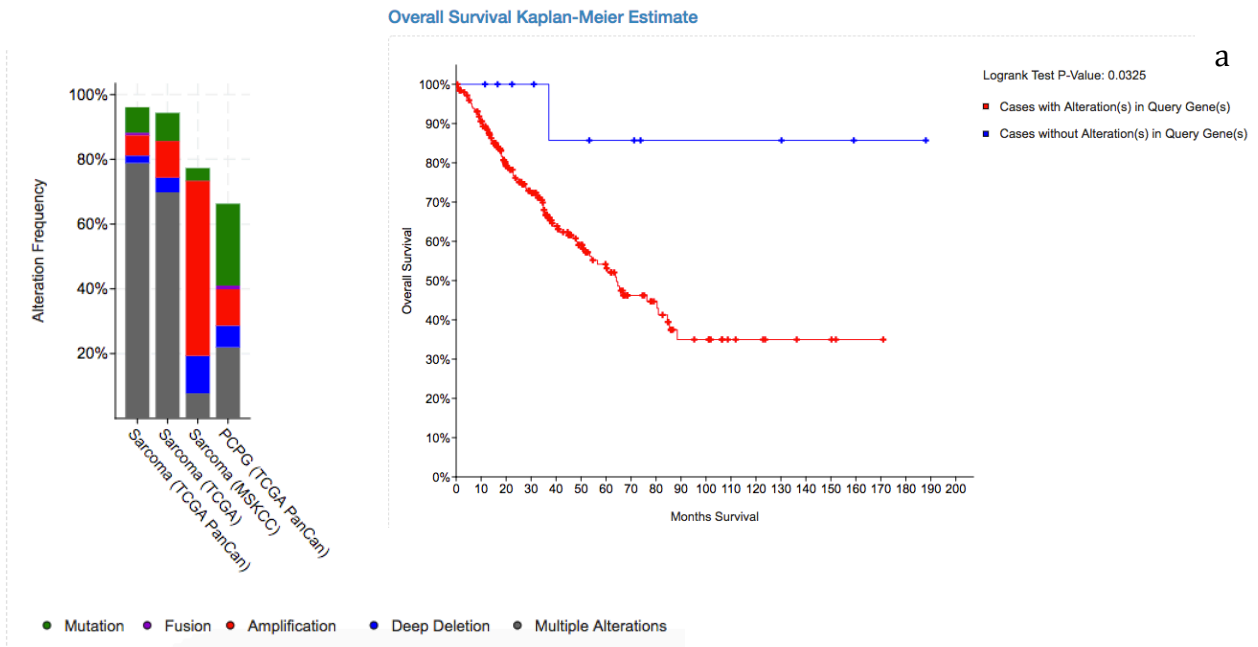
Figure 1.9 Correlation between immune-gene alterations and survival of Sarcoma Patients. Two subgroups of patients were generated on the basis of the presence or absence of genetic alterations within 730 immune-gene list from PanCancer IO360™ panel to investigate differences between Overall Survival **(a)** and Disease/Progression-Free Survival **(b)**. The column graphs on the left depict the overall genetic alteration frequency of the immune panel between the two subgroups of patients. Different columns' colours show the frequency of different types of genetic alterations (mutation, fusion, amplification, deep deletion or multiple alterations).

Discussions

1)   Current immuneterapeutic drugs can interfere with immune cells in the tumor microenvironment to facilitate control of the tumor[12,13]. Targeted therapies for PD-L1 and Programmed Cell Death Protein 1 (PD-1) suggested that in patients with PD-L1-expressing tumors, the immune reactions leading to the cancer-killing by eliciting an immune response could be properly functioning and patients might elicit a rapid and durable response to PD-L1 and PD-1 inhibition. A meta-analysis of 4174 patients with advanced or metastatic cancers showed that efficacy of PD-1 and PD-L1 inhibitors in patients that were PD-L1 positive and PD-L1 negative were significantly different (p=0.02). In fact PD-L1 positive patients had significantly prolonged survival (n=2254, HR 0.66, 95% CI 0.59-0.74) than PD-L1 negative patients (1920, 0.80, 0.71 to 0.90)[14]. However, a small minority of PD-L1-negative tumors responded to PD-L1 or PD-1 monotherapy[15,16].

2)   It is noteworthy that expression of PD-L1 is not always a predictor of efficacy of PD-1 or PD-L1 inhibitors; currently there is still a matter of debate on the correlation between PD-L1 expression and inhibitor therapy outcome[17]. This is probably because of the many variables that could affect reliability of quantification: heterogeneity in immunohistochemistry staining, mainly attributable to multiple antibodies and clones that have different affinities and specificities for the ligand; different platforms and staining protocols; tumor heterogeneity itself could cause a huge variation in gene expression profiles. Besides the urgent need for an international standard method for PD-L1 expression analysis, more reliable biomarkers of response to immune therapies are also needed. This investigation aimed to look at biomarkers for overall survival and disease/progression free survival and showed that an immune signature of 730 genes from the Nanostring panel could be a potential tool to be validated and explored in clinical settings.

3)   Immune checkpoint inhibitors are, moreover, just the tip of the iceberg in discovery and development of targeted therapies exploiting the immune system to fight cancer. Many other immune targets could be used for making new drugs that modulate the immune system, with drug

combinations potentially eliciting a better immune response than single drugs. Combining a Cytotoxic T-lymphocyte-associated Protein 4 (CTLA-4) targeted therapy (ipilimumab) with a PD-1 targeted inhibitor (nivolumab), for example, can enhance immune activity in patients relative to either therapy alone[18]. Anti-CTLA4 could lead to enhanced priming and activation of antigen-specific T cells as well as potentially clear regulatory T cells from the tumor microenvironment. CTLA4-targeted therapy assisted recognition of cancer cells by the immune system in PD-L1-negative patients[19]. This proves that there are molecular pathways in the immune system complementary for the proper functioning of the immune system against cancer. Besides checkpoint inhibitors (monoclonal antibodies), options for cancer immunotherapy are as follows: non-specific immunotherapies (interferons and interleukins); oncolytic virus therapy; T-cell therapy (CAR-T); T-cell receptor therapy; TILs therapy; bispecific monoclonal antibodies, designed to target more than one different epitope at once, some of which attach to both a cancer cell and an immune system cell to trigger an immune reaction against cancer; cancer vaccines; colony-stimulating factors, strengthening the immune system by boosting the production of white blood cells in the bone marrow; drugs such as imiquimod, lenalidomide, thalidomide and pomalidomide that kick-start the immune system against some solid tumors.

4)   Personalized medicine has an increasingly crucial role in cancer diagnosis, prognosis and treatment. The recent introduction of various drugs into routine clinical practice has positively changed the cancer landscape. Moreover, many clinical trials are currently investigating the synergistic effect of the combination of immune checkpoint inhibitor and targeted therapy on driver mutations with promising initial results[20,21]. The essence of precision medicine is "individualized treatment" through genotyping of patients and "dedicated drug/s". Clinical oncology is entering the precision medicine era as treatment decisions are based on the targeting of tumor molecular abnormalities using a "signature" to select patients who will benefit from a new, targeted drug and a combination with chemotherapy, hormone therapy or radiotherapy. The gene panel that we used

made of 730 genes could potentially be investigated also in this context to create a tool to discriminate patients based on disease/progression free survival to one of these targeted therapies.

5) Investigation of tumor-immune phenotypes is key to better understanding and more effective tailoring of immunotherapies[22] and drugs that somehow involve the immune system. Our first chapter's investigation focused on immune profiling of solid tumors through an *in silico* approach to better understand the role of prognostic markers of the immune system in solid tumors and it demonstrated that alterations in the gene panel made of the 730 immune-related genes correlated with worse survival of patients with nine different types of solid tumors.

Chapter 2: Tumour infiltrating lymphocytes and PD-L1 expression as potential predictors of outcome in patients with malignant pleural mesothelioma

Rationale

1)   Malignant Pleural Mesothelioma (MPM) is a rare and aggressive form of tumour. Some mesotheliomas have been proven to be highly immunogenic. Here, we investigated the correlation between tumour infiltrating lymphocytes (TILs) or Programmed Cell Death Ligand 1 (PD-L1) expression with overall survival (OS) in patients with MPM.

2)   In our study, 62 Paraffin-embedded formalin fixed (PEFF) samples analyzed for TILs and PD-L1 expression. Patients were divided in 4 groups according to a cut-off of the percentage of TILs found per sample as measured by immunohistichemistry: "0" or absent (between 0% and 5%), "1" or low (between 6% and 25%), "2" or moderate (between 26% and 50%) and "3" or high (more than 51%). OS was then correlated with different TILs' expression patterns. Moreover, PD-L1 expression was assessed within the tumour as well as in the adjacent stroma on the same samples.

3)   In the results section of this chapter, it is evinced that higher expression of peritumoral TILs (Group 2 + 3) versus Group 0 and 1 correlated with improved OS (p-value = 0,02). On the contrary PD-L1 expression seemed to be inversely correlated with clinical outcomes, even in the absence of statistical significance (HR: 1.76; p=0.083 95% IC: 0.92-3.36 in areas within the tumour; HR: 1.60; p= 0.176 95%; IC: 0.80-3.19 in areas within the stroma). No relationship between TILs and PD-L1 expression was identified.

4)   In conclusion to this chapter, our research supports the use of TILs and PD-L1 expression as potential outcome predictors in patients with MPM. The use of TILs and PD-L1 as biomarkers for checkpoint inhibitors efficacy warrants future investigation.

Materials and methods

Mesothelioma Patients and Sample Collection

1)   PEFF specimens were obtained from 62 patients diagnosed with MPM between April 2005 and December 2016 from the archive of the Institute of Pathological Anatomy of Trieste. All patients presented histologically proven diagnosis of MPM. Patients with epithelioid histotype and with tumour sample ≥2 cm were selected. The epithelioid histotype was selected because it was the most frequently occurring one for MPM, making it feasible to enrol a decent sample population for statistical comparisons. The chosen size answered the need for having fragments that are large enough to execute immunostaining and make percentage counts for TILs and PD-L1.   The institutional review board approved the study, and patients provided informed written consent. Histopathological diagnoses were determined by two pathologists at our institution and clinicopathological information was collected from patients' charts. A digital microscope D-Sight Fluo 2.0 (A, Menarini, Diagnostics, S.r.l, Firenze, Italy) was used for acquisition of photomicrographs in preparation for histological examination.

Evaluation of TILs

1)   An automatic stainerBenchMark ULTRA (Ventana Medical System, Inc.) was used for the immunohistochemical analysis. The antigen was retrieved with cell conditioning buffer 1. Next, endogenous peroxidase was inhibited with H_2O_2 at 3% (Bioptica) for 10 min. Samples were incubated with primary antibody anti-CD3 (2GV6) (Roche-Ventana), Rabbit Monoclonal Pre-diluted (0.4ug/mL), for 20 min at 36°C. The antibody was exposed with *ultra*View Universal DAB Detection Kit (Cat No. 760-500). As counterstaining Mayer haematoxylin was used for 4 min.

2)   Two investigators, blinded to the patients' clinicopathological data, assessed expressions of TILs according to the standardized method developed by the International TILs Working Group in 2015 [23]. Four different sub-areas were investigated for TILs: intraepithelial, stromal, peritumoural

and perivasal. To investigate TILs, the number of cells CD3-immunoreactive per microscopic field (5X and 10X) were counted in ten independent regions having the most abundant immunoreactive cells. We used ten slides for each sample. We selected the slides having the five highest numbers of TILs. We averaged the scores of the five highest to obtain the final score per each slide. Patients were divided in 4 groups according to a cut-off of the percentage of TILs found per sample as measured by immunohistichemistry: "0" or absent (between 0 and 5%), "1" or low (between 6 and 25%), "2" or moderate (between 26 and 50%) and "3" or high (above 51%).

Evaluation of PD-L1

1)   Levels of PD-L1 were evaluated on 4-micron layers obtained from FFPE mesothelioma specimens of tumour and stromal regions. Immunohistochemical staining were performed using monoclonal antibody DAKO 22c3 diluted 1:50 and EnVision FLEX + detection kit (Agilent platform, DAKO).

2)   For each region Intensity of staining (values from 1 to 3) and Extension (percentage of positive cells on total surface) were evaluated. The results were combined obtaining two scores (tumour and stromal region) for each patient to define positive and negative PD-L1 tumours: samples which scored "0" were considered negative, while those which scored more than "1" were considered positive.

Statistical analysis

1)   For statistical analysis purposes, a preliminary data exploration was performed. Numerical variables were expressed as median and range and compared by non-parametric tests (Mann–Whitney U-test). Qualitative data were expressed as frequencies and organized into contingency tables; the association between categorical variables was investigated by means of the Fisher's exact test or Person's Chi-square. Time dependent variables were calculated according to the

Kaplan–Meier method. For the statistical analysis the significance level was established at p<0.05. All data were analysed with STATA software. The overall survival (OS) was defined as the time from the date of diagnosis to death from any cause; patients who were still alive were excluded from survival analysis. For the analysis of TILs, samples groups «0» and «1» were put together due to the low number of «0» events and compared with group «2» and «3». For the analysis of PD-L1, patients were divided in positive and negative according to the description above.

Results

Patient characteristics

1)   A total of 62 patients were retrospectively evaluated. Of those patients, 51 (82%) were male. Their median age was 76.3 years (range 37–92); Eastern Cooperative Oncology Group performance status was 0, 1, or 2 in 13 (21%), 42 (68%), and 7 (11%) patients, respectively; all patients were diagnosed with epithelioid MPM histologic subtype. Fourteen (22%) patients had received one line of chemotherapy (Pemetrexed [Alimta]), and 4 (6.4%) patients had received radiotherapy only. Characteristics of patients are reported in **Table 2.1**. The OS, evaluated from the time of diagnosis until the time of death, was available for 50 patients, as the remaining 12 were still alive at the time this study was conducted. The median OS for all patients was 18 months (14-24 95%IC). After screening for the highest quality of stained samples, quantification of TILs expression was done on 51 recovered samples, while the expression of PD-L1 was analysed on the total 62 samples.

TILs and Overall Survival

1)   Results of the expression of peritumoural TILs in the different subgroups are presented in **Table 2.2 and Figure 2.1.** For peritumoural regions there was a statistically significant increase in groups «2» plus «3» compared to groups «0» plus «1» (p-value = 0,02; **Figure 2.2**). In particular, patient groups «2» plus «3» had a median OS of 19 months (95% CI 9-25) in comparison to 14 months (95% CI 6-16) for patients in groups «0» plus «1». No statistical differences in OS were seen in relation to the infiltration location, intraepithelial, stromal, and perivasal regions.

PD-L1 and survival

1)   Among 62 patients, tumour PD-L1 was positive in 25 (40.3%) and stromal PD-L1 was positive in 19 (30.2%) patients respectively. Patients who expressed PD-L1 had a lower OS in comparison to patients with no PD-L1 expression, although this difference did not reach statistical significance in neither tumour, nor stromal PD-L1. Hazard Ratio for intratumour PD-L1: 1.76

(P=0.083 95% IC: 0.92-3.36); Hazard Ratio for stromal PD-L1: 1.60 (P=0.176 95% IC: 0.80-3.19). Although in the absence of statistical significance when we looked at intra-tumour PD-L1 expression, patients without expression of PD-L1 had an OS of 18 months (95% CI 9-24) in comparison to 12 months (95% CI 7-16) for patients with PD-L1 expression. When we looked at PD-L1 distribution in the stroma, patients without expression of PD-L1 had an OS of 16 months (95% CI 10-22) in comparison to 14 months (95% CI 7-18) for patients with PD-L1 expression.

Correlation between the expression of PD-L1 and TILs

1) The correlation between PD-L1 and TILs was explored in 51 patients who had available data for both PD-L1 and TILs expression. We did not find any correlation between expression of TILs and that of PDL1. Among the 18 tumor PD-L1 positive patients, TILs «2» or «3» was observed in 8 (44.4%) patients and TILs «0» or «1» was observed in 10 (55.6%), while among the 33 stromal PD-L1 negative patients, TILs «2» or «3» was observed in 15 (45.4%) patients and TILs «0» or «1» was observed in 18 (55.6%) p=1.

2) Among the 13 stromal PD-L1 positive patients, TILs «2» or «3» was observed in 7 (53.8%) patients and TILs «0» or «1» was observed in 6 (46.2%), while among the 38 stromal PD-L1 negative patients, TILS «2» or «3» was observed in 15 (45.4%) patients and TILs «0» or «1» was observed in 18 (55.6%) p=0.53.
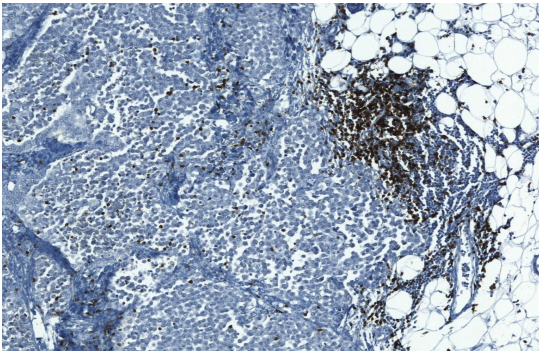
A   B
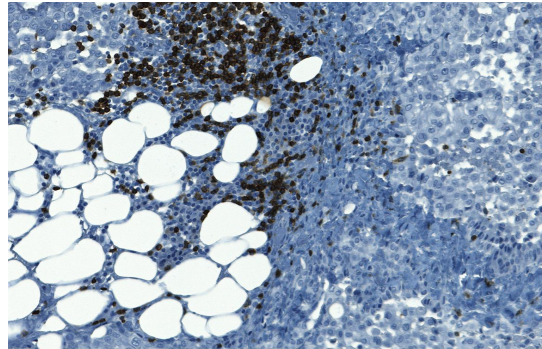
Figure 2.1. Intratumoral TILs in Malignant Pleural Mesothelioma (MPM).
Peritumoral TILs (in dark-brown) in MPM samples were detected with a D-sight microscope.
Photomicrographs were taken at 10X (**A**) and 20X (**B**).

Table 2.1. Characteristics of patients

Characteristic	Number of patients (n =62)
Age, years	
Median	77.5
Range	(37-92)
Sex	
Male	51
Female	11
ECOG PS	
0	13
1	42
2	7
Radiotherapy	
Yes	4
No	58
Chemotherapy	
Yes	14
No	48
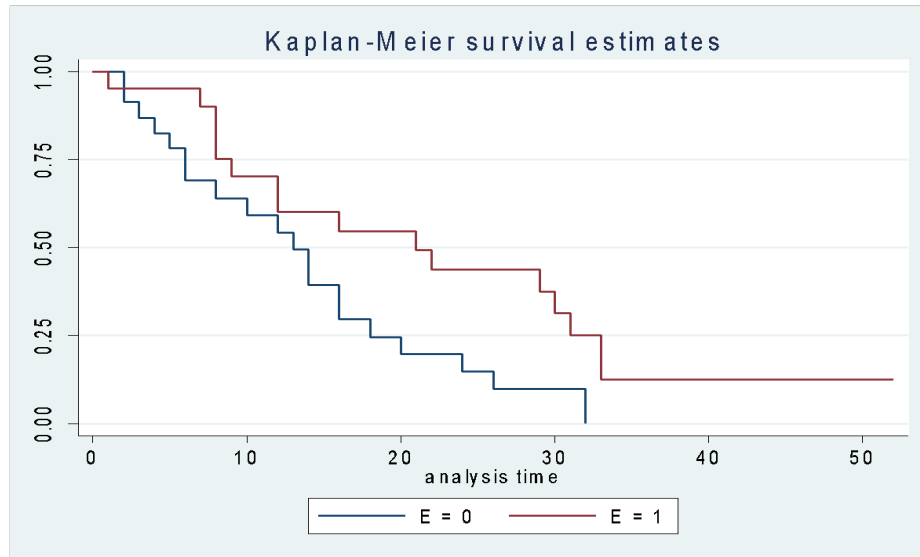
Figure 2.2. Overall survival (OS) of MMe patients correlation with peritumoral TILs.
The expression of peritumoral TILs was correlated with median OS through this Kaplan Meier curve. From the comparison of higher level of peritumoural TILs (groups «0» and «1» (E0)) to the with the lower level of peritumoral TILs (groups «2» and «3» (E1)) the median OS was significantly higher in group E1 compared to group E0, by means of Two-Tailed, Student's T-Test (P = 0.02). Higher expression of TILs correlated with a higher patients' survival.

Table 2.2. Frequency, Percentage and Cumulative Percentages of TILs for each of the four subgroups.

E	Frequency	Percentage (%)
0	4	7.8
1	24	47.1
2	15	29.4
3	8	15.7
Total	51	100

Discussions

The results from the MMe samples show that high expression of peritumoral TILs is associated with improved survival, according to the literature [24–28].

An interesting new hypothesis is emerging: the presence of TILs in the peritumoral region could be creating a protective shield which stops tumour cells from spreading to other organs, thus providing another possible explanation for their clinical activity supporting, thus, the hypothesis better clinical outcomes. It is likely that MPM patients would respond to an immunotherapy stimulating peritumoral TILs. In support to this idea, several studies showed that TILs are important predictors for successful neoadjuvant chemotherapy in breast cancer patients. From them it is possible to evince that a high level of TILs is an independent predictor of pathological Complete Response (pCR) [29–32]. A meta-analysis further proved that TILs can be used as a prognostic factor to predict responsiveness to chemotherapy and survival of patients with breast cancer[33]. Overall, this is supporting the idea that pre-treatment with a stimulator of a host immune response could modulate the effectiveness of chemotherapy against tumour cells. The same dynamics could be taking place in our mesothelioma study, where having high TILs could lead to a better response to Alimta therapy- the chemotherapy that all the MPM patients had received. Thus, high TILs could confer improved responsiveness to chemotherapeutic agents. In line with this, a combination of chemotherapy with an enhancement of the immune response could improve pCR and survival outcomes, opening the door to more clinical trials to test this hypothesis.

The quantity of TILs, not merely their presence or absence, has been shown to be a predictor of OS in a large study of ovarian cancer. The study assessed samples from 5,577 patients using immunohistochemistry, dividing them into four groups [negative (0), low (1-2), moderate (3-19) and high (>20) on the basis of the estimated number of CD8-positive TILs]. The median survival was 2.8 years in patients without TILs, compared to 3 years in those with low levels, 3.8 years for patients with moderate levels and 5.1 years for patients with high levels (p= 4.2x10-16)[34]. The study pointed to the quantity of TILs as a prognostic factor in itself as well as key in unravelling tumour

heterogeneity's outcome in this type of cancer. Similarly, in our MPM study, higher percentages of TILs correlated with improved OS. In fact, the subgrouping of our MPM samples into four populations on the basis of expression of TILs showed that there is a tendency to a better outcome with increasing percentage of TILs. Unfortunately, due to the nature of the disease, the patient sample was too loo to allow individual group comparisons.

When we looked at PD-L1 expression, although patients expressing PD-L1 showed a lower OS in comparison to patients who did not express PDL1, this difference did not reach statistical significance in neither tumour nor stromal PD-L1, probably due to the small patient sample and low number of PD-L1 positive cases.

PD-1 is an immonoinhibitory receptor expressed in many immunological cells, including activated T-cells, B-cells and natural killer cells [35,36] and highly expressed in MPM in comparison to normal mesothelium [37–39]. Our study supports a line of already established evidence where a high expression of PD-L1 correlates with a worse prognosis. Cedrès *et al.* demonstrated that median survival of PD-L1 positive MPM patients was 4.79 months compared to the 16.3 months of the PD-L1 negative patients (p = 0.012) [39]. Similarly, Mansfield *et al.* proved that high expression of PD-L1 was significantly correlated with worse survival in mesothelioma patients (risk ratio 1.71, 95% confidence interval 1.03-2.78 [p=0.04] and risk ratio 2.18, 1.08-4.23 [p=0.03])[38]. Our results are in accordance with these studies, thus corroborating the hypothesis that PD-L1 expression in MPM represents a negative prognostic marker. However, these results failed to reach statistical significance. Moreover, it is still being debated whether PDL1 expression is required to predict clinical response to anti-PDL1 inhibitors. The pitfall of PDL1 as a predictive marker of therapeutic efficacy lays in the many variables, which can impact on the reliability of quantification measurements[14,17]. First of all the heterogeneity in the immunohistochemistry staining, mainly related to the use of multiple antibodies and clones with different affinity and specificity for the ligand. Second, the staining protocols and the platforms used. Lastly, the very tumour heterogeneity

which causes huge variation in gene expression profiles. In response to this, some studies even consider a cut-off of 5% of PDL1 positive tumour cells.

It is clear how internationally approved guidelines are needed to standardise PDL1 expression analysis.

As a matter of fact, since data from literature shows that MMe are immunogenic, immunotherapy represents a valid opportunity in the treatments of this rare disease[40]. Most common strategies include immune-checkpoint targeting drugs in combination with standard chemotherapy. Another immunotherapy for MPM is vaccination; for example, engineered Listeria-based vaccine has been used to induce immune system's T-cells to target cancer cells that express mesothelin, a glycoprotein highly expressed on the surface of MPM cells. Data from a phase Ib clinical study made of 38 MPM patients demonstrated that using this approach in combination with standard chemotherapy proved to be well-tolerated as well as elicited a partial response in 59% and stable disease in 35% of the patients [41]. Interestingly, T-cell stimulation via CD3, CD28 and/or CD40 halved the inhibitory effect of TGF-β1 over lymphocyte proliferation and production of TNF and IFN-γ [42].

Preliminary results from an on-going phase Ib trial (KEYNOTE-028, NCT02054806) are encouraging: anti PD-1 pembrolizumab (Keytruda) is well-tolerated and seems to have a good anti-tumour activity in patients with PD-L1-positive malignant mesothelioma [43]. Pembrolizumab was FDA-approved in 2017 for the treatment of non-small cell lung cancer[44], making it a feasible option for mesothelioma as well. The single agent nivolumab has clinical efficacy and a manageable safety profile in pre-treated patients with mesothelioma irrespective of PD-L1 expression[45]. The COMFIRM trial also provided evidence of the potential benefit of the use of nivolumab in the treatment of relapsed mesothelioma Trials[46]

Chapter 3: Tumour infiltrating lymphocytes and immunerelated genes as predictors of outcome in pancreatic adenocarcinoma.

Rationale

1)   In this chapter we investigated the correlation of pancreatic ductal adenocarcinoma patient prognosis with the presence of tumour infiltrating lymphocytes and expression of 521 immune system genes.

2)   To achieve this purpose intratumoral CD3+, CD8+, and CD20+ lymphocytes were examined by immunohistochemistry in 12 Pancreatic Adenocarcinoma (PDAC) patients with different outcomes who underwent pancreaticoduodenectomy. The results were correlated with the gene expression profile using the digital multiplexed NanoString nCounter analysis system (NanoString Technologies, Seattle, WA, USA).

3)   In the result section of this chapter we evinced that twenty immune system genes were significantly differentially expressed in patients with a good prognosis relative to patients with a worse prognosis: TLR2 and TLR7 (Toll-like receptor superfamily); CD4, CD37, FOXP3, PTPRC (B cell and T cell signalling); IRF5, IRF8, STAT1, TFE3 (transcription factors); ANP32B, CCND3 (cell cycle); BTK (B cell development); TNF, TNFRF1A (TNF superfamily); HCK (leukocyte function); C1QA (complement system); BAX, PNMA1 (apoptosis); IKBKE (NFkB pathway). Differential expression was more than twice log 2 for TLR7, TNF, C1QA, FOXP3, and CD37.

4)   In conclusion in this chapter we demonstrated that tumour infiltrating lymphocytes were present at higher levels in samples from patients with better prognosis. Our findings indicate that tumour infiltrating lymphocyte levels and expression level of the genes listed above influence pancreatic ductal adenocarcinoma prognosis. This information could be used to improve selection of best responders to immune inhibitors.

Material and methods

Pancreatic Adenocarcinoma Patients and sample collection

1)   Fresh PDAC specimens were obtained from patients (n=12) undergoing surgical resection at the Department of Medical, Surgical & Health Sciences, Cattinara Teaching Hospital, Trieste University, between 2005 and 2015. These experiments were approved by Trieste University Institutional Review Board. Tissue specimens were snap-frozen in liquid nitrogen and stored at −80°C.

2)   Formalin-fixed, paraffin wax-embedded sections were used for immunohistochemical staining. All 12 paraffin wax blocks were confirmed to contain tumour tissue by two pathologists, comprising six pancreatic adenocarcinomas with a good prognosis and six pancreatic adenocarcinomas with a bad prognosis.

3)   The following clinical data were collected: patient age, gender, and outcome; the presence/absence of metastasis; tumour location, size, margin status, TNM stage, degree of differentiation, invasion degree and location (lymph node, bile duct/duodenal serosa, hepatic, portal vein, vascular, perineural), schedule of chemotherapy, neoadjuvant and/or adjuvant chemotherapy, chemotherapy toxicity, and treatment follow up. Patients were informed about the project and gave written consent for study participation.

Follow up

1)   OS was measured from the time of surgery to the time of death or the last follow up visit. Dates of death were obtained from patient hospital records or follow up telephone calls. A more in-depth analysis of the 12 patients revealed two groups with different DFS and/or OS: six patients with an OS between 25 and 66 months were classified as "good cases", while six with OS between 2 and 9 months or DFS between 1 and 2 months were classified as "worse cases". **Table 3.1** summarizes the clinical-pathological data of the two groups of patients.

Table 3.1. Summary of the clinical-pathological information of patients

	Good cases	Bad cases
Num. Pts	6	6
Age	65.3	63.8
Gender		
Man	4	3
Woman	2	3
ECOG		
0	2	0
1	2	3
2	1	2
Not reported	1	1
TNM		
T		
T0	0	0
T1	1	2
T2	0	4
T3	4	0
T4	1	0
N		
N0	2	2
N1	4	4
M		
M0	6	6

PanCancer Immune Profile Panel multiplex gene expression profiling

1)   Total RNA from fresh frozen tumour tissues was extracted using Qiagen RNeasy (Qiagen Inc., Toronto, ON, Canada) as per the manufacturer's instructions. A NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to measure RNA concentration and purity. All RNA samples included in the study passed the quality control requirements (as verified by RNA integrity number or OD 260/280 ratio) of the platform. Using 100 ng total RNA from each sample as input, according to the manufacturer's instructions, the digital multiplexed NanoString nCounter analysis system (NanoString Technologies, Seattle, WA, USA) was used for gene expression profiling. Tumour RNA samples were analysed using nCounter PanCancer immune profile panel consisting of 770 human immune-related genes (Nanostring Technologies).

2)   In this assay, colour-coded barcodes are used to represent single-target transcripts in the reaction. An overnight hybridisation reaction was used to incorporate the resulting material, carried out by combining 20 ml of nCounter Reporter probes in hybridisation buffer, 5 ml of nCounter Capture probes and 5 ml of the total RNA sample for a total reaction volume of 30 ml. The hybridisations were incubated at 65 °C for 16–20 h. An excess of probes is provided during overnight hybridization to ensure that each target finds a probe pair. Target abundance values can then be determined through the nCounter Digital Analyzer by counting the individual fluorescent barcodes. A high-density scan was performed for each assay (encompassing 600 fields of view). After hybridisation, the cartridges were analysed in the Digital Analyzer that counts (representing the number of molecules) and arranges the barcodes.

Immunohistochemistry

1)   An automatic stainer (BenchMark ULTRA, Ventana Medical System, Inc.) was used for the immunohistochemical test. The antigen was retrieved with cell conditioning buffer 1. Next,

endogenous peroxidase was inhibited with H_2O_2 at 3% (Bioptica) for 10 min. Samples were incubated with primary antibody anti-CD3 (2GV6) (Roche-Ventana), anti-CD8 (SP57) (Roche-Ventana), Rabbit Monoclonal Pre-diluted (0.4μg/mL), for 20 min at 36°C; anti-CD20 (L26) (Roche-Ventana), Mouse Monoclonal Pre-Diluted (0.4μg/mL) for 24 min at 36°C. The antibody was exposed with ultraView Universal DAB Detection Kit (Cat No. 760-500). As counterstaining, Mayer haematoxylin was used for 4 min.

2) TIL levels were assessed by two investigators blind to the patients' clinical-pathological data using the standardized method coded in 2015 by the International TILs Working Group[23]. TILs were investigated per microscopic field (5X and 10X) and an average over ten independent regions having the most abundant immunoreactive cells was calculated for each slide.

IHC Statistical analysis

1) For IHC statistical analysis, a preliminary data exploration was performed. Numerical variables were expressed as median and range and were compared by non-parametric tests (Mann–Whitney U-test). Qualitative data were expressed as frequencies and organized into contingency tables; the association between categorical variables was investigated by means of Fisher's exact test or Pearson's Chi-square. Time-dependent variables were calculated according to the Kaplan–Meier method. For the entire statistical analysis, the significance levels were established at $p < 0.05$. All data were analysed with STATA software.

Immune Profile Panel multiplex nanoString Statistical Analyses

NanoString data analysis

1)   nSolver (NanoString Technologies) was used for the normalization of raw data as previously reported [47]. The raw NanoString counts were initially subjected to normalization for all target RNAs in all samples based on built-in positive controls. This step accounts for post-hybridization processing, inter-sample and experimental variation such as hybridization efficiency. The geometric mean of each of the controls was calculated, indicating the overall assay efficiency. For the mRNA content normalisation, housekeeping genes were then used. To facilitate downstream statistical analysis, values < 0 were blanketed and considered equal to 1. After initial normalisation steps, data were imported to GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) to be processed for statistical analysis. Multiple t-tests were performed with correction for multiple comparisons through the Sidak–Bonferroni method. A difference in expression with a P-value of ≤ 0.05 was considered statistically significant.

Results

Immune-related gene expression analysis in pancreatic adenocarcinomas with good versus worse prognosis

1)   Prognosis of primary PDAC patients was determined using clinical data and Kaplan-Meier curves (Figure 3.1 and Supplementary Table 3.1). Three primary PDAC patients with a good prognosis and three with a worse prognosis were then chosen for mRNA analysis by PanCancer Immune Profile Panel multiplex gene expression analysis. Fig. 3.2 shows the differential gene expression. Among the immune system genes showing statistically significant (p <0.01) differential expression between pancreatic adenocarcinoma with a good and worse prognosis, differential expression of *TLR7*, *TNF*, *C1QA*, *FOXP3*, and *CD37* was more than twice log 2: +2.76 log 2 ± 0.58 (p <0.00896), +2.39 log 2 ± 0.389 (p <0.00356), +2.19 log 2 ± 0.43 (p <0.00697), +2.07 log 2 ± 0.372 (p <0.00513), and +2 log 2 ± 0.297 (p <0.00254), respectively (Supplementary Figure 3.1). *BTK (+1.91 log 2 ± 0.309 (p<0.0035)), CD4 (+1.86 log 2 ± 0.235 (p<0.00138)), HCK (+1.86 log 2 ± 0.304 (p<0.00364)), PTPRC (+1.83 log 2 ± 0.259 (p<0.00211)), CCND3 (+1.67 log 2 ± 0.337 (p<0.00777)), STAT1 (+1.59 log 2 ± 0.238 (p<0.00626)), IKBKE (+1.51 log 2 ± 0.282 (p< 0.00585)), IRF8 (+1.43 log 2 ± 0.246 (p<0.00439)), TNFRF1A (+1.39 log 2 ± 0.298 (p<0.00954)), TLR2 (+1.34 log 2 ± 0.147 (p<0.000799)), BAX (+1.31 log 2 ± 0.246 (p<0.00598)), IRF5 (+1.27 log 2 ± 0.193 (p<0.00272)), PNMA1 (+0.986 log 2 ± 0.201 (p<0.00799)), ANP32B (+0.92 log 2 ± 0.163 (p<0.00484)), TFE3 (-0.37 log 2 ± 0.0783 (p<0.00919)),* and mRNA also showed statistically significant (p <0.01), but less than twice log 2, differential expression between pancreatic adenocarcinomas with good and worse prognosis (Table 3.2).
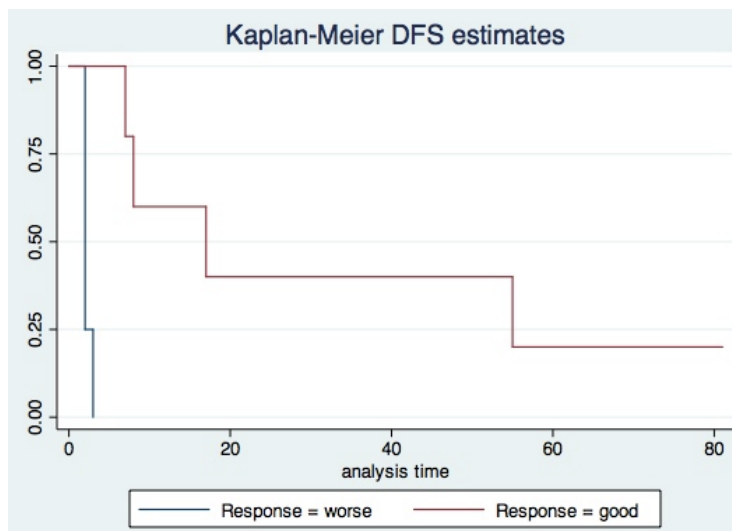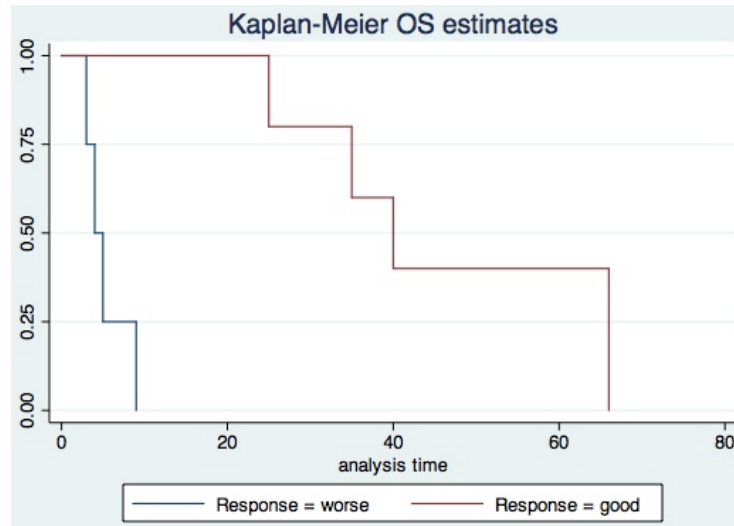
Figure 3.1. Overall survival (OS) and Disease Free Survival (DFS) for the two groups of patients with pancreatic adenocaricinoma. Kaplan-Meir curves show the difference of OS and DFS in the two groups of patients named Good (red line) and Worse (blue line).
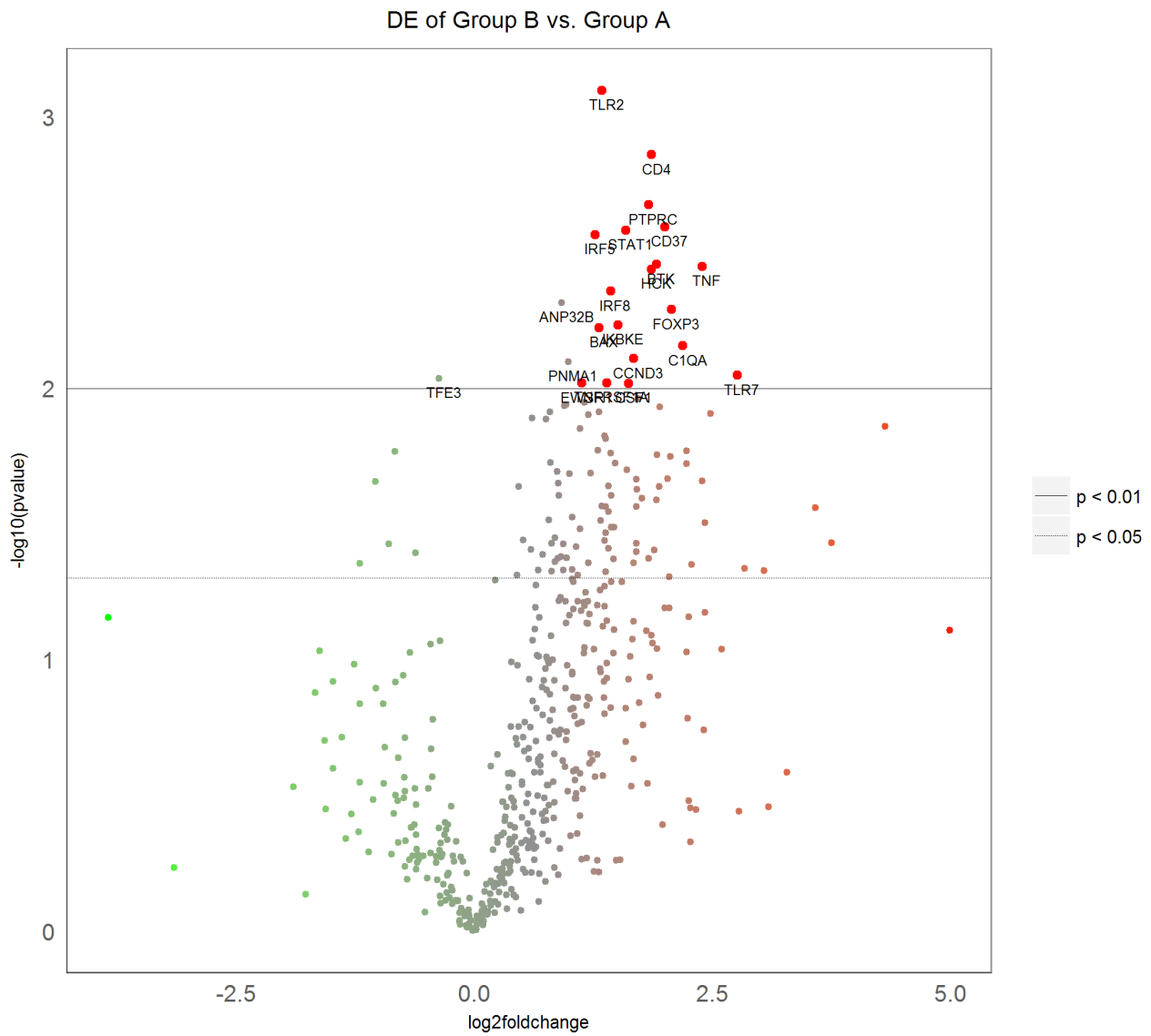
Figure 3.2. The volcano plot shows the differential expression between "good cases" and "bad cases". Larger red dots represent only those genes whose expression is at least twice log2 higher between "good cases" (Group B) and "bad cases" (Group A) and p-value <0.01.

Table 3.2. Top 20 genes differentially expressed between "good cases" and "bad cases".

Genes	Differential expression between "good cases" and "bad cases" (Log2 fold change)	Std error	P-Value
TLR2-mRNA	1.34	0.147	0.000799
CD4-mRNA	1.86	0.235	0.00138
PTPRC-mRNA	1.83	0.259	0.00211
CD37-mRNA	2	0.297	0.00254
STAT1-mRNA	1.59	0.238	0.00262
IRF5-mRNA	1.27	0.193	0.00272
BTK-mRNA	1.91	0.309	0.0035
TNF-mRNA	2.39	0.389	0.00356
HCK-mRNA	1.86	0.304	0.00364
IRF8-mRNA	1.43	0.246	0.00439
ANP32B-mRNA	0.92	0.163	0.00484
FOXP3-mRNA	2.07	0.372	0.00513
IKBKE-mRNA	1.51	0.282	0.00585
BAX-mRNA	1.31	0.246	0.00598
C1QA-mRNA	2.19	0.43	0.00697
CCND3-mRNA	1.67	0.337	0.00777
PNMA1-mRNA	0.986	0.201	0.00799
TLR7-mRNA	2.76	0.58	0.00896
TFE3-mRNA	-0.37	0.0783	0.00919
TNFRSF1A-mRNA	1.39	0.298	0.00954

2)   Gene expression analysis indicated that the pancreatic adenocarcinoma group with a good prognosis showed higher levels of the following cell types compared to the group with a worse prognosis (Fig. 3.3): CD45-expressing cells, Tregs, DCs, macrophages, NK CD56dim cells, T-cells, exhausted CD8$^+$ cells, cytotoxic cells, mast cells, CD8$^+$ T cells and neutrophils (Fig. 3.3A and 3.3B). Box plot representations indicate that the following subtypes of cells exhibit particularly different levels: CD45-expressing cells, dendritic cells, macrophages, natural killer cells, the family of T cells (Fig. 3.3 C) and exhausted CD8$^+$ and Treg cells (Supplementary Figures 3.1-3.3).
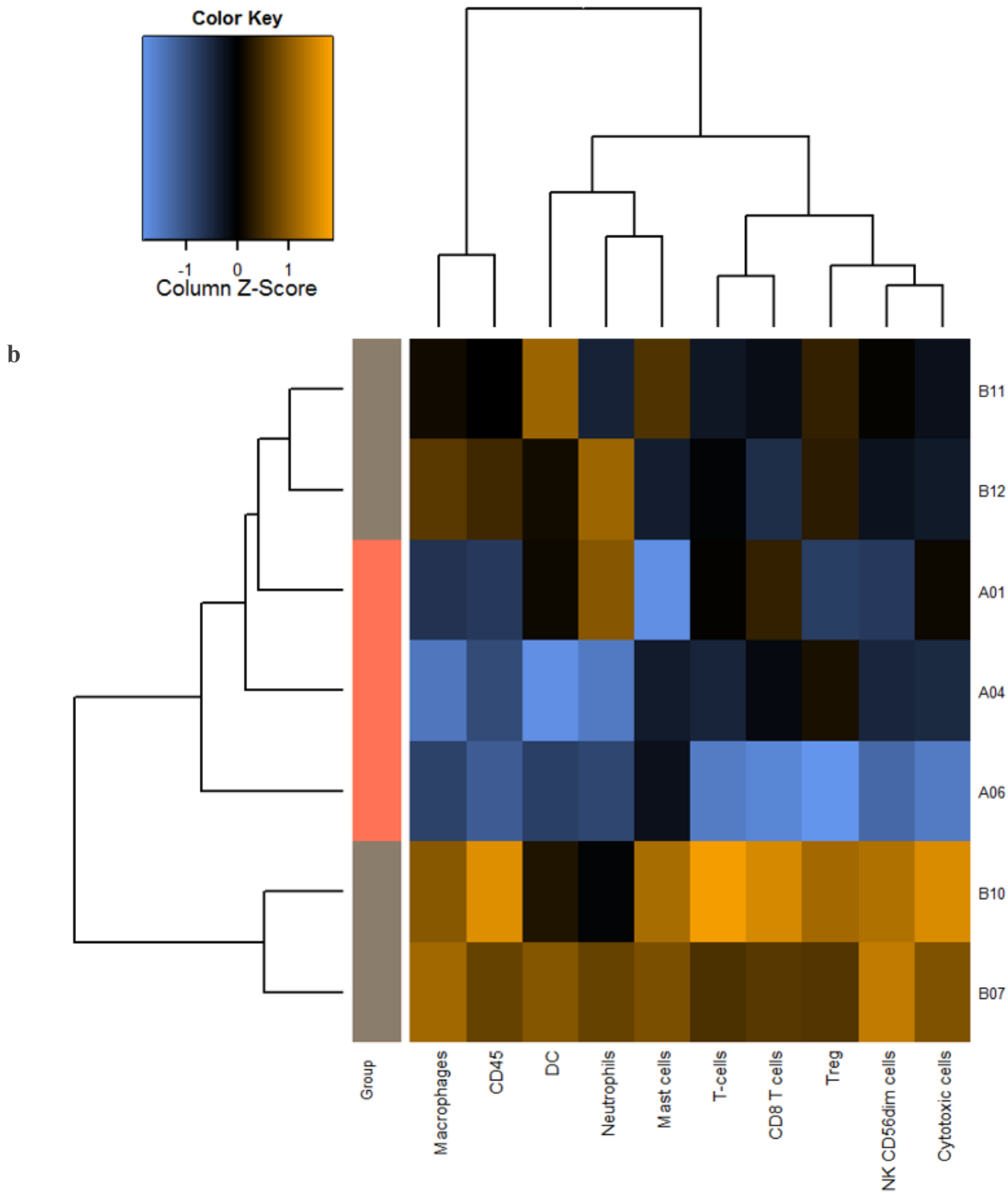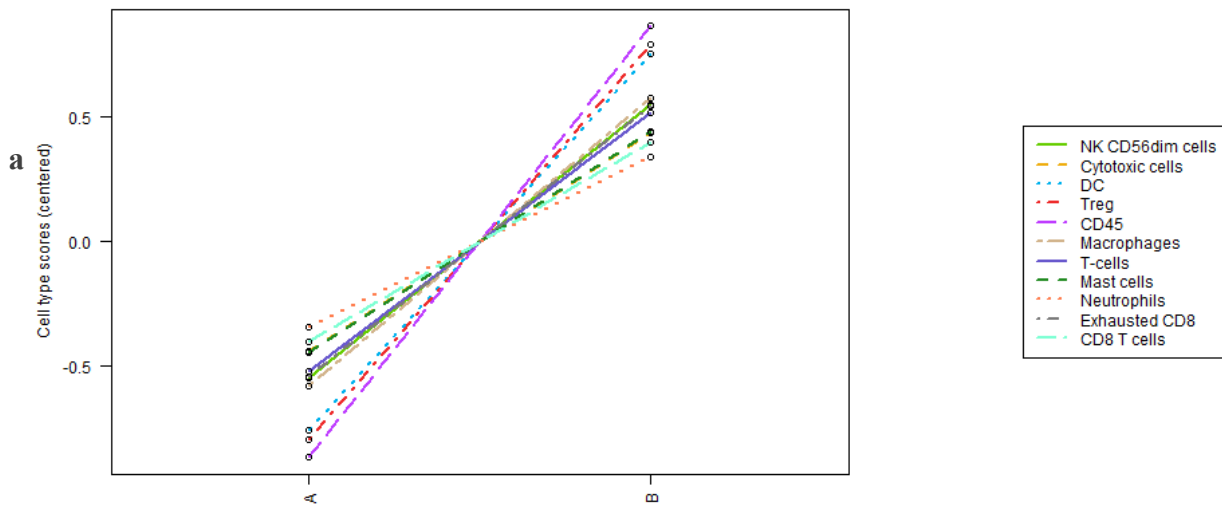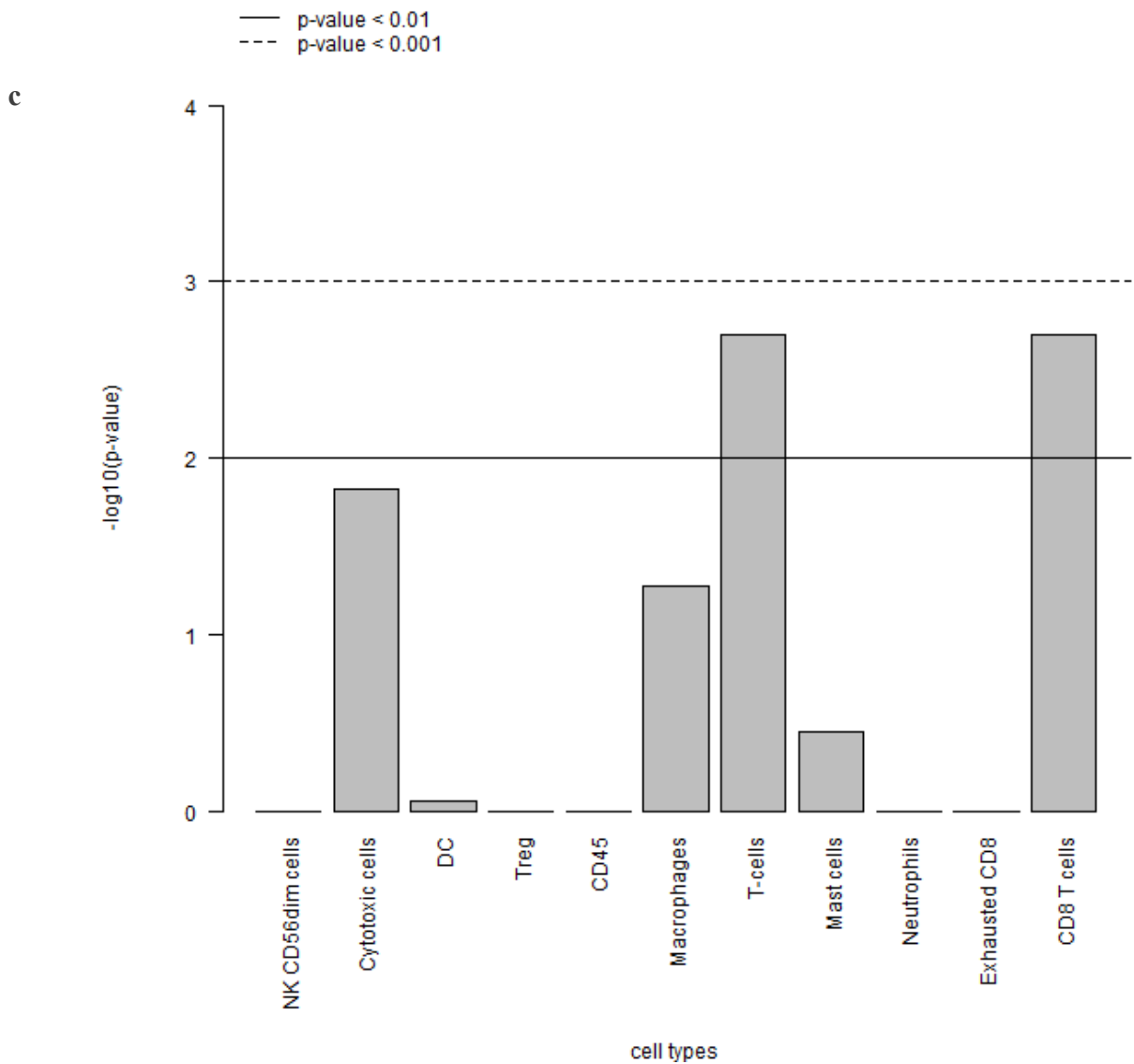
Figure 3.3. a) Trend plot summarizing the expressions of cell type scores from "bad cases" (A) to "good cases"(B). **b)** Heat-map showing the expressions of the different cell-types in "bad cases" (orange label) and "good cases" (grey label). **c)** Bar plot of the log10 p-value from the differential expression of the scores of the cell-types between "good cases" and "bad cases".
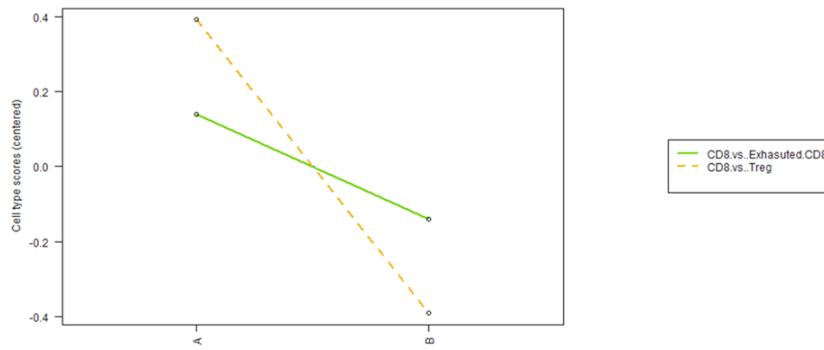
Supplementary Figure 3.1. Differential gene expression between the good and worse prognosis PDAC patient groups. Volcano plot displaying each gene's -log10 (p-value) against log2 fold change: a) TLR7, b) TNF, c) C1QA, d) FOXP3 and e) CD37. Highly statistically significant genes fall at the top of the plot, and highly differentially expressed genes fall to either side. Genes within the selected gene set are highlighted in orange. Horizontal lines indicate various False Discovery Rate (FDR) thresholds.
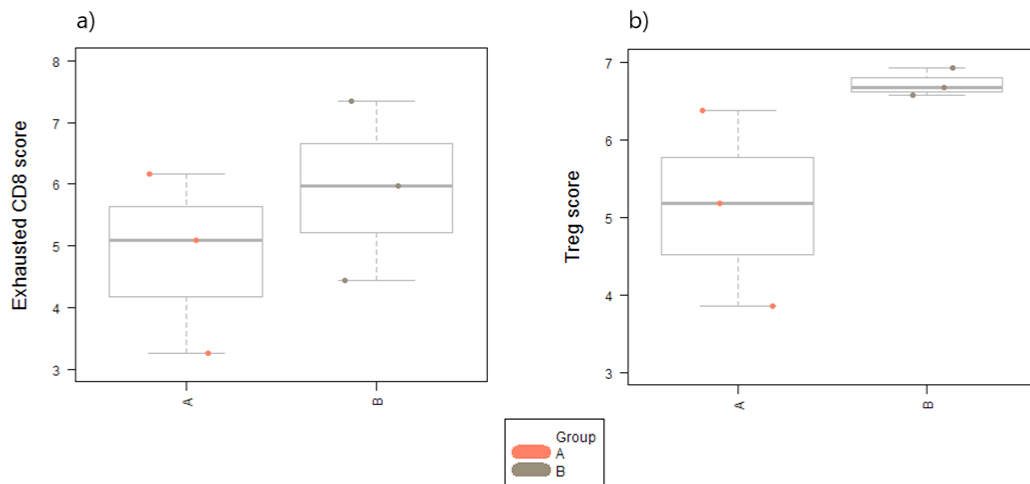
Supplementary Figure 3.2. Exhausted CD8+ and Treg cell profiling in pancreatic adenocarcinomas with good versus worse prognosis. Box plots show levels of exhausted CD8+ cells (a) and Tregs (b) in patients with worse prognosis (group A) and patients with worse prognosis (group B). Even though scores seem overlapping, the average score for both sets of cells is higher in group B than in group A, probably due to the fact that longer-surviving PDAC patients had higher levels of intratumoral TILs.
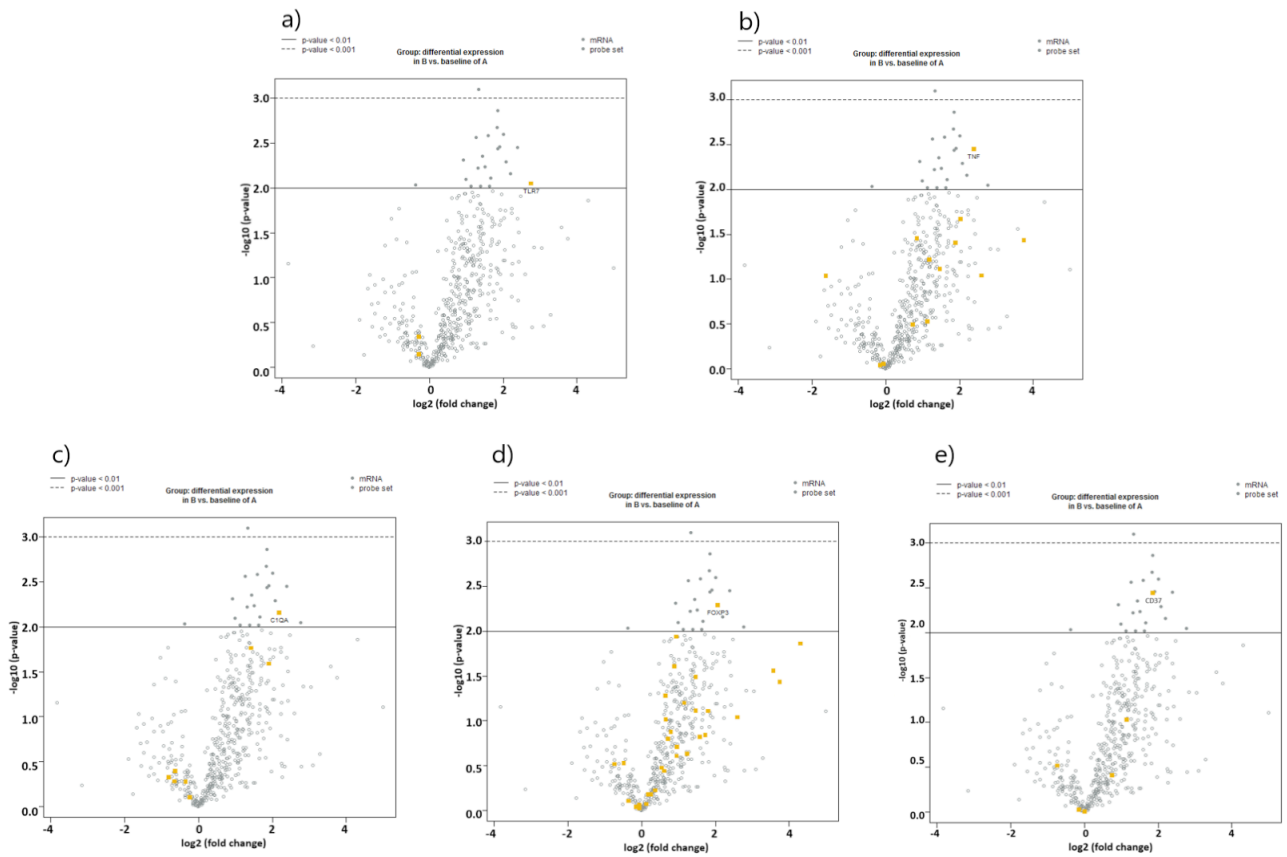
Supplementary Figure 3.3. Relative cell type abundance measurements between group A and B. The diagram shows the abundance of exhausted CD8+ cells and Tregs compared to levels of CD8+ cells. In agreement with the previous figure, levels of exhausted CD8+ cells (green line) and Tregs (dashed orange line) are reported to be lower in the group with a worse prognosis (group A) than in the group with a better prognosis (group B) when compared with the total level of CD8+ cells.

Tumour immune cell profiling in pancreatic adenocarcinomas with good versus worse prognosis

1)   In agreement with existing data [48–52], tumour immune cell marker levels were higher in good prognosis cases compared to worse prognosis cases (**Table 3.3**). The CD3 level was statistically higher in the good prognosis group compared to the worse prognosis group (p=0.0267, **Table 3.4 and Fig. 3.4**). Despite the fact that the number of CD8$^+$ and CD20$^+$ cells has been found to be higher in patients with good prognosis in our study, no statistically significant difference was found between the two subgroups of immune cells (p = 0.119 and p = 0.925, respectively) (**Table 3.4 and Fig. 3.4**). TILs' marker levels were not assessed in one case due to calcification in pancreatic ductal tissue. **Fig. 3.5** shows the detection of TILs in the two sets of six PDAC patients. Staining for TILs was visually negative in the adenocarcinomas with a worse prognosis while it was positive for the adenocarcinomas with a good prognosis.

Table 3.3. Expression levels of the following TILs subpopulation: CD3, CD8 and CD20.

	Sample number	CD3		CD8		CD20	
Good cases	1	10	8	10	9	2	3
	2	10	8	5	3	0	1
	3	23	16	6	12	0	0
	4	10	8	10	8	0	1
	5	27	16	15	13	5	4
	6	16	15	15	11	2	3
Worse Cases	1	6	7	5	9	1	2
	2	Not performed					
	3	13	11	10	6	2	3
	4	9	8	13	13	1	2
	5	9	4	3	4	2	3
	6	9	5	1	3	0	0

Table 3.4. Statistical difference of TIL levels between the two groups. The table summarizes the statistical difference of TIL levels between the "worse case" and "good case" groups (non-parametric Mann Whitney test or "U-test").

	Median "worse cases"		Median "good cases"		p-value
	N. pts		N. pts		
CD3	6	12.25 (9-35)	5	7 (6.5-12)	0,0267
CD8	6	9.25 (4-14)	5	7 (2-13)	0,119
CD20	6	1.5 (0-4.5)	5	1.5 (0-2.5)	0,925
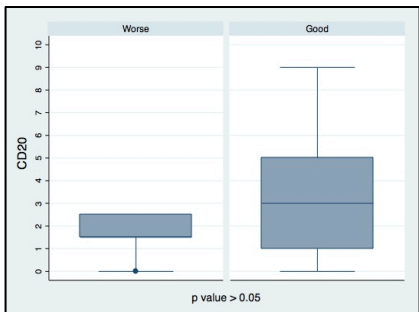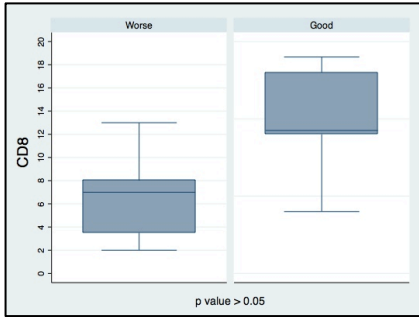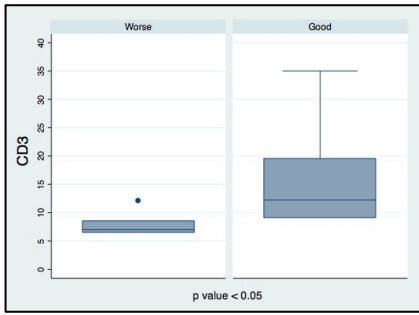
Figure 3.4. Expression of TILs markers between "bad cases" and "good cases" of pancreatic adenocarcinomas are represented. The box plots elicit the expression levels of TILs CD3, CD8 and CD20 subpopulations between the group of "bad cases" and the group of "good cases". The lower table summarizes the statistical difference of TILs between the two groups of patients "good cases" and "bad cases" adopting no-parametric Mann Whitney test or "U-test".
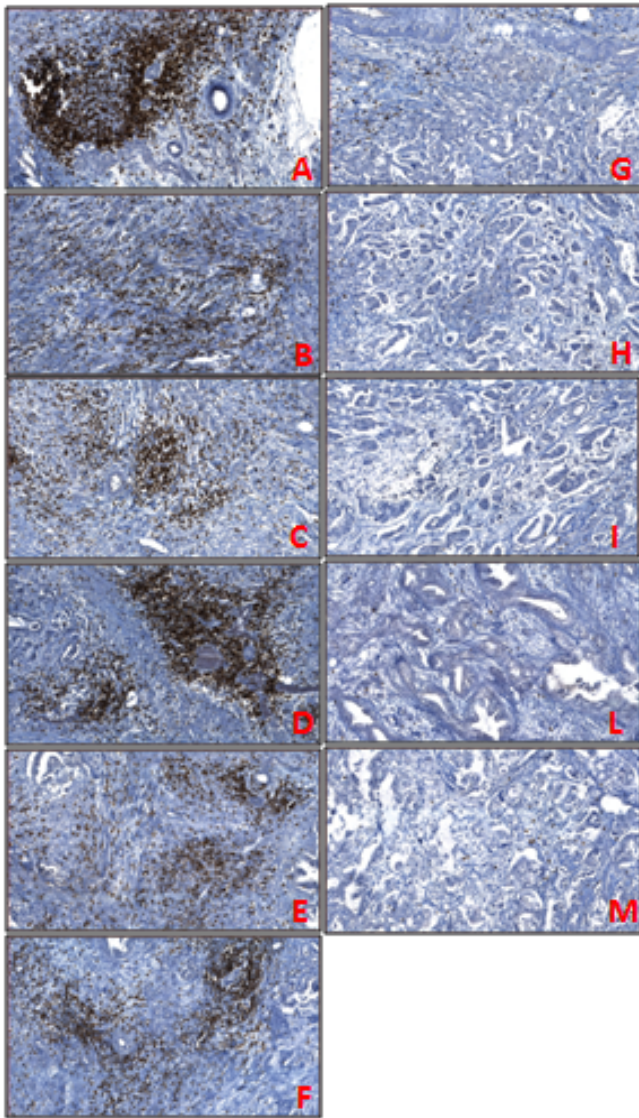
Figure 3.5. Expression levels of TILs anti-CD3 across the two groups of patients: samples A-F were collected from "good cases"; whereas samples G-M were obtained from "bad cases".

Supplementary Table 3.1. Dataset used for Kaplan-Meyer curves.

ID	date diagnosis	date of metastasis	DFS (months)	exitus	date of death	OS (months)
	18/09/2013	21/11/2013	2	yes	07/01/2014	4
	08/11/2013	20/01/2014	2	yes	10/08/2014	9
Worse cases	16/01/2014	20/02/2014	1	no		
	16/02/2015	08/04/2015	2	yes	25/07/2015	5
	22/06/2015	27/08/2015	2	yes	25/09/2015	3
	26/10/2015	06/11/2015	1	no		
	05/03/2008	01/12/2011	45	yes	07/03/2012	48
	19/05/2009	01/10/2010	17	yes	05/09/2012	40
Good cases	22/09/2009	01/04/2014	55	yes	17/03/2015	66
	20/10/2011	28/05/2012	7	yes	29/09/2014	35
	21/02/2013	29/10/2013	8	yes	07/03/2015	25
	27/01/2009	29/09/2013	56	yes	03/07/2014	66

Discussions

There are three major barriers impeding immune therapy in PDAC: 1. The mutational load in PDAC is much lower than that of lung cancers and melanoma; 2. PDAC has a strong immunosuppressive microenvironment which is composed of a dense desmoplastic reaction having remarkable infiltration of tumourigenic MDSCs and macrophages [53]; 3. The PDAC microenvironment has a very low number of infiltrating T cells, insufficient to provide a significant T-cell response.

In the current study, PDAC samples from patients with a good prognosis had higher levels of TILs compared to a group of patients with a worse prognosis, as assessed via immune marker levels. Even though the patient numbers are small and the selection of good prognosis or worse prognosis somewhat arbitrary (based on clinical data and Kaplan-Meier curves) (Fig. 3.1), the correlation is consistent with previous reports suggesting that TIL levels provide a robust predictor of outcome in pancreatic cancer [51,54]. Consistent with data reported by Stromness *et al*, we point out that in some samples of the "Good" prognosis group, CD3+ cells tend to organize in tertiary lymphoid structures (TLS) within tumour stroma[55]. Although only a few information is known about TILS, these formations are commonly found in solid tumour with a better prognosis, suggesting its possible role in T-cell regulation of *in-situ* immune response[55]. Furthermore, our study revealed a significant (p-value <0.001) differential expression of 20 immune system genes between PDAC patients with good and worse prognoses. Among these genes, expression of five (*TLR7, TNF, C1QA, FOXP3, CD37*) was more than twice log 2 higher in the good prognosis group relative to the worse prognosis group. Expression levels of these five genes could constitute a molecular signature of likely outcome and could therefore be useful for clinical applications.

FOXP3 is a well known marker of Tregs, with a pivotal role in the development and differentiation of these cells to promote tumour immune escape [56,57]. In line with our results showing that higher *FOXP3* is associated with better clinical outcomes, is data from colorectal cancer. In fact a meta-

analysis of 18 studies comprising 3627 colorectal cancer patients with colorectal cancer showed that higher *FOXP3* was associated with better overall survival (HR= 0.76, 95% CI= 0.58-1.01, P =0.058), with a p-value on the threshold of significance[58]. Conversely to our results, *FOXP3* has been reported to be an important tumour suppressor gene in breast cancer [59–63], gastric adenocarcinoma [64,65], prostate cancer [66], and non-small cell lung cancer [67]. These findings indicate that the roles of *FOXP3* in tumours are diverse and situation-dependent.

C1QA encodes the A-chain polypeptide of complement subcomponent C1q and plays an important role in counteracting tumour cells [68,69]. Teschendorff and Caldas *et al* showed that overexpression of *C1QA* in ER-negative basal-like breast cancer patients is associated with better prognosis [70]. It was shown more recently that lower *C1QA* expression could be linked with worse outcomes in patients with ER-negative breast cancer [71]. Nonetheless, Bulla *et al* recently showed that C1q can exert functions unrelated to complement activation, contributing to extracellular changes within the tumour microenvironment and supporting tumour growth and invasion [72]. This last finding is supported by Winslow *et al* [73].

TNF has long been considered a key regulator of the inflammatory and immune response to cancer, promoting either death or survival under different circumstances [74]. Although several anti-TNF therapies have been developed with different binding and pharmacokinetic profiles [75], TNF is used in current therapies to fight cancer, notwithstanding its toxicity [76]. TNF has proved to have an effect on metastatic melanoma treatment and unresectable soft tissue therapies [77,78]. There is evidence of TNF's role in promoting regression of unresectable hepatic metastasis from colorectal cancer [79] and in causing tumour necrosis via its pro-coagulant effect [80].

TLR7 is of special interest in cancer therapy on account of its strong stimulation of IL-12 and type-I interferons, which are important cytokines and effectors of T and NK cell functions [81,82]. TLR7 ligands can not only activate directly NK cells and cytotoxic T-cells [83,84], but also hamper the suppressive function of myeloid-derived suppressor cells [85,86] and interfere with the migration of

Tregs into the tumour [87]. TLR agonists are clinically approved or under clinical evaluation for cancer immunotherapy [88–90].

CD37 belongs to the tetraspanin superfamily of transmembrane proteins that regulate protein adhesion, trafficking, and migration and that are emerging controllers of both humoral and immune control, especially stimulating dendritic cell migration and B cell survival [91–93]. The contribution of CD37 to antitumour immunity has been known since the finding that $CD37^{-/-}$ mice have impaired antitumour responses [94]; however, the role of CD37 in the tumour microenvironment is not clear and further investigations are needed. Tetraspanins in the tumour microenvironment may have therapeutic potential via stimulation or inhibition of immune cell functions, depending on the immune cell type [95].

There are numerous biomarkers that have proved to be clinically useful for other cancers such as lung cancer [96], colorectal cancer [97], breast cancer [98] and melanoma [99], but clinical application of biomarkers for PDAC has been somewhat limited. Indeed, the only FDA-approved PDAC marker, the serum protein CA 19-9, was approved in the 1980s [100]. At least 10% of patients do not express CA 19-9, however, and its level is easily affected by metabolic abnormalities. Several studies have identified biomarkers that could be used as predictors of clinical outcome for PDAC [101–104], but none of these involves the immune-related gene signature revealed here, which is unique.

Chapter 4: The effect of everolimus on immune cell subsets in patients with mBC: potential implications on clinical outcome.

Rationale

1.   In this last chapter I have investigated immune-related biomarkers for predicting response to mTOR inhibitor everolimus therapy in HR+/HER2-. Neutrophil and platelet to-lymphocyte ratios (NLR and PLR), immune pathways, and TILs were analysed in correlation with everolimus responsiveness in 2131 metastatic patients from the BALLET study, in 23 patients receiving neoadjuvant everolimus and in 15 metastatic patients at the local institution, respectively.

2.   In the 27 HR+/HER2- patients of the MREC study receiving neoadjuvant everolimus of most of the immune system pathways were activated in everolimus responders vs. non-responders.

3.   In the BALLET study quartiles of patients with lower NLR levels in the blood had higher survivals compared to patients with higher NLR: NLR ≤ 2.3 vs. NLR >2.3; NLR ≤ 3.2 vs. NLR > 3.2; and NLR ≤ 4.4 vs. NLR >4.4 (p=0.19, p=0.12 and p=0.01).

4.   In the smaller population of 15 metastatic patients from Cremona Hospital, Fluorescence-Activated Cell Sorting (FACs) analyses showed that everolimus responders vs. non-responders had higher levels of CD3+ T-lymphocytes at baseline (p=0.0343) and during treatment (p=0.0233), higher levels CD8+ and CD4+ T-lymphocytes at baseline (p=0.0172, p=0.0005, respectively) and during treatment (p=0.0102, p=0.0032, respectively); while they had slightly lower levels of regulatory T-lymphocytes and Natural Killers (NKs) (p=0.0588 and p=0.0411, respectively). IHC further confirmed that higher TILs correlated with improved survival in primary and metastatic lesions.

Material and Methods

Clinical Section

1)   The window of opportunity trial, based on the administration of 5mg everolimus in neo-adjuvant setting, obtained the ethical approval from Northern and Yorkshire MREC (MREC reference 04/MRE03/89). All patients gave informed consent. A total of 32 post-menopausal women diagnosed with operable ER-positive early breast cancer were recruited receiving treatment daily for 14 days prior to primary surgery [105].

2)   The mTOR study (code 12063/2015) was approved by Ethical Committee Val Padana-Cremona. Patients provided informed written consent and the study was conducted in conformity with the 1964 Declaration of Helsinki and its later amendments. Pathologists at ASST-Cremona determined histopathological diagnoses, and clinic information was collected prospectively from patients' charts in the Breast Unit –ASST of Cremona. A total of 15 post-menopausal women, diagnosed with metastatic ER-positive BC, were treated daily with 10 mg of everolimus alone for 21 days (window of opportunity trial) followed by the combination with exemenstane (25 mg) Tissue samples were taken when possible from the metastasis for immunohistochemical analysis before starting treatment and blood samples were taken before and after everolimus administration for flow cytometer analysis. Responsiveness to everolimus was measured by [18]Fluorodeoxyglucose (FDG)-Positive Emission Tomography (PET)/Computed Tomography (CT) after 1 month of treatment. Patients were considered responsive to everolimus when a reduction of Maximum Standardized Uptake Value (SUV_{max}) was present; whereas with a detection of increase or stability in SUV_{max}, the patients were classified as non-responsive. The characteristics of the patients are summarized in **Table 4.1**.

3)   The BALLET study was a European, phase IIIb, open-label, single-arm, multicenter expanded access clinical investigation (EudraCT Number: 2012-000073-23), which has been previously described[106]. Informed consent was formally obtained from all patients and the study was conducted accordingly to Good Clinical Practice guidelines and the Declaration of Helsinki.

The protocol was independently approved by ethics committee review board at each site. The information about the neutrophil and lymphocyte status has been collecting at basal and at the time of progression from the combination of everolimus/exemestane, when the information was available. The characteristics of the patients are summarized in **Table 4.2**.

ID	Histotype	Grading	T	N	ER	PgR	HER2	Ki67	Date of Progression	Metastatic site
MS	Ductal Infiltrating	3	2	1	75	30	0	38	14/07/2015	bone
LP	Ductal Infiltrating	3	1C	0	90	90	0	7	15/09/2015	hepatic
PM	Ductal Infiltrating	NE	1C	1	90	0	0	15	15/09/2015	hepatic
AO	Lobular Infiltrating	2	2	1	90	60	1	7	22/09/2015	hepatic
GM	Lobular Infiltrating	3	4	1	70	0	0	9	05/10/2015	bone
MGR	Ductal Infiltrating	2	2	0	0,9	0,9	0	45	12/10/2015	bone
MGC	Ductal Infiltrating	2	1C	0	75	75	0	60	30/10/2015	pleura
MEM	Ductal Infiltrating	3	2	NE	95	5	0	15	30/10/2015	hepatic
RL	Lobular Infiltrating	2	1B	3	90	80	1	10	04/02/2016	bone
PB	Lobular Infiltrating	NE	4	NE	95	70	0	25	04/02/2016	hepatic
FR	Ductal Infiltrating	2	1C	2	90	90	0	15	04/02/2016	pleura
SO	Ductal Infiltrating	2	3	1	90	50	0	30	11/02/2016	bone
DS	Ductal Infiltrating	3	4D	NE	95	0	0	30	17/02/2016	skin-bone-hepatic
RA	Ductal Infiltrating	2	1c	0	90	75	0	20	07/06/2016	nodal
AG	Ductal Infiltrating	2	2	0	95	95	1	40	20/06/2016	bone-hepatic
MS	Ductal Infiltrating	2	2	1	95	30	0	13	12/09/2016	hepatic
SN	Ductal Infiltrating	3	2	1	95	10	0	40	21/09/2016	brain
RS	Ductal Infiltrating	2	1C	3	75	75	0	5	19/12/2016	nodal

Table 4.1. **Clinico-pathological features of metastatic BC patients according to response.** The table shows the clinical pathological characteristics of the HR+/HER2- breast cancer patients from Cremona's Hospital.

Table 4.2. Circulating immune markers and endothelial cells' bar plots. Quantification of immune markers and CECs at basal (**a**) or intermediate (**b**) levels of everolimus therapy from the blood of everolimus responsive (0) vs. everolimus nonresponsive patients (1).

Characteristics	Overall	Quartile 1 NLR $\leq$ 2.3	Quartile 2 2.3<NLR $\leq$ 3.2	Quartile 3 3.2<NLR $\leq$ 4.4	Quartile 4 3NLR $\geq$4.4	P value*
Basophils, median	113	0.02 (0-0.2)	0.02 (0-0.19)	0.03 (0-0.11)	0.02 (0-0.6)	0.82
Eosinophils, median	113	0.01 (0-0.58)	0.08 (0-0.35)	0.07 (0-0.32)	0.09 (0-2)	0.63
Lymphocytes, median	114	2 (0.76-4.74)	1.51 (0.83-4.69)	1.36 (0.74-1.93)	0.82 (0.36-16.7)	0
Monocytes, median	114	0.44 (0.14-1.31)	0.58 (0.2-1.76)	0.53 (0.2-1.22)	0.65 (0.15-6.6)	0.21
Neutrophiles, median	114	3 (1.24-8.91)	4.23 (1.9-12.79)	4.89 (2.68-8.43)	5.73 (3.12-74)	0
Platelets, median	114	213 (56-442)	240 (160-445)	266.5 (92-517)	261 (118-636)	0.32

Immunohistochemistry

1)   Tissue from tumor specimens was obtained biopsying the metastasis for 15 patients with mBC and then were embedded in paraffin and fixed in formalin (FFPE) for immunohistochemical analysis.

2)   CD3$^+$/CD8$^+$ and CD3$^+$/CD4$^+$ T cells were evaluated on H&E-stained sections following the recommendations of the International TILs working group [23]. CD3$^+$/CD8$^+$ and CD3$^+$/CD4$^+$ T cells were predominantly located within the stromal tissue of the tumour and therefore assessed in this compartment. The mononuclear inflammatory infiltrate was assessed in predefined categories, no TILs (0%), 1–10%, 11–25%, 26–50% and >51%, by two pathologists blinded to any single case. Regions with non-invasive carcinoma, normal breast epithelium or necrosis were excluded from the evaluation. Standard immunohistochemistry was performed on FFPE for HER-2, Oestrogen Receptor (ER), Progesteron Receptor (PgR), Ki67 and CD31 staining using standard protocols as described elsewhere [107–109].

Flow cytometry analysis

In mBC patients, whole blood was taken before and after treatment to analyse circulating cells and their changes under therapy. Flow cytometry analysis was performed using the dual-laser flow cytometers Becton Dickinson (BD) FACSCanto™ and BD FACSCanto II™, with BD™ Cytometer Setup and Tracking (CS&T) control in order to make the signals reproducible and comparable regardless of the variation in environmental conditions. Acquisition of at least 1.5 x 10^6 events was assessed by BDFACSC Diva software. The lymphocytes subpopulations (B, NK, T with CD4 and CD8 subpopulation) were analysed by BD Multitest 6-Color TBNK kit (Becton Dickinson™) containing FITC-labelled CD3 (SK7clone), CD16 (B73.1 clone) and CD56 (NCAM 16.2 clone) conjugated with PE, CD45 conjugated with PerCP-Cy5.5 (2D1 clone), CD4 conjugated with the fl6-Color TBN PE-Cy7 (SK3 clone), CD19 APC (clone SJ2SC1) and CD8 conjugated with APC-Cy7 (SK1 clone). Leucocytes were identified by CD45 expression and SSC/FCS morphological

parameters. T lymphocytes were sorted by CD3 expression and then split into CD4 and CD8 populations. CD3 negative cells were split into B lymphocyte (expressing CD19) and NK cells (CD16 and CD56 positive). Subpopulations absolute count was done by the "trucount tube" (BD™) containing a known number of beads. T-reg cell (CD4 positive, bright CD25 positive and CD127 negative) were sorted using single Becton Dickinson monoclonal antibodies: CD3 conjugated with the flcell (CD4 p FITC (SK7 clone), CD25 conjugated with PE (2A3 clone), CD4 (clone SK3) and CD127 conjugated with V450 (HIL-7R-M21 clone), CD45 conjugated with V500 (clone HI30). Circulating Endothelial Cells (CEC) are an uncommon finding in peripheral blood and are characterized by CD45 negativity and CD31 and CD 146 positivity. CEC sorting was performed using a three colours panel: CD31 conjugated with the flan uncommon FITC (WM59 clone), CD146 conjugated with PE (P1H12 clone), CD45 conjugated PerCP-Cy 5.5 (2D1 clone).

Statistical analysis

The MREC Study:

The gene expression data used in this study derived from 27 breast carcinomas (pre- and post-treatment) receiving 14 days pre-operative everolimus [8]. Microarray data were processed starting from the authors' raw data, and processed as described [8]. Briefly, bioinformatics analyses were performed using R [110], version 3.4.2 and BioConductor [111], release 3.6. Class comparison analysis was performed using the Bioconductor package. The probes from Illumina profile expression data were normalized using quantile normalization within the beadarray package [112] and batch processing effects were corrected using the combat tool [113]. Pairwise Significance Analysis of Microarrays (SAM) method [114], implemented with siggenes package, was used to identify the differentially expressed probes and to predict false discovery rate (FDR). The data on the reduction in the percentage of Ki67 positive cells after treatment was used to separate responders (R) from non responders (NR). The web-based pathway analysis tool IPA (Ingenuity Systems, www.ingenuity.com) was used to identify the most significant canonical signalling pathways to the

input dataset of differentially expressed genes (p<0.05). Based on this gene array we performed 4 comparisons: 1) responders (14 patients) vs. non-responders (9 patients) pre-treatment; 2) responders (13 patients) vs. non-responders (8 patients) post-treatment; pre-treatment vs. post-treatment in responders (13 matched patients); pre-treatment vs. post-treatment in everolimus non-responders (8 matched patients).

The mTOR Study:

Circulating immune cells, the $CD3^+/CD8^+$, $CD3^+/CD4^+$ and CD31 expression were analysed as continuous variables. Non-parametric statistical methods (Mann–Whitney test for unpaired data, Wilcoxon's matched-pairs signed-rank test for paired data, Spearman Rho for simple correlation analysis) were used in the primary analyses of the data. All tests were two-sided; $P<0.05$ was considered as statistically significant. Statistical analysis was performed on an IBM-compatible personal computer using Statistica software (Statsoft, Tulsa, OK, USA) for Windows (Microsoft, Redmond, WA, USA) software.

The BALLET Study:

Neutrophil-to-lymphocyte ratio (NLR) was calculated by dividing the absolute neutrophil count by the absolute lymphocyte count after extrapolation from BALLET database. Pre-treatment percentage of neutrophils and NLR were considered. The value of NLR that best discriminated between good and poor outcome, which is the most significant p value according to the long-rank test, was determined by testing all possible cut-offs. The Kaplan-Meier method was used to measure survival, and differences in survival time between patient subgroups were analysed using the Long-rank test. Statistical software SPSS (15.0 version; SPSS Inc., Chicago, IL, USA) was used for all the analyses. Statistical significance was established at the $p < 0.05$ level, and all analyses were two-sided.

Results

Immune-related pathways differentially represented in tumors according to response to everolimus in neoadjuvant setting

1)   To investigate whether the immune profile could be related to response to everolimus treatment, we analysed the gene ontology profile of early BC before and after 14 days pre-operative treatment with everolimus and separated patients into two groups according to response to therapy as illustrated in a previously published work[115]. Differentially expressed genes ($p<0.05$) were analysed for enrichment in canonical signalling pathways by the ingenuity test. Among the 2063 genes found differentially expressed between everolimus "responders" and "non-responders" before treatment, several pathways were found to be associated with the immune system, as top scoring ($p<0.001$) (**Figure 4.1 a**), with the majority of innate and adaptive immunity related genes up-modulated in everolimus-responsive compared to everolimus-unresponsive tumours.

2)   After treatment, the majority of pathways found differentially expressed in responders compared to non-responders were those typically represented in epithelial cells and associated with response to everolimus, such as PI3K, actin cytoskeleton and Extracellular signal-regulated kinases (ERK), with the majority of genes down-modulated in responsive tumors **(Figure 4.1 b)**. The only immune-related pathway that remains significantly positively enriched in responsive tumors is the one related to antigen presentation.

a

b
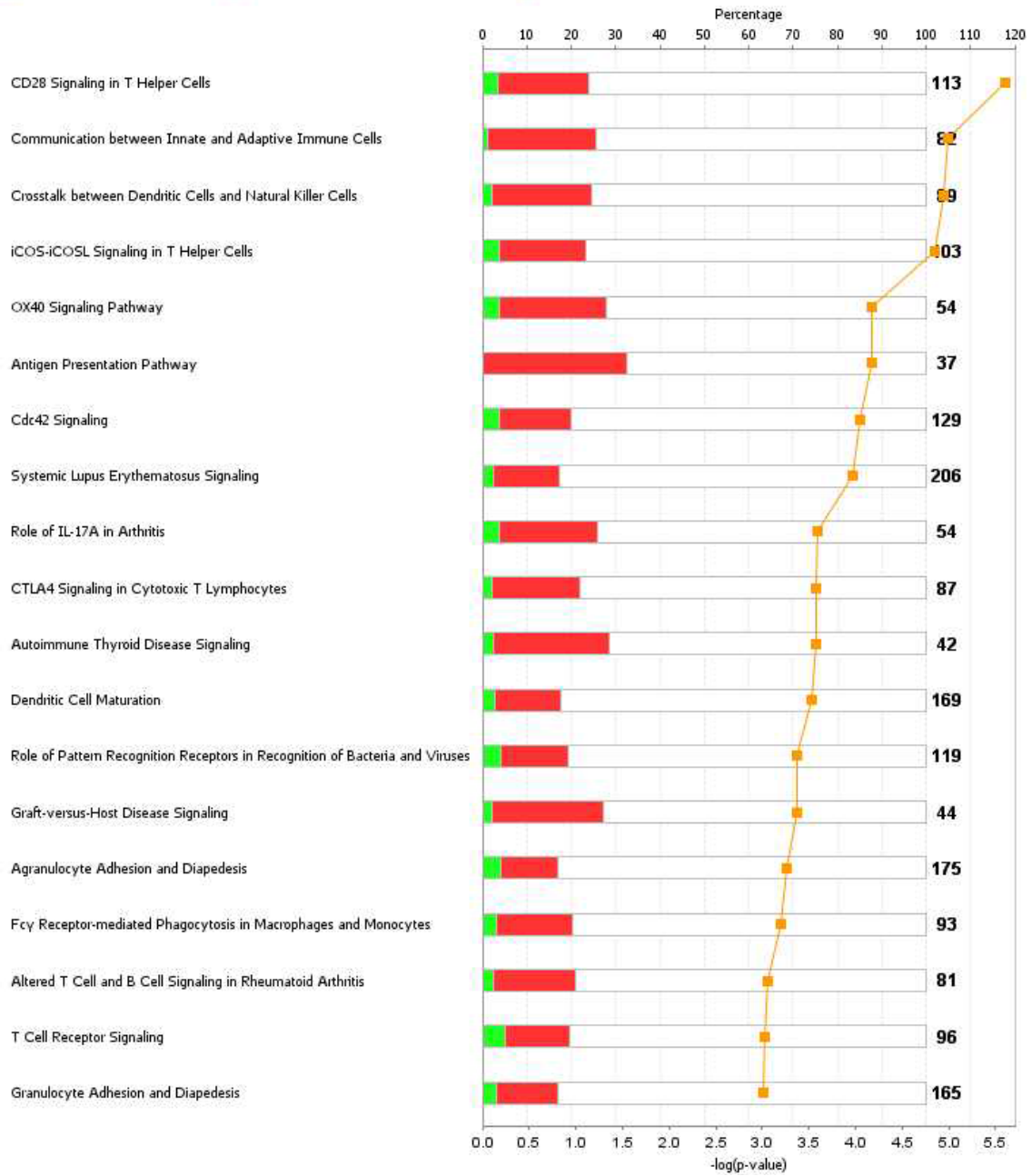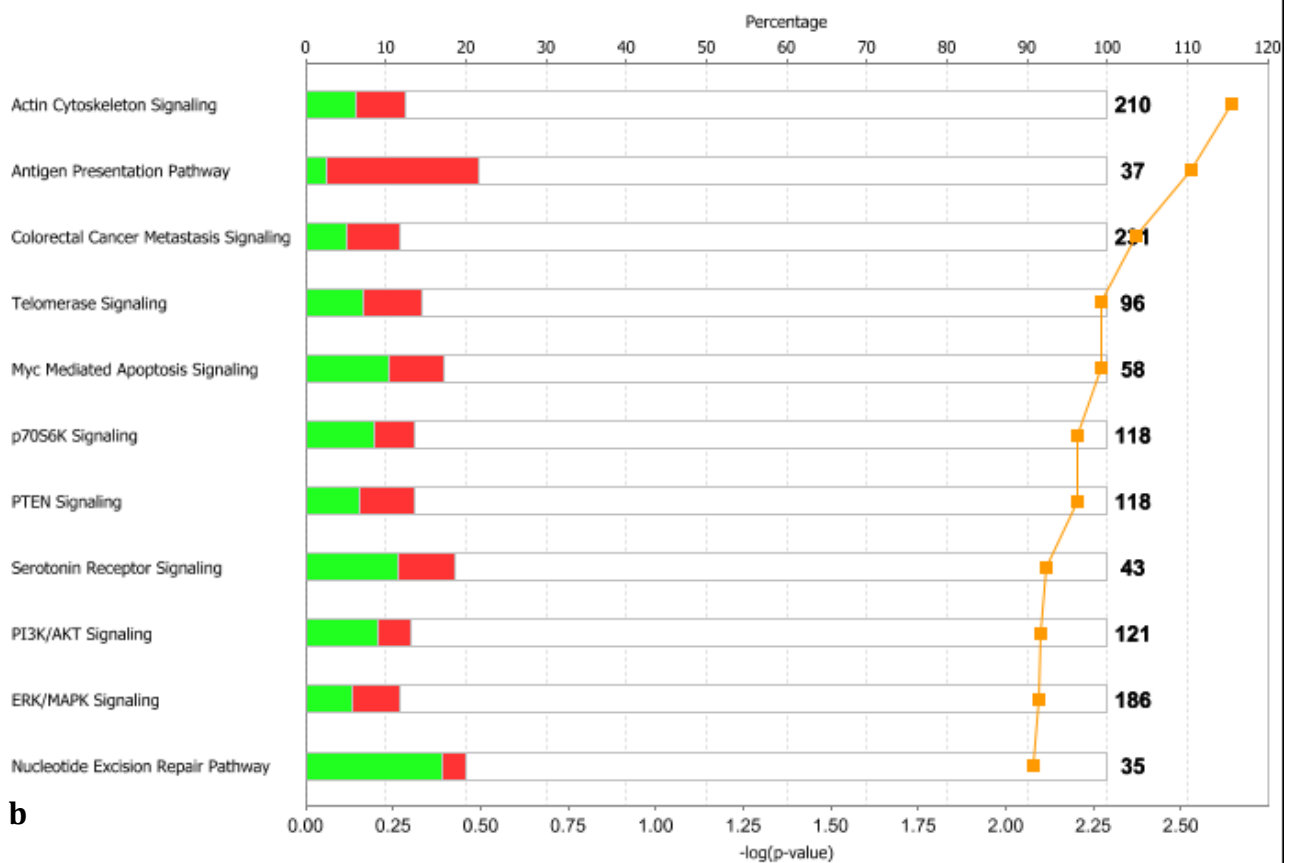
Figure 4.1. Immune-related gene expression analysis. Gene classification accordingly to canonical signalling pathways using Ingenuity Pathway Analysis (IPA) in the neoadjuvant sample population. Green bars denote the percentage of downregulated and red bars the percentage of upregulated differentially expressed genes in responsive compared to non-responsive tumors out of the total number of genes present in the IPA database (shown in black to farthest right) within each pathway before treatment (**a**). Green bars denote the percentage of downregulated and red bars the percentage of upregulated differentially expressed genes in responsive compared to non-responsive tumors out of the total number of genes present in the IPA database (shown in black to farthest right) within each pathway after treatment (**b**).

Circulating cells in patients according to response to everolimus in the metastatic setting

1)   Based on the association between expression of immune–related genes in tumors responsive to short term everolimus in neo-adjuvant setting, we investigated whether the amount of circulating immune cells could predict response to 10 mg everolimus administered alone in a cohort of 15 patients with mBC. While no difference in the number of $CD45^+$ total lymphocytes at baseline or during treatment was found between responders and non-responders, the levels of T-lymphocyte $CD3^+$ were higher in responders versus non-responders at both baseline (p=0.0343) and during treatment (p=0.0233). Likewise, the levels of T-lymphocytes $CD3^+/CD8^+$ and $CD3^+/CD4^+$ were higher in responders compared to non-responders at baseline (p=0.0172, p=0.0005, respectively) and during treatment (p=0.0102, p=0.0032 respectively). On the contrary there was no statistically significant difference in the number of B-lymphocytes $CD19^+$ between responders and non-responders at both basal and intermediate stages of treatment. T-regulatory lymphocytes $CD4^+/CD25^+/CD127^-$ levels were lower in responders compared to non-responders at baseline (p=0.0754) as well as during treatment (p=0.0588), in the absence of statistical significance. $CD16^+/CD56^+$ NK cells were not different at baseline, but after treatment responsive tumours have slightly lower circulating NK cells compared to non-responders (p=0.0411). Interestingly, the higher number of circulating CD4+ and CD8+ T cells was associated with higher pre-treatment infiltration of these cells in the tumor microenvironment of responsive tumors compared to non-responders (Table **4.3**). Based on the demonstrated performance of CECs, as measured by flow cytometry in the blood, as non-invasive biomarker mirroring the occurrence of angiogenesis in the tumor[116], CECs ($CD45^-/CD31^+/CD146^+$) were analysed in the circulation of patients before and 21 days after everolimus treatment in the metastatic setting (Table 4.3, Figure 4.2). As shown in **Table 4.3 and Supplementary Figure 4.1**, no significant differences were apparent between responders and non-responders before treatment. However, after treatment with everolimus a significant reduction in CECs number was observed only in responders, resulting in a highly significant amount between responders and non-responders (p=0.0002). Notably, responders showed a higher

tumor vascularisation at baseline illustrated as higher CD31+ vasculature, compared to non-

responders (**Figure 4.2**).

| | BASALE | | | INTERMEDIO | | |
	median (95% CI)			median (95% CI)		
	Responders	Non Responders	p value	Responders	Non Responders	p value
CD3+ T-lymph.	1525 (845-1869)	961 (639-1432)	0,0343	1332.5 (589-2112)	782 (652-1304)	0,0233
CD3+ CD4+ T-helper lymph.	944 (753-1350)	616 (388-913)	0,0005	1115.5 (278-1477)	490.5 (236-691)	0,0032
CD3+ CD8+ T-lymph.	644 (335-845)	369.5 (117-682)	0,0172	488.5 (265-988)	315 (122-560)	0,0102
CD45+ total lymph.	1670 (1050-2820)	1645 (850-2360)	0,5967	1509 (830-3070)	1390.5 (930-2010)	0,2568
CD19+ B-lymph.	118.5 (80-637)	162.5 (56-443)	0,9397	187.5 (112-571)	125 (85-351)	0,1038
CD16+ CD56+ NK cells	249.5 (116-418)	322.5 (62-573)	0,4057	158 (123-656)	297.5 (114-570)	0,0411
CD4+ CD25+ CD127- T-reg lymph.	38.75 (27-90.38)	59 (22-151)	0,0754	37.75 (23-52.5)	69.95 (19.46-112)	0,0588
WBC	6.01 (3.6-6.98)	5.38 (3.91-7.55)	0,8798	4.1 (2.44-7.51)	4.29 (3.1-5.18)	0,4055
CEC (CD45- CD31+ CD146+)	13 (1-50)	7 (0-36)	0,344	2.5 (1-5)	10 (5-16)	0,0002

Table 4.3. Circulating immune markers and endothelial cells expression levels and p-values.
Quantification of immune markers and CECs at basal or intermediate levels of everolimus therapy
from the blood of everolimus responsive vs. everolimus nonresponsive patients. The p-values were
calculated by unpaired t-test.
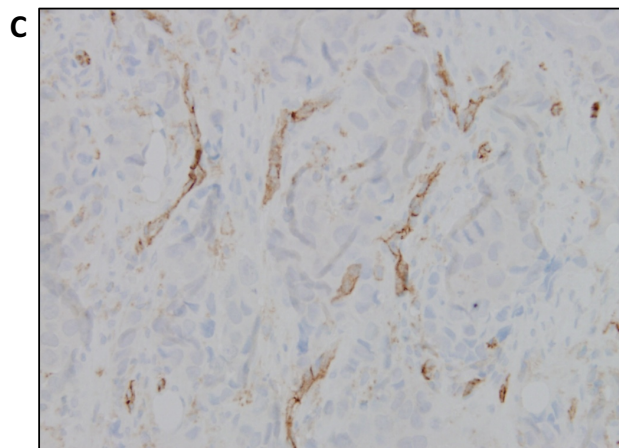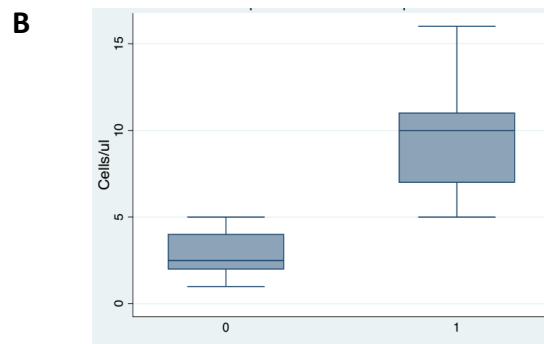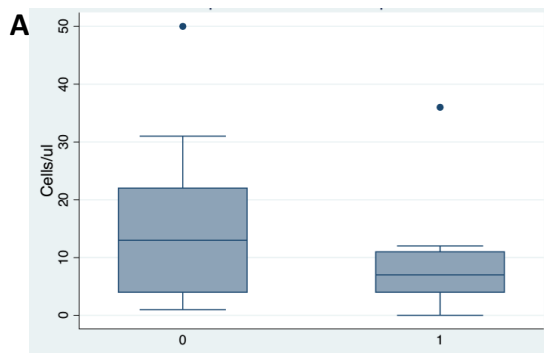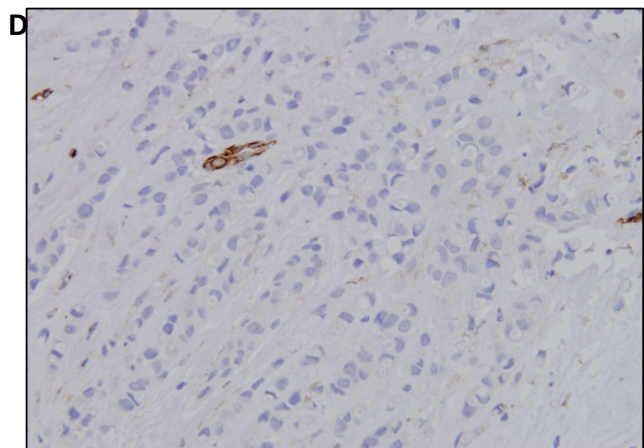
RESPONDERS   NON RESPONDERS

Figure 4.2. **Immunohistochemistry of Circulating Endothelial Cells (CECs) in breast cancer responders against non-responder patients from the Cremona's study.** Barplot **A** shows the expressions levels of CEC before (0) and after everolimus therapy (1) in everolimus responders (n=7). Barplot **B** shows the expressions levels of CEC before (0) and after everolimus therapy (1) in everolimus non-responders (n=8). IHC photomicrographs indicating angiogenesis marker CD31 in breast cancer everolimus-responsive (**C**) and everolimus-nonresponsive (**D**) from samples of patients from Cremona's Hospital.

IHC on primary and metastatic lesions in everolimus responsive vs. non-responsive tumours

1)   IHC was conducted to confirm the presence of immune-cells in HR+ BC in both primary and metastatic lesions. As shown in **Figure 4.3**, staining for $CD3^+/CD8^+$ or $CD3^+/CD4^+$ T cells, T cells were higher in responders vs. non-responders either in primitive or metastatic lesions.
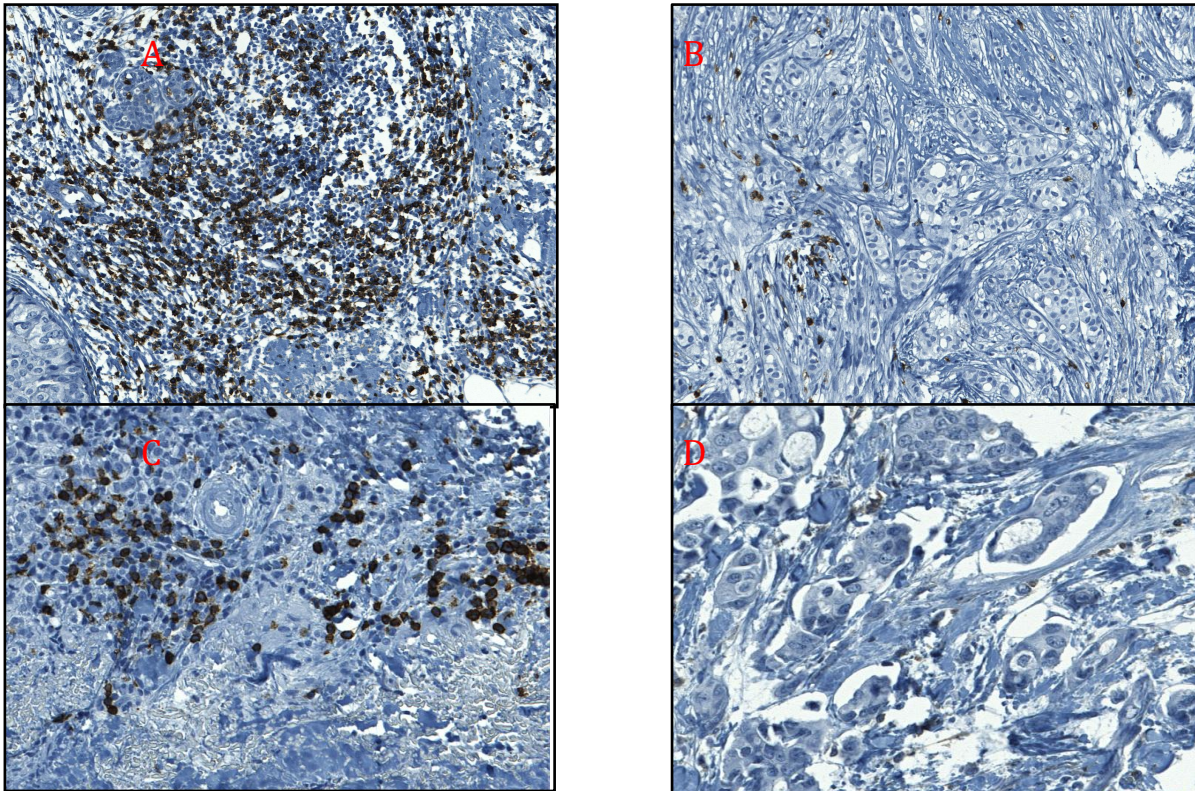
) of Tum HC
, CD3/CI
nsive (B

Prognostic significance of the N/L Ratio in BALLET Study

1)   A post-hoc analysis was conducted from the neutrophils and lymphocytes values derived from the BALLET study in order to investigate a correlation with survivals of patients. NLRs were calculated based on four cut-off values and patients discriminated based on four quartiles accordingly to Santoni *et al* [117]. As shown in **Table 4.2** the following NLR-based quartiles were generated: quartile 1 (NLR ≤ 2.3), quartile 2 (2.3 < NLR ≤ 3.2), quartile 3 (3.2 < NLR ≤ 4.4), quartile 4 (NLR > 4.4). The differences in survivals were analyzed through Kaplan Meier curves comparing NLR ≤2.3 vs. NLR > 2.3; NLR ≤ 3.2 vs. NLR> 3.2; and NLR ≤ 4.4 vs. NLR> 4.4 (p=0.19, p=0.12 and p=0.01, respectively; **Figure 4.4**). From each comparison it was possible to evince that lower NLR corresponds to better survival outcomes in mBC treated with everolimus. However, such difference was statistically significant only from the comparison between the first quartile vs. the last quartile (p=0.01; **Figure 4.4**). There were no significant correlations between levels of eosines and patients' survival outcomes (Supplementary Figure 4.2).
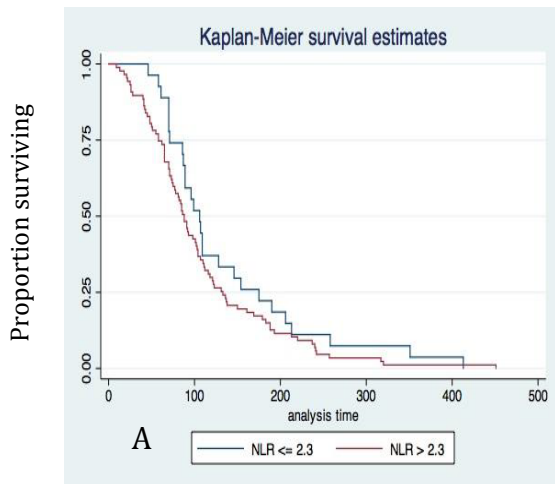
Log rank test
p=0.19
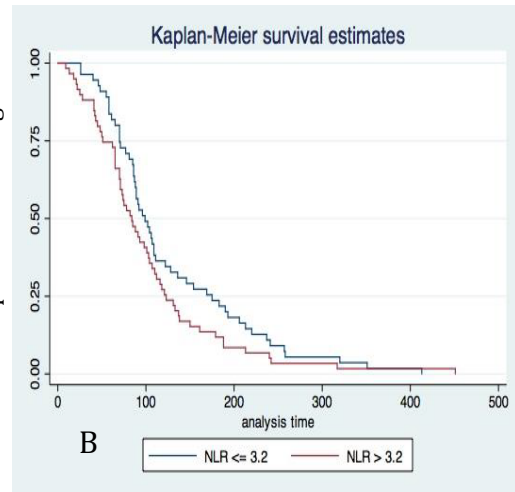
Log rank test
p=0.12
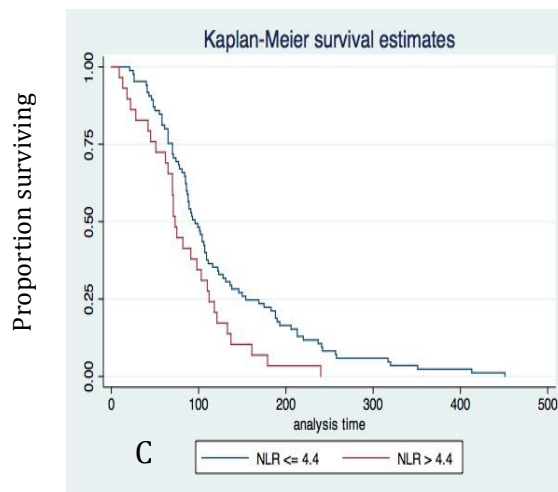
Log rank test p = 0.01

Figure 4.4. NLR significantly correlated with improved survival of patients from in the Ballet Study. In **A** the category of patients with NLR ≤2.3 was compared to the category of patients with the NLR >2.3 (p=0.19); in **B** the category of patients with NLR ≤3.2 was compared to the category of patients with the NLR >3.2 (p=0.12); in **C** the category of patients with NLR ≤4.4 was compared to the category of patients with the NLR >4.4 (p=0.01).
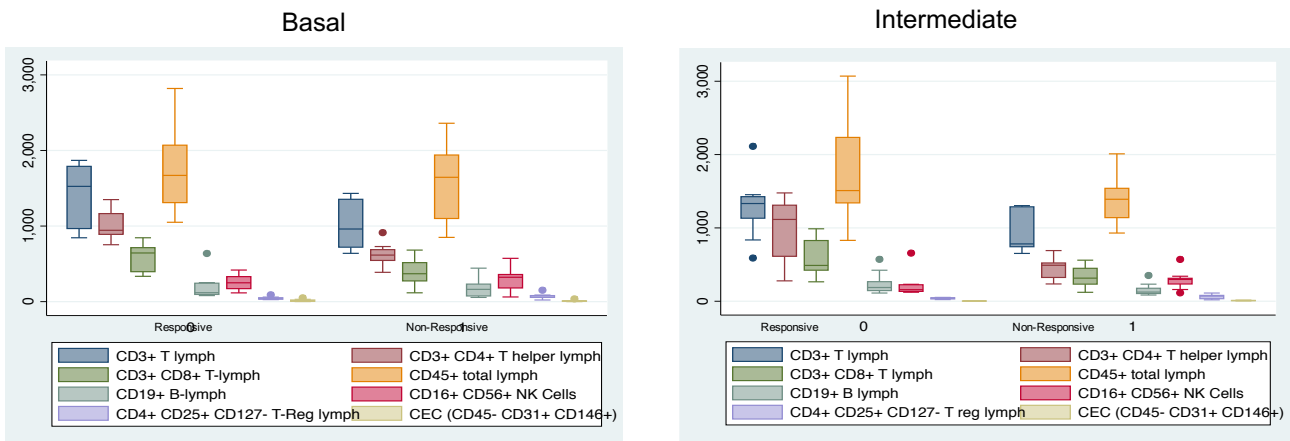
Supplementary Figure 4.1. Expressions of Lymphocytic subpopulations from the BALLET study.

Discussions

The combination of everolimus with exemestane has been FDA approved for the treatment of postmenopausal hormone-receptor positive MBC in second line setting after the positive results from the BOLERO 2 study showing improved PFS vs. a control for everolimus (11 mo vs. 4.1 mo; p<0.0001)[118]. However, there is a lack of biomarkers that could predict the efficiency of everolimus treatment. The mTOR/PI3K pathway through vascular endothelial growth factor (VEFG) is related to both angiogenesis and immune suppression in tumor microenvironment[119]. Conversely, it is well known that mTOR inhibitor everolimus has a strong immune-suppression activity in normal tissues and that it is therefore used after transplantations to prevent organs to be rejected[120,121]. Therefore it appears that everolimus acts in opposite ways in suppressing the immune system between normal tissues and the tumor microenvironments.

More reliable biomarkers of everolimus for treatment of mBC could be intrinsic in such pathways, which could be crucial in predicting response to this therapy and to help thereby guiding clinicians in the decision process of giving such type of drug. In this investigation we have identified immune system biomarkers correlated with improved response to everolimus in BC, in various sample groups and by using different approaches.

Firstly, enrichment of immune pathways from the gene expression of 2063 genes from the MREC study constituted of 27 everolimus-treated BC, which had been previously studied by another group[115], through functional annotation bioinformatics microarray analysis (DAVID) platform, here was re-investigated to find immune-related enriched pathways from the microarray data utilizing a different software, namely the Ingenuity Pathway Analysis (IPA). Our re-analysis confirmed that before treatment in everolimus responders, pathways associated with immune response were differentially expressed vs. non-responders. Moreover, after treatment in everolimus responders, IPA showed that mTOR-PI3K pathway-related genes were down-modulated in patients who

responded to the mTOR inhibitor everolimus. Intriguingly, after everolimus treatment enriched immune pathways in responders vs. non-responders did not remain enriched, besides those related to antigen presentation.

The IPA of the gene array showed that many immune-related pathways were significantly up-regulated in everolimus responders compared to non-responders before treatment. On the other hand, after treatment only those genes belonging to the antigen presentation family were found up-regulated in responders during treatment (HLA-A, HLA-B, CIITA, HLA-F, HLA-DQA2, PSMB6, TAP2, TAPBP). Interestingly, the higher expression of immune-related genes in basal samples could be indicative of a role that the immune system has in modulating everolimus' response.

Secondly, neutrophils to lymphocytes ratios (NLR)s in blood samples derived from the BALLET study, made of 2131 mBC patients treated with everolimus, proved that lower NLRs corresponded with better survival outcomes. It remarkable to observed that only the difference between first and last quartiles was such as to reach statistical significance (p=0.01) and be hence considered of value. Therefore, NLR biomarker could have a clinical application when considering such differences between NLRs belonging to extreme quartiles, in order to determine everolimus responders at priori. NLRs have long been observed to be correlated also in another typologies of solid tumors, however in the opposite direction. In other words, NLRs has been observed to be lower in renal cancer (RC) patients who were bad-responders[117]. In fact, it was demonstrated in literature that high levels of NLR proved to be a good predictor of poor clinical outcome in everolimus treated patients with RC [117]. Santoni *et al* was the first group that investigated the correlation between NLR and solid tumors. It must be considered the fact that such study was conducted in the context of the immune system's microenvironment of RC. The divergence with our data could possibly be due to a different immune microenvironment in RC compared to BC. Moreover, our investigation on NLR was conducted over a larger population size. The results that

were presented here from the BALLET study were the first one of this kind conducted in the context in a large population size of mBC patients treated with everolimus.

Thirdly, some of the most promising T-lymphocyte subpopulations that could be interesting biomarkers for response to everolimus observed in the previously mentioned two groups of patients were finally investigated in a small cohort of 15 BC patients treated with everolimus at Cremona Hospital. FACs showed that both at baseline and during everolimus treatment T-lymphocytes CD3+ and/or CD8+, T-helpers CD3+/CD4+ were significantly higher in everolimus responders vs. non-responders. On the other hand, only after everolimus treatment, NK cells were significantly lower in everolimus responders compared to non-responders. Tregs CD4+/CD25+/CD127- difference between responders and non-responders was on the border of significance after treatment and non-significantly different before treatment everolimus responders vs. non-responders. To confirm the previous observation on NLR on the large group, we investigated the prognostic value of this ratio also in this smaller group of patients (Supplementary Table 4.1). However, the data from this study was just indicative that low NLR could be an indicator of a better survival in BC patients treated with everolimus. In fact NLR in this relatively smaller sample sized did not reach statistical significance like it did for the BALLET study, when considering difference between first and last quartiles.

The expression of Circulating Endothelial Cells (CECs) can be an indicator of angiogenesis. CECs have been proven to significantly correlate with plasma levels of VCAM-1 and VEGF[116], many of whose downstream pathways are also inhibited by the mTOR inhibitor everolimus.
Our, immunohistochemical analysis in the 15 mBC patients re-confirmed that T-lymohocyte subpopulations (CD3+/CD8+ and CD3+/CD4+) were expressed at higher levels in everolimus responders and that CECs were expressed at lower levels in everolimus responders vs. non-responders. Such data was confirmed using both primary and metastatic lesions.

The immune-suppressive activity of everolimus in the context of solid tumors has been shown to be beneficial in both kidney [120] and heart transplantations [121] and became part of clinical practice. Sabbatini *et al*. have previously shown in kidney transplant recipients, receiving daily amounts of everolimus, that the mTOR inhibition was able to suppress the immune system avoiding therefore organ rejection. In fact everolimus decreased neutrophils and CD8+ T-cells, the activity of CD9+ and CD4+ cells, and increased Treg Foxp3[120]. Similarly Vitiello *et al.* proved in cardiac transplant recipients that everolimus reduced at the same time VEGF and IL8, while preserved the release of the anti-inflammatory cytokine interleukine-1 receptor(IL-1RA)[121]. Likewise Girdlestone *et al.* proved that mTOR-inhibited mesenchymal stromal cells become 5 times more potent at inhibiting T-lymphocyte proliferation [122].

As to correlation between the immune system and cancer, this is the first study to the best of our knowledge investigating it in the BC context. There are other studies proving the opposite in other solid tumours, showing that everolimus down-modulates the immune system worth to mention. In fact, Beziaud et al. treating renal cell carcinoma (mRCC) with everolimus similarly to Sabbatini *et al.*, Vitiello et al. and Girdlestone et al. showed a down-modulation of the immune system. Sabbatini *et al.* proved that there was an expansion of Foxp3 in patients receiving long-term everolimus treatment. Furthermore, they proved that the efficacy of the everolimus therapy was dependent on the addition of mycophenolic acid (MFA) that could act in the down-modulation of Tregs [120]. When cells were treated with everolimus, Tregs increased and with them, there was a consequent decrease of T-lymphocytes. In fact after treatment with everolimus CD8+ CD45RO+ /Tregs ratio significantly decreased, suggesting that Tregs negatively influences CD8+ T cells upon everolimus treatment. When the authors tested in mice the contemporary mTOR inhibition with temsirolimus and induced Tregs depletion, they showed that the survival of mice significantly increased compared to control mice. Therefore a valid alternative could be that of treating patients with a combination of rapamycinand Tregs inhibitors[123]. However, this work has some limitations.

First of all, the retrospective nature of the three studies is a limitation. In fact it restrains the measurement of additional statistical values and the possible choice of more variables, such as time-to-drug exposure, at the decision of the investigators. Secondly, the lower number of patients in the local study (15 patients) and the neoadjuvant study (27 patients) is another limitation. In fact, future randomized clinical trials confirming the role of NLR in predicting the outcomes of the patients to everolimus treatment are warranted. Furthermore, larger clinical trials confirming the immune pathways and immune molecules correlated with patients who respond better to everolimus are needed and could pave the way to the development of a new tool capable of predicting everolimus response in HR+/HER2- BC based on a panel of immune-related biomarkers.

In conclusion, this study showed that lymphocytes subpopulations (T-lymphocytes, T-helpers, T-regs and NKs), NLR and CECs could be an interesting marker predictive of the functioning of everolimus in BC patients. Future clinical trials to confirm such hypothesis are warranted.

Supplementary Table 4.1. Neutrophilic and lymphocytic levels in the HR+/HER2- population from the hospital of Cremona.

Cox Regression Analysis		95% CI		
Variable	HR	Min	Max	p-value
Delta NLR* 1 month	2.2	0.52	9.3	0.282
Delta NLR 3 months	3.26	0.54	19.54	0.18
Delta Lymphocytes 1 month	0.74	0.18	3.07	0.676
Delta Lymphocytes 3 months	0.75	0.21	2.73	0.66
Delta Neutrophils 1 month	2.35	0.37	14.83	0.36
Delta Neutrophils 3 months	0.19	0.03	1.27	0.08
Delta Leucocytes 1 month	1.94	0.27	13.99	0.51
Delta Leucocytes 3 months	0.25	0.03	2.05	0.19

Kaplan-Meier survival estimates

Log rank test p = 0.06
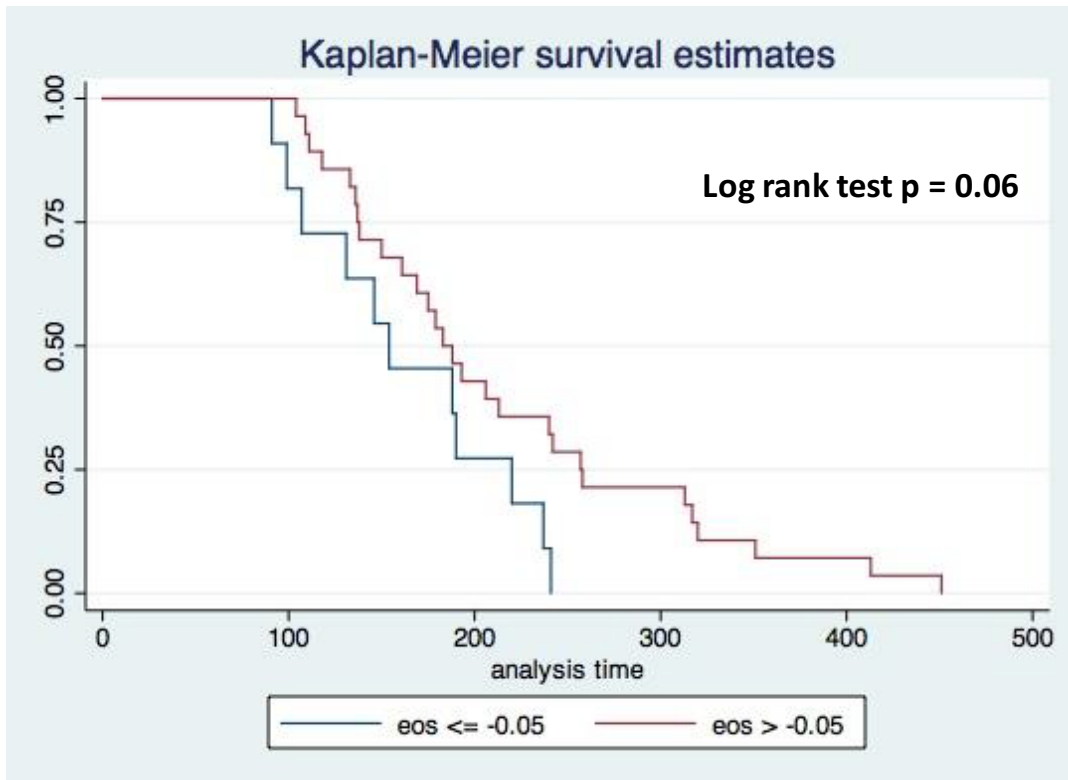
eos <= -0.05   eos > -0.05

Supplementary Figure 4.2. Eosin levels correlation with survival of Patients from the BALLET study. The survival rates of patients from two groups, one with lower levels of eosines (eos≤ -0.05) and one with survival rates with higher levels of eosines (eos≥ -0.05), were compared via Kaplan-Meier curves. The data lacked statistical significance by means of Two-Tailed, Student's T-Test (p=0.06).

Conclusive remarks and future perspectives

In chapter 1, the aim was to give an overview of the role of the immune system in solid tumors by the use of an *in silico* approach. I conducted a meta-analysis to interrogate cBioPortal's multiple large-scale cancer genomic datasets with an immune-related gene list made of 730 genes (PanCancer IO 360[TM] panel).

Even with the intrinsic limitations related to the retrospective nature of the analysis, my data confirms that the presence of genetic alterations affecting genes involved with immune evasion and tolerance is associated with a worse prognosis in patients with solid tumors. It is well known that consideration of PD-L1 alone is not always robust enough (due to known limitations of the immunohistochemistry procedure) in patient selection via prediction of response to PD-L1 inhibitors[124]. The gene-panel, besides giving prognostic information, could help clinicians in selecting patients who would benefit from checkpoint inhibitors. Thus, it could be useful to have a toolkit including other biomarkers besides PD-L1; the immune-related gene signature could be a solution to this problem. The IO360[TM] signature incorporates many genes implicated in the immune system; it could therefore assist oncologists to make well-informed decisions and improve outcomes when matching checkpoint inhibitors with patients.

In chapter 2 the aim of my study was to investigate the baseline expression of TILs and PD-L1 in MPM and to correlate them with OS. Our results show that high expression of peritumoral TILs is associated with improved survival, according to the literature [24–28]. In conclusion, the data here reported suggests that only the high expression of TILs in the peritumoral regions of samples from MPM patients with epithelioid histotype correlated with a significantly improved survival. The same could not be evinced for TILs in the intraepithelial, stromal and perivasal regions, whose expressions did not correlate with survival. Moreover, neither the expression of PD-L1 was statistically correlated with survival outcomes in the patients' cohort.

These data support the potential use of immune checkpoint inhibitors targeting the PD-1/PD-L1 pathway in malignant pleural mesothelioma (MPM) patients.

In chapter 3 the aim of our study was to investigate the baseline expression of TILs and 770 genes involved with the immune system in PDAC patients and to correlate them with OS.

Immune cells within the cancer infiltrate may have a role in fighting cancer growth via antigen restricted tumouricidal responses or they may promote tumour progression by suppressing the immune system [125].

The key findings from this study, that longer-surviving PDAC patients had higher levels of intratumoral TILs and overexpressed five immune markers (*TLR7*, *TNF*, *C1QA*, *FOXP3*, *CD37*), could have two main uses. Firstly, TIL levels and marker gene panel expression could be used for clinical outcome prediction, stratification and treatment design for PDAC patients. A previous study showed that a signature comprising another 15 genes was an independent prognostic factor in two cohorts of PDAC patients. In contrast to our results, higher expression of these 15 genes was associated with poor OS [101]. Similarly, Sergeant et al identified high co-expression of *TGF-β1* and a panel of cell motility genes as independent predictors of worse clinical outcome [102], while Van den Broek et al discovered that high expression of *ABCB1* and *CXCR4* correlated with worse clinical outcome [103]. Furthermore, decreased levels of *DPEP1* and increased expression of *TPX2* were independently associated with poor survival [104]. Presumably, a wide panel of validated gene signatures would be most useful for outcome prediction, stratification and therapeutic decision-making.

Secondly, our findings could be useful in developing new PDAC treatments, for example in combination with current immunotherapeutic strategies. Expression of the target genes identified here could be induced together with therapies modulating the tumour microenvironment to relieve immunosuppression, and/or approaches to break down the desmoplastic barrier surrounding PDAC

to facilitate target access for infiltrating T cells or therapeutic molecules [125]. Such strategies could be effected in combination with recently reported gene therapy and oncologic vaccination approaches [126–128].

In summary, our data indicate that a gene signature comprising at least *TLR7*, *TNF*, *C1QA*, *FOXP3*, and *CD37* could be useful to improve the prediction of OS in PDAC patients. Together with an assessment of TIL levels, such an immune system gene panel constitutes a potential prognostic tool to permit a risk-based stratification of pancreatic tumour patients into personalized treatment protocols towards improving the current abysmal clinical outcome of these patients.

In chapter 4 the aim of our study was to investigate the correlation between the expression of TILs and NLR with survival of breast cancer patients treated with mTOR inhibitor everolimus and to evince a possible role of the immune system markers and the efficacy of everolimus.

After the encouraging results coming from BOLERO-2 randomized phase 3 clinical trial that treatment with everolimus plus exemestane significantly improved PFS of patients compared to placebo with exemestane in HR+/HER2- BC, everolimus can be very useful. [118]. Among the various oncogenic pathways the mTOR-axis interacts with is the one of angiogenesis: an activation of the mTOR-axis increases the secretion of VEGF [129]. The expression of CECs can be an indicator of angiogenesis. CECs have been proven to significantly correlate with plasma levels of VCAM-1 and VEGF[116], many of whose downstream pathways are also inhibited by the mTOR inhibitor everolimus.

In this chapter firstly we first showed through an IPA of a gene array of 2063 genes of the MREC study in 27 everolimus neoadjuvantly treated HR+/HER2- BCs that many immune-related pathways were significantly expressed at higher levels in everolimus responders compared to everolimus non-responders, such as TNF, IFNG, and TCR. Antigen presenting genes (HLA-A, HLA-B, CIITA, HLA-F, HLA-DQA2, PSMB6, TAP2, TAPBP) were expressed at higher levels in everolimus responders during treatment.

In the second part of this chapter we presented a re-analysis of the BALLET study, phase IIIb, open-label, single-arm, multicenter expanded analysis study, made of 2131 patients, to prove that lower NLR levels significantly correlated with higher patients' survivals . This proves that low NLRs, an indicator of a relatively active immune system with high lymphocytes and low neutrophiles, could be a good biomarker to predict survival of HR+/HER2- patients treated with everolimus. On the other hand, literature from RCs treated with everolimus did not prove the same for NLR predictive capacity [117]. This difference could be attributable to the fact that RC and BC have different immune contextures and that the RC study was a much smaller study (n=97) [117]. In fact our study is novel, as it looks NLRs predictive role in everolimus-treated HR+/HER2- BC patients for the first time.

Thirdly, in this conclusive chapter we have investigated some of the most promising immune-markers in 15 HR+/HER2- BC patients treated with everolimus at Hospital of Cremona. Our FACs data showed that T-lymphocytes CD3+ and/or CD8+, T-helpers CD3+/CD4+ were significantly higher in everolimus responders vs. non-responders before and after treatment. IHC further proved that higher TILs correlated with improved survival in primary and metastatic lesions. Just after treatment, NK cells were slightly lower in everolimus responders compared to non-responders. Statistical significance was not reached for Tregs levels.

There was a similar trend to the BALLET study of the correlation between lower NLR levels and improved survivals and the NLR levels of the smaller study of the Hospital of Cremona's study; however the correlation did not reach statistical significance in the smaller study as it actually did in the larger one so the same conclusions could not be drawn for the smaller study as for the study made of 2131 patients.

In conclusion, to the best of our knowledge this is the first study investigating the correlation between the immune system and response to everolimus treatment in the context of breast cancer. The data presented here shows that an active immune system could be a potentially good predictor

of response to everolimus in breast cancer patients and clinically useful tools to best predict best everolimus responders may be designed and refined based on this data and on future clinical trials investigating the potential of that we present here.

Everolimus could be used together with other immune therapies in patients with an overall active immune system to even further improve immune response.

Among the latest, currently FDA-approved, immunotherapies there is Avelumab (Bavencio, MSB0010718C), which is an IgG1 fully human anti-PD-L1 antibody. Avelumab inhibits PD-L1/PD1 interaction in order to disinhibit T-cells and removes the suppression of T-cell activity, which response can be assessed by evaluating the release of interferon-γ[130]. Moreover, also the interaction of PD-L1 with a second inhibitory receptor, B7.1, is inhibited by avelumab; this receptor might be expressed on T cells and APCs [131]. By blocking the interaction between B7.1 and PD1 on T-cells and with PD-L1 on APCs within the lymph nodes or tumour microenvironment, avelumab might reactivate T cells and the production of cytokines [132].

Furthermore, because of its native IgG1 crystalliable fragment (Fc) domain, avelumab conserves the ability to engage natural killer cells with the Fc-γ receptor to induce tumour-targeted antibody-dependent cell-mediated cytotoxicity (ADCC) *in vitro*[133].

This capacity to promote innate immune interactions against tumour cells makes avelumab exclusive among anti-PD-L1 or anti-PD1 antibodies in already approved or ongoing clinical trials.

A randomized phase III clinical trial called JAVELIN Gastric 300 (NCT02625623) has been comparing the use of third line avelumab vs. chemotherapy. This clinical investigation was a randomized, multicenter, open-label, phase III study constituted of 371 patients with advanced GC/GEJC who progressed to two lines of therapy, who were not selected for PD-L1 expression. All the patients received Best Supportive Care (BSC) as background therapy and were grouped according to a geographic region (Asia vs. non-Asia). However, this study did not meet its primary

endpoint of improving OS, with median OS of 4.6 vs 5.0 months (HR= 1.1 [95% confidence interval (CI) 0.9-1.4]; p = 0.81) in avelumab vs chemotherapy arms. Neither the secondary endpoints were met of PFS, with a median PFS of 1.4 months vs 2.7 months (HR= 1.73 (95% CI 1.4-2.2); p>0.99] nor ORR, with an ORR of 2.2% in avelumab (n=4; 95%, CI 0.6-5.4) versus the 4.3% (n=8; 95% CI 1.9-8.3)[134].

Another randomized, phase III clinical trial made of 499 patients with GC/GEJ patients, named JAVELIN Gastric 100 (NCT02625610), has been testing the efficacy of switch-maintenance treatment. This study has been comparing avelumab vs. continuation of capecitabine + oxaliplatin (XELOX) or leucovorin + 5-FU + oxaliplatin (FOLFOX) in patients with advanced GC/GEJC who did not progress to first-line chemotherapy. The aim of this clinical trial is to test whether avelumab is capable of providing durable antitumor activity after tumour shrinkage and immunogenic priming obtained because of first-line chemotherapy, with less tumour toxicity burden because of the additional chemotherapy. The primary endpoint is to demonstrate the superiority of avelumab maintenance therapy compared to continuation of 1L chemotherapy in terms of OS in all randomized or PD-L1 positive tumour-bearing patients. Secondary objectives include the demonstration of the superiority of avelumab vs continuation of 1L chemotherapy in terms of PFS, ORR, safety and tolerability and quality of life[135,136].

As a consequence in future there is a need to have accurate predictive biomarkers that could help clinicians to decide whether patients would best respond or not to checkpoint inhibitors such as avelumab. Since in a large cohort of 2220 lung cancer patients in a phase III, multicenter, open-label study showed that patients respond better to checkpoint inhibitors when they have a high tumour burden, even better than in patients with high PD-L1[137], it might interesting in future to test tumour burden as a predicting biomarker for the efficacy of immunotherapy in GC/GEJC.

During my third year of Ph.D I contributed with my Professor to write a clinical trial, called INvEST (ImmuNE profile in Solid Tumor), to investigate biomarkers of immunotherapy response in solid tumors. This clinical trial is an observational, multicenter, retrospective study for the identification of the prognostic and predictive value of immune profiling (PD-L1, TILs /TReg, Immuno-gene-profile and Tumor Mutation Burden) evaluated on archival tumour samples (from FFPE) of solid tumors (Mozart Program- Dipartimento d'Eccellenza DSM-UniTS)

This clinical trial has been accepted by the Ethical Committee of Cremona and it is under revision of the Ethical Committee of Trieste. As an outcome of this approval, Avelumab has been also tested in breast cancer and biomarkers of efficacy of immunotherapies have been tested in the Cremona Hospital's clinical setting.

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List of publications coming from this work:

1. D'Angelo A, Sobhani N, et al, Tumour Infiltrating Lymphocytes and Immune-Related Genes as Predictors of Outcome in Pancreatic Adenocarcinoma. Plos One. In Press.

2. Roviello G, Conter FU, Mini E, Generali D, Traversini M, Lavacchi D, Nobili S, Sobhani N. Nanoparticle albumin-bound paclitaxel: a big nano for the treatment of gastric cancer. Cancer Chemotherapy Pharmacology 1-9, (2019).

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