Modification of Breast Cancer Milieu With Chemotherapy Plus Dendritic Cell Vaccines: An Approach To Select Best Therapeutic Strategies

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Research article

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Abstract

Background

The addition of Dendritic cell vaccines (DCV) to neoadjuvant chemotherapy (NAC) could induce immune biomarker changes in those patients with residual disease (RD) by transforming tumor microenvironment.

Methods

Core-diagnostic biopsies and surgical specimens from 80 patients (38 in the Vaccinated Group plus NAC (VG) and 42 in the Control Group (CG) treated only with NAC) were selected. We quantify TILs (CD8, CD4 and CD45RO) using Immunohistochemistry (IHC) and the Automated Cellular Imaging System (ACIS III) in the core-diagnostic biopsies and in the surgical specimen, to compare the amount of TILs in each group.

Results

A CD8 rise in TNBC samples was observed after NAC plus DCV, changing from 4.48% in the biopsy to 6.70% in the surgical specimen, not reaching statistically significant differences (p = 0.11). TNBC patients in the CG showed a TILs drop from 2.71% in the biopsy to 0.18% in the surgical specimen (p = 0.5). We also found that a 66.7% (4/6) of TNBC patients from VG registered an increase in TILs after treatment as compared with 20% (1/5) of TNBC patients in the CG (p=0.24). This phenomenon is not observed in the other biologic subtypes.

An association between before NAC CD8 TILs (4% cut-off point) and pathological complete response in VG was found in univariate and multivariate analysis (OR=1.41, IC95% 1.05-1.90; p=0.02, and OR=2.0, IC95% 1.05-3.9; p=0.03, respectively).

Conclusion

Our findings suggest that patients with TNBC especially benefit from the stimulation of the antitumor immune system by using DCV pulsed with tumor antigens.

Trial registration: NCT01431196. Registred 19 May 2016. EudraCT 2009- 017402-36.

Background

Immune infiltration in breast cancer (BC) milieu has driven to the achievement of a better outcome of these patients with standard chemotherapy, but also has enhanced the incorporation of immunotherapy as a therapeutic strategy. Thus, higher levels of tumor infiltrating lymphocytes (TILs) on the core diagnostic biopsy have been related to increased pathological complete responses (pCR) with neoadjuvant chemotherapy (NAC) and longer event-free survival (EFS) and overall survival (OS) in the

more aggressive BC subtypes [1–5]. However, the presence of residual disease (RD) after NAC implies further adjuvant chemotherapy that could be avoided if pCR would have been reached. Additionally, the rise of TILs in RD after NAC has been linked to a better outcome and it could be considered a surrogate marker for long-term treatment efficacy in Triple Negative Breast Cancer (TNBC) patients in the absence of pCR [5–7].

Aside from these data, the development of new immune strategies that convert cold into hot immune enriched tumors are mandatory. The study of biological changes in BC patients with RD after NAC opens a translational window in breast tumors and its milieu in this population with a worse prognosis. Immune checkpoint inhibitors (CPI) have been evaluated in BC with outstanding results in combination with chemotherapy in the neoadjuvant arena [8–14] although with an increased toxicity profile, and without information regarding biological impact on tumor milieu. Dendritic cells, as the main directors of the immune system, have been studied for their role in the antigenic cross presentation, a key step for T cytotoxic responses. Dendritic cell vaccines (DCV) in BC patients have demonstrated a safety profile and an outcome improvement in different scenarios [15, 16]. So far, the implementation of dendritic cell therapy together with NAC in naïve BC patients could be a big challenge by increasing host immunity and immunogenic cell death in tumoral cells and generating potential modifications in tumor milieu.

The aim of this study is to evaluate if the addition of DCV to NAC could induce immune biomarker changes in those patients with RD by transforming tumor microenvironment, in order to identify which group of patients could benefit from this strategy and to select the best therapy in the maintenance scenario in upcoming studies.

Materials And Methods

Patients

All patients were treated with sequential NAC consisting of 4 cycles of dose-dense epirubicin plus cyclophosphamide with G-CSF support followed by 4 cycles of docetaxel each 21 days according to standard protocols. Patients in the experimental group also received intradermal DCV loaded with autologous tumor lysate as described in our previous work [16, 17]. Surgical management was performed after NAC and was followed by radiation therapy ± hormonal therapy if needed. Table 1 provides demographic and clinical patient characteristics.

Table 1
Patient characteristics corresponding to control and vaccinated groups.

Characteristics	Control group	Vaccinated group	p
Age in years ++	55,31	45,68	0,02
(range)	(26–84.35)	(36.15–74.48)	
Menopause Δ	17 (38.64 %)	26 (66.66 %)	
Pre-menopause			
Peri-menopause	2 (4.55 %)	2 (5.12 %)	0,02
Menopause	25 (56.82%)	11 (28.20%)	
Biologic Subtype*	14 (31,1 %)	10 (26,3 %)	
Luminal A			
Luminal B	18 (40 %)	12 (31,6 %)	0,45
Triple Negative	13 (28,9 %)	16 (42.1 %)	
Pathological response*	4 (8,9 %)	10 (26,3 %)	0,03
pCR			
non-pCR	41 (91,1 %)	28 (73,7 %)	

++ Median values. Δ According the number of patients. *According the number of tumor samples. pCR pathological complete response.

Samples and criteria for analysis

Core-diagnostic biopsies and surgical specimens from 80 patients (38 in the Vaccinated Group (VG) and 42 in the Control Group (CG)) were selected. Among the CG, two patients had multifocal/multicentric tumors in the breast. We were able to quantify TILs according to the characteristics of the core-diagnostic biopsies in the CG as follows: CD8 (37 samples), CD4 (39 samples) and CD45RO (36 samples). With respect to the surgical specimen, we studied 35 specimens for each marker. Regarding VG; 35, 34 and 34 core-diagnostic biopsies were available for CD8, CD4 and CD45ro biomarker, respectively. Surgical specimens were 27, 28 and 26 for CD8, CD4 and CD45RO markers, respectively. Samples were classified by biological subtype according to the 14th St Gallen International Breast Cancer Conference [18]. Total pCR was considered as no infiltrating residual tumor (ypT0/Tis ypN0) in both breast surgical specimen and lymph nodes according to American Joint Committee on Cancer 8th Edition. Recommendations from the International Immuno-oncology Biomarker Working Group on BC were used in order to evaluate TILs in the stromal compartment related to tumoral cells. The denominator used to determine the percentage of stromal TILs was the area of stromal tissue occupied by lymphocytes, not the number of stromal cells [19]. Moreover, these recommendations were applied to immunohistochemistry to be able to measure

CD8, CD4 and CD45RO lymphocytes in the stromal tumor area. After NAC markers were only measured on the samples with RD.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using formaldehyde-fixed and paraffin-embedded tissue sections from 3 to 4 mm thick. The tissue sections were stained by Autostainer Link48 using a monoclonal Mouse Anti-Human CD8 (Dako® Clone C8/144B), CD4 (Dako® Clone 4B12) and CD45RO (Dako® Clon UCHL1). Antigen retrieval was performed by PT link in high pH at 98 degrees for 5 minutes.

Immunohistochemistry measurement and scoring

IHC was quantified using Automated Cellular Imaging System (ACIS III)[20,21]. The ACIS III system is an automated, bright-field microscope with a patented image processing and analysis software based on color detection and pattern recognition to evaluate cells or tissue sections stained with IHC. Each slide was scanned at 10X. Stromal areas were evaluated according to tumor burden within each sample. A minimum area of 0.23 mm^2 and a maximum area of 3.10 mm^2 by sample with an average area of 1.50 mm^2 were analyzed. The score of each sample was obtained dividing the number of brown pixels (stained by IHC) by the total stromal area of the selected fields. Then, the result was transformed to a percentage value. Only areas with invasive breast carcinoma and without any processing defect were analyzed (Figure 1).

Statistical analysis

Non-continuous data were compared by the Chi-square test and Fisher test. Normality was tested using Shapiro–Wilks Test. The Mann – Whitney U-test and the Wilcoxon test were employed to study unpaired non-parametric variables and paired non-parametric variables, respectively. Sensitivity, specificity, predictive values, logistic regression and receiver operator characteristic (ROC) curves were calculated. Data were analyzed with the SPSS statistical software (version 20.0 for Windows) and OpenEpi: Open Source Epidemiologic Statistics for Public Health, Version 3.01. P-values ≤ 0.05 were considered significant, and 95% confidence intervals were calculated.

Results

TILs quantification before and after chemotherapy and in paired samples

Basal TILs before NAC were well balanced and expressed as percentage in the CG versus VG regarding CD8 (1.43 % versus 1.33 %; p = 0.75); CD4 (2.33 % versus 2.10 %; p = 0.77); and CD45RO (1.11% versus 0.78 %; p = 0.74), respectively. CD8 TILs decreased after NAC ± DCV in tumors as compared to initial values as follows: median CD8 was 1.43% (0.03%-13.29%) before NAC as compared to median CD8 of 0.58% (0.03%-39.28%) after NAC (p = 0.51) in the CG; and 1.33% (0.19%-16.26%) versus 0.59% (0.14%-11.66%) (p = 0.42) in the VG (see Table 2). The biologic subtype with the highest rate of CD8 TILs

in paired samples in both groups was the TNBC ($p < 0.0001$). When data were analyzed according to biologic subtype, a trend to a CD8 rise in TNBC samples was observed after NAC plus DCV, changing from 4.48% (0.48–16.26) in the diagnostic biopsy to 6.70% (0.76–11.66) in the surgical specimen, not reaching statistically significant differences ($p = 0.11$). On the contrary, TNBC patients in the CG showed a TILs drop from 2.71% in the biopsy to 0.18% in the surgical specimen ($p = 0.5$). Differences in the CD8 before and after NAC in both groups are described in Table 2. We also found that a 66.7% (4/6) of TNBC patients from VG registered an increase in TILs after treatment as compared with 20% (1/5) of TNBC patients in the CG ($p = 0.24$). This phenomenon is not observed in the other biologic subtypes.

Table 2
Quantification of CD8, CD4 and CD45RO TILs according to biologic subtypes in tumor samples, showing median values and ranges.

TILs	Pre-Treatement % (range)	Post-Treatement % (range)	p
CD8 CG General	1.43 (0.03–13.29)	0.58 (0.03–39.28)	0.51
CD8 CG LA	0.89 (0.29–3.66)	0.59 (0.19–2.23)	0.79
CD8 CG LB	1.98 (0.03–8.49)	0.81 (0.10-17.09)	0.69
CD8 CG TN	2.71 (0.03–13.29)	0.18 (0.03–39.28)	0.50
CD8 DCV General	1.33 (0.19–16.26)	0.59 (0.14–11.66)	0.42
CD8 DCV LA	0.90 (0.25–1.29)	0.45 (0.15–1.62)	0.13
CD8 DCV LB	1.08 (0.19–3.06)	0.56 (0.14–2.65)	0.07
CD8 DCV TN	4.48 (0.48–16.26)	6.70 (0.76–11.66)	0.11
CD4 CG General	2.33 (0.04–21.56)	0.47 (0.00- 38.29)	0.04
CD4 CG LA	0.54 (0.04–3.35)	0.18 (0.00- 2.82)	0.15
CD4 CG LB	2.65 (0.10-21.56)	0.65 (0.03–13.47)	0.17
CD4 CG TN	4.31 (0.49–18.41)	2.33 (0.02–38.29)	0.68
CD4 DCV General	2.10 (0.01–58.91)	0.61 (0.04–19.92)	0.24
CD4 DCV LA	0.62 (0.01–2.61)	0.25 (0.04–1.89)	0.20
CD4 DCV LB	1.09 (0.15–9.88)	0.46 (0.05–11.21)	0.33
CD4 DCV TN	10.44 (1.18–58.91)	7.71 (0.45–19.92)	0.91
CD45RO CG General	1.11 (0.03–15.86)	0.31 (0.01–24.44)	0.24
CD45RO CG LA	0.34 (0.03–1.79)	0.15 (0.01–2.79)	0.37
CD45RO CG LB	1.94 (0.05–15.86)	0.72 (0.03–24.44)	0.30
CD45RO CG TN	1.29 (0.04–8.74)	0.52 (0.27–2.78)	1.00
CD45RO DCV General	0.78 (0.02–32.31)	0.29 (0.02–25.58)	0.34
CD45RO DCV LA	0.55 (0.02–2.37)	0.13 (0.02–1.99)	0.16
CD45RO DCV LB	0.49 (0.03–3.25)	0.27 (0.09–11.27)	0.76
CD45RO DCV TN	2.85 (0.37–32.31)	2.26 (0.25–25.58)	0.50

DCV: Dendritic Cell Vaccine; CG: Control Group; LA: luminal A, LB: luminal B, TN: Triple negative.

Regarding CD4 TILs, a significant decreased infiltration was shown in the CG with the effect of NAC (p = 0.04) when compared paired-samples that was not present in the experimental group (p = 0.24) (Table 2).

TILs in the core-diagnostic biopsy: identifying target population to DCV

An association between CD8 TILs before NAC and pCR in VG (see Table 1 for pCR) was found in univariate and multivariate analysis (OR = 1.41, IC95% 1.05–1.90; p = 0.02, and OR = 2.0, IC95% 1.05–3.9; p = 0.03, respectively). However, this association disappeared in the CG (univariate analysis OR = 1.18, (IC95% 0.90–1.55; p = 0.22); and in the multivariate analysis, OR = 1.11 (IC95% 0.68–1.81; p = 0.65)). No association between CD4 or CD45RO before NAC and pCR in both univariate and multivariate analysis was observed in the CG (CD4 univariate analysis OR = 1.11 (IC95% 0.94–1.32; p = 0.18); CD4 multivariate analysis OR = 1.09 (IC95% 0.83–1.44; p = 0.51); in the VG CD4 univariate analysis OR = 1.10 (IC95% 0.98–1.23; p = 0.08); multivariate analysis OR = 0.96 (IC95% 0.79–1.17; p = 0.71). Regarding the univariate analysis of CD45RO marker in the CG we found an OR = 1.04 (IC95% 0.78–1.40; p = 0.75); in the multivariate analysis, an OR = 0.87 (IC95% 0.51–1.49; p = 0.62) was shown; and in the VG univariate analysis an OR = 1.05 (IC95% 0.95–1.15; p = 0.33) was described; lastly, in the multivariate analysis in the experimental group the OR = 0.82 (IC95% 0.66–1.02; p = 0.07). The association between TILs and pCR disappears when the samples are organized by biologic subtype.

Related to pCR, ROC curve establishes a cut-off point of 4% in diagnostic samples for CD8 TILs score. With this cut-off point, a 92.6% sensitivity (IC95% 76.63–97.94), 75% specificity (IC95% 40.93–92.85), 92.6% positive predicted value (PPV) (IC95% 76.63–97.94) and a 75% negative predicted value (NPV) (IC95% 40.93–92.85) were obtained in the VG. Using the same cut-off point in the CG, a similar sensitivity of 87.9% (IC95%:72.67–95.18) and a PPV of 93.55% (IC95%: 79.28–98.21) was reached, although the specificity and NPV were 50% (IC95%: 15–85) and 33.33% (IC95%: 9.6–70), respectively. Differences between both groups were not statistically significant (p = 0.20). No comparable results were found with CD4 and CD45RO TILs (Table 2).

Discussion

To our knowledge, this is the first report that identifies good responders to DCV in combination with NAC related to CD8 expression in the needle core biopsy with the established 4% cut-off point. Moreover, this is the first time that biomarker changes in the tumor and its milieu are reported in RD in naïve BC patients treated with standard NAC ± active cell based-immunotherapy, by showing maintained CD4 levels and a trend to a rise in CD8 infiltration (only in TNBC). Clinical impact of these findings could be relevant to improve selection of: 1) patients that could benefit from the addition of DCV to NAC; and 2) the best maintenance therapy in TNBC subtypes with RD based on the expression of CD8 and CD4 markers to choose standard capecitabine [8, 22] versus immunotherapy. Modifications on the tumor niche that reflects immune activation after DCV helps to be open wide to less toxic and more specific immunotherapy.

Regarding TILs, TNBC patients reach higher levels than the luminal subtypes in our study, as described before [5]. This could be in part due to specific mutational signatures, copy number variations, stromal metagenes and clonal heterogeneity of TNBC subtype [23–25].

We have shown a non-significant increase in stromal TILs after combined therapies with NAC plus DCV in up to 66% of TNBC patients, but not in the luminal subtypes. Probably our results are not significant due to the small sample size, being one limitation of our study. This fact could illustrate a reinforcement of the immune niche produced by DCV in BC patients. It can be suggested that vaccines could produce an increase in CD8 + T cells post-treatment in TNBC. Waks *et al*. found that luminal BC are less enriched in CD8 TILs than other BC subtypes and this cell population decreased after NAC [26]. Nowadays, there is not enough knowledge about how immunotherapy could change BC microenvironment. Results of the studies that have worked with paired samples (before and after conventional NAC) remain controversial, suggesting different roles of immune cell populations in carcinogenesis, response to therapies, tumor progression and a crosstalk among the tumor and the microenvironment. While some reports described a reduction of CD8 TILs in cancer milieu after NAC [26–29], others shown an increase on TILs count [6, 30, 31] or an inversion on the CD4/CD8 ratio [4]. Luen *et al*. described an increase in TILs level in 48% of the patients and a decreased in 47% of the patients in RD after NAC [32].

The input of immunotherapy in BC patients in the neoadjuvant scenario looks for a global improvement within the tumor (higher pCR), the niche (hottest tumors) and the systemic immune surveillance. Dendritic cell-based adjuvant immunotherapy has already shown a gain in CD8 T cells in peripheral blood in non-luminal BC with an encouraging PFS improvement, suggesting benefits in the systemic immunity [15]. Vaccination is more effective in the prevention of tumor growth, and probably the clinical advantage could be more relevant in patients with a small tumor burden and with a preserved immune system (naïve of therapies) than in large tumor burden patients, including metastatic scenario, and with an exhausted immune system. Thus, immune cell profile of primary versus metastases of BC patients is different [33, 34].

Current efforts have been made to standardize the quantification of TILs and to produce reliable results [35–38]. Some authors pointed out that the reproducibility of TILs evaluation improves when the categories are simplified to low versus high TILs, based on the concept of lymphocyte-predominant breast cancer phenotype [39] but the standard cut-off points need to be defined. The incorporation of digital analysis (ACIS III) provided a better quantification of the stromal TILs avoiding interobserver bias. Additionally, other aspect to be considered is the selection of the area to be evaluated and how many fields should be selected. Recent works support the fact that the average lymphocyte score from a single biopsy of a tumor is reasonably representative of the whole cancer [40]. Our results showed that a cut-off point of 4% CD8 in the diagnostic specimen could predict a better pathologic response when DCV are added to conventional NAC. As it can be observed in Fig. 1, this cut-off could be considered a relatively high density of CD8 + cells in the stromal tumor. This optical microscope immunohistochemical image could be used as a reference of what we consider as the minimal density of cells which correlates with response to vaccines.

Qingzhu *et al*., found in their meta-analysis that memory T lymphocyte infiltration of a tumor site could serve as a indicator for OS and DFS prediction in patients with malignant tumours [41]. Also, Yajima *et al*., find a relationship between high CD45RO (i.e memory T lymphocyte and marker of activated T cells) expression and a lower pathological stage in BC patients [42]. The importance of CD4 TILs is not clear in BC scenario. No association between CD4 and pCR was found in our study, although in the CG CD4 TILs significantly decreased in patients after NAC ($p = 0.04$) as compared to those who received the experimental therapy which remained stable ($p = 0.24$) (Table 2). However, most of the studies correlate CD4 or CD4/CD8 ratio with an increased pCR and better survival [34, 5], and this fact strengths the role of DCV.

Although more studies are needed to establish the benefit of DCV addition to chemotherapy, our results suggest that we are moving in the right direction. Identification of predictive and prognostic biomarkers to select patients that could benefit from the addition of immunotherapy to the standard systemic chemotherapy is key to develop more efficient therapeutic strategies [44]. The relationship between lymphocyte-predominant breast cancer patients in the case of the TNBC [45] and the pCR, PFS and OS is clearly established [46–48]. Nonetheless, an outstanding selection of good responders to immunotherapy is complex [49] and the study of biomarkers in RD after NAC plus immunotherapy as well asin peripheral blood is mandatory to improve this limitation.

Evaluation of tumor responses to immune strategies by imaging techniques has become tricky because changes in tumor burden need new response criteria based on special guidelines (iRECIST) [50]. In this way, biologic markers in the blood, the tumor and its milieu could contribute to a more specific information than imaging markers regarding patient selection for immune strategies in the early BC arena.

Conclusion

A 4% cut-off point of CD8 TILs in a TNBC subtype could help to establish which patients can benefit from DCV added to NAC. In the same way, a maintained expression of CD4 as well as an increased infiltration of stromal CD8 after DCV therapy could improve responses to further therapies, as seen in other solid tumors after relapse to IMT strategy when patients were treated with second line chemotherapy in the advanced scenario [6, 7]. Our findings suggest that patients with TNBC especially benefit from the stimulation of the antitumor immune system by using DCV pulsed with tumor antigens. Deeper studies based on immune profiling genomic panels in the tumor and immune cell populations on peripheral blood are still ongoing within our patients and could help us to elucidate the biological behavior of BC as well as the benefits from adding immunotherapy to conventional NAC. Promising combined immune approaches potentiating immune system with DCV added to the blockade of immune checkpoints together with NAC should be tested in clinical trials in this selected population.

Declarations

Ethics approval and consent to participate

This study was approved by the Navarra University Ethics Committee (approval no.)

Consent for publication Not applicable.

Availability of data and materials

The datasets generated and analysed during the current study are available from the corresponding autor on reasonable request.

Competing interest

M. Santisteban has received honoraria from Roche, Pfizer and Novartis and travel support from Roche, Pfizer and Miltenyi. A. López-Díaz de Cerio and S. Inogés have travel support from Miltenyi. No other disclosures were reported. All remaining authors have declared no conflict of interest.

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Authors´ contributions

L.M collected data, built the dataset, performed the analysis and helped write and revise the manuscript. A. L.J collected data and helped revise the manuscript. A. C collected data and helped revise the manuscript. P. S collected data and helped revise the manuscript. B.P.S helped to perform the statistical analysis and revise the manuscript. S.I made the dendritic cell vaccines and helped revise the manuscript. A.L.D.C. made the dendritic cell vaccines and helped revise the manuscript. F.G helped to perform the statistical analysis. O. F.H helped recruit patients and collect data. S.D.L.C helped recruit patients. L.H. collect data. MD. L collect data. M.I designed the samples assessment, collect data and helped write and revise the manuscript. M.S. designed the clinical trial, collected data and helped write and revise the manuscript.

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Figures

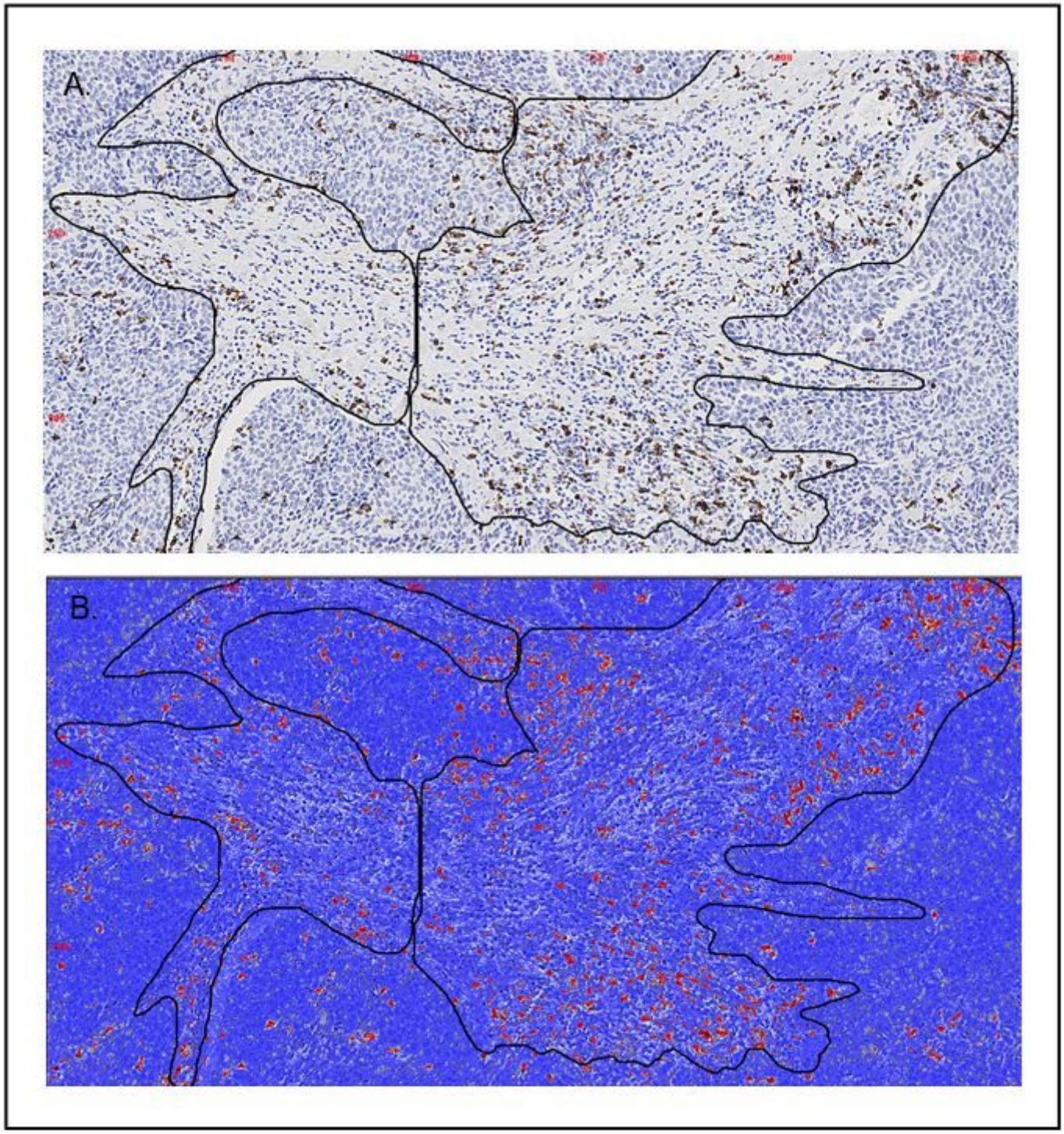

Figure 1

Immunohistochemistry measurement and scoring. A) Image analysis by ACIS III of a biopsy, two stromal areas selected (0.43mm2). B) TILs quantification by ACIS III, in red CD8 lymphocytes 5%. Scanned at 10X. Magnification of 50%.