PROYECTO DE INVESTIGACIÓN

NOTCH PATHWAY IN CIRCULATING MYELOID-DERIVED SUPPRESSOR CELLS (MDSCS) AS PREDICTIVE BIOMARKERS OF RESPONSE TO IMMUNE CHECKPOINT INHIBITORS (CPIS) IN ADVANCED TRIPLE-NEGATIVE BREAST CANCER (TNBC)

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INTRODUCTION

Immunotherapy in Triple Negative Breast Cancer (TNBC):

TNBC, defined by the lack of estrogen and progesterone receptors as well as HER2, accounts for 15% to 20% of all breast cancers and typically displays aggressive behavior, including earlier recurrence and metastasis. In advanced scenarios standard treatment is based on cytotoxic agents including taxanes, anthracyclines or platin compounds. Several targeted therapies, including bevacizumab, have failed to achieve survival benefits and reveal the complexity of this disease. There is an urgent need for effective therapeutic agents and targets[1].

Immunotherapy has prolonged survival in other solid tumors and represents a promising treatment strategy for TNBC. Several characteristics make TNBC more likely to respond to immunotherapy than other breast cancer subtypes. (1) TNBC has more tumor-infiltrating lymphocytes (TILs), TILs contain multiple types of immune cells, including B-lymphocytes, T-lymphocytes, and macrophages. TILs stimulate the tumor cell microenvironment to produce an immunosuppressive tumor microenvironment or an immune cancer-promoting tumor microenvironment by initiating inflammatory reactions. It has been shown that TILs expression correlates with better responses to ICIs in other tumors, and high levels of TILs in TNBC associate with improved prognosis in early-stage TNBC. (2) TNBC has higher levels of PD-L1 expression on both tumor and immune cells, providing direct targets for ICIs and correlating with response to anti–PD- 1 therapies. (3) TNBC has a greater number of nonsynonymous mutations, which give rise to tumor-specific neoantigens that activate neoantigen-specific T cells to mount an antitumor immune response, which can be strengthened by ICIs.

Activity of ICIs as single agent in TNBC is still low, with response rates ranging from 5% to 23% depending on prior treatment exposition and PD-L1 expression. Combination of PD-1/L1 inhibitors plus chemotherapy have demonstrated impressive activity in metastatic and early TNBC. Two ICIs (pembrolizumab and atezolizumab) have provided consistent gain in PFS for mTNBC with different degrees of PD-L1 expression. The IMpassion130 phase III trial in treatment-naïve mTNBC showed that atezolizumab added to nab-paclitaxel resulted in a clinically meaningful Overall Survival (OS) improvement of 7 months in the PD-L1–positive subgroup but not in the entire cohort. In a similar scenario, the KEYNOTE-355 trial has provided a 6 months improvement in median PFS for pembrolizumab in combination with different chemotherapy regimens for tumors with PD-L1 expression >10%, but OS data are still immature8. In early stage, two large neoadjuvant trials with pembrolizumab or atezolizumab had both shown a significant increase on pathological complete responses independently of the PD-L1 status. Provided the consistent benefit of ICIs in several TNBC subgroups, developing a more accurate biomarker- driven selection for immunotherapy remains a crucial challenge. Currently, there is no reliable biomarker available that allows select population to respond. Expression of PD-L1 and PD-L2 in tumor cells and/or in the microenvironment, TILs, tumor mutational burden (TMB), mismatch repair status, gene expression profiles (GEP), intrinsic molecular subtypes profiling, tumor microenvironment immune types (TMIT), gastrointestinal microbiome are some of biomarkers that have been correlated with the outcome of treatment with ICIs. Several have shown correlation with response to anti-PD-1/PD-L1 treatment, but not to OS. Out of the complexity and validation of these techniques, any of them has been prospectively validated.

Notch pathway and antitumor immune response evasion:

Notch signaling is an evolutionary conserved cell-to-cell communication system that is involved in a number of pivotal cellular processes, such as cell fate specification, differentiation, proliferation, cellular death and function of many cell types, including immune cells (1). Mammals have four Notch receptors (Notch1 through 4) that are bound by five ligands of the Jagged (Jagged1 and Jagged2) and the Delta-like (DLL1, DLL3, and DLL4) families (1). Binding of the Notch receptors on CD8^{+} T cells to DLL ligands on antigen-presenting cells induces antitumor cytotoxic responses, whereas binding of Notch to Jagged members resulted in suppressive signals (2). The mechanism for this opposite effect remains unclear with possible explanations, including different kinetics of Notch activation or selectivity of DLL and Jagged ligands for Notch receptors. Although the effects of Notch signaling in innate and adaptive immune responses are the focus of active research, the mechanisms leading to the expression of Jagged molecules in tumors and the potential effect of they in resistance to immunotherapy remain largely unknown. There are accumulating evidence that underlines the direct

involvement of the Notch signaling in normal mammary gland growth and breast carcinogenesis. Notch 1 and Notch 4 are categorized as breast oncogenes (3). Breast cancer clinical outcome was accompanied by high expression of Notch pathway and Notch-2 was identified as a tumor suppressor in many studies. Higher Notch-1 and Notch-3 expression have been observed in TNBC (4-6). Overexpression of Notch receptors or the Notch ligands have also been linked to poor prognosis in TNBC (6, 7). In addition, in breast cancer, the Notch pathway is especially active in the cancer stem cells (CSC) subpopulation and the use of gamma secretase inhibitor or anti-Notch 4 antibody results in a decreased cellular self-renewall (6-8).

Notch cascade plays an important role in breast cancer cell growth, migration, invasion, and metastasis, and its aberrant activation is associated with a poor prognosis, resistance to treatments, and relapse, particularly in TNBC.

Notch pathway and differentiation of myeloid cells in cancer.

There is now ample evidence supporting the critical role of myeloid cells in tumor progression. The major changes that are observed in the myeloid compartment include accumulation of myeloid-derived suppressor cells, defects in differentiation of dendritic cells, and accumulation and polarization of tumor-associated macrophages (1,2).

Myeloid cell development in bone marrow is regulated by complex network of cytokines and by the direct physical interaction between hematopoietic progenitor/stem cells (HPC) and stromal cells (3). Signaling through the Notch family of transcriptional regulators plays a major role in the direct interaction between HPC and stroma (4).

In the tumor context there are studies demonstrating that Notch signaling inhibition in HPC, suppressor myeloid cells and dendritic cells is directly involved in the abnormal differentiation of myeloid cells. This effect seems to be mediated by a serine/threonine kinase-dependent phosphorylation of Notch, which leads to an alteration in the interaction of the NICD and its CSL transcriptional repressor, preventing the transcription of its target genes (5). Thus, an increase in CK2 activity in HPC blocks Notch signaling, inhibits differentiation towards mature dendritic cells, and leads to a MDSCs accumulation.

Other works suggest the potential role of Jagged-1 and Dll-1 ligands in MDSCs generation in the bone marrow. Similar to T cells, DLL and Jagged ligands induced opposite effects in myeloid cells. Myeloid precursors cultured with fibroblasts expressing DLL1 differentiated into functional DCs, whereas activation of Notch through Jagged promoted immature myeloid cells and induced IL10. A potential explanation for the opposite roles of DLL and Jagged in myeloid cells is their differential effects on Wnt pathway (4-6).

On the other hand, immune-suppressive effects of Myeloid suppressor cells (MDSCs) are relatively well studied in tumor-bearing mouse models (7). Myeloid cells, including MDSCs and macrophages, have been linked with cancer stemness (8,9). However, the non-immunologic effects of MDSCs are poorly understood in human breast cancer. It has been reported that peripheral blood MDSCs correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin–cyclophosphamide chemotherapy (10). In line with this, Peng et al. have found high numbers of MDSCs in breast cancer tissues. They reported that MDSCs directly promote and maintain the CSC pool through two integrated signaling pathways: IL6/STAT3 and NO/NOTCH signaling pathways (11).

Among many other functions, the Notch pathway and its ligands have been involved in the development of the antitumor adaptive immune response (CD4+ T cells, CD8+ T cells, dendritic cell) as well as the immunosuppression in the microenvironment to evade the immune response (MDSC, M2 macrophages, Treg lymphocytes, immune checkpoints receptors like PD-1/PD-L1).

Preliminary results in lung cancer model:

The role of pretreatment circulating MDSC subpopulations on ICIs outcomes has been explored by our team in advanced NSCLC patients. A total of 86 NSCLC patients treated with a standard of care ICI regimen (pembrolizumab or atezolizumab), as well as 10 healthy donors were recruited in 3 centers between 02/2018 and 10/2019. Pretreatment immunophenotyping of monocytes, early-MDSC (e-MDSC: CD14-/CD15- /HLA-DR-/CD33+/CD11b+), monocytic-MDSC (M-MDSC: CD14+/CD15-/HLA-DR-/CD33+/CD11b+) and polymorphonuclear-MDSC (PMN-MDSC: CD14-/CD15+/HLA-DR-/CD33+/CD11b+) was prospectively performed by flow cytometry in fresh whole blood. All patients have received at least one previous platinum-based combination therapy for stage III-IV NSCLC and were valid for the analysis; median age 62 years; 82 (95%) smokers, 52 (60%) non-squamous; 69 (93%) ECOG 0-1; 43 (50%) PDL1 ≥ 1% and 65 (76%) treated in a second line setting. Median PFS (mPFS) was 2.79 months (m) [95% CI, 1.3- 4.2] and mOS 11.6 m [9.3-13.9]. Overall, 3m-death rate was 18.6%. Pretreatment high-NLR (NLR > 3) (62/86; 72%) was correlated with poor PFS (p=0.03), OS (p=0.01) and a 3m-death rate of 22.6%. High level of global MDSC before anti-PD-1/PD-L1 therapy, defined by the median value as a cut-off (>6.3%), was associated with poor OS (p=0.03). Pretreatment low e-MDSC (<21.7%), was also associated with poor PFS (p=0.01), OS (p=0.006) and a 3m-death rate of 23.8%. There was no correlation between M-MDSC, PMN- MDSC and clinical outcomes. The gene expression analysis (n=103 patients) showed that patients with a "high" expression of Jagged1, Notch1 and Hes1 in circulating MDSC had a shorter survival than patients with a "low" expression (Jag1 p = 0, 04 for PFS; Hes1 p = 0.009 for PFS and Notch1 p = 0.022 for OS). Our study suggests that a baseline circulating low level of e-MDSC and high-NLR are associated with early failure to ICI and poorer survival. The role of e-MDSC appears interesting as a potential predictive and prognostic biomarker in NSCLC patients treated with anti-PD1/PD-L1. Dynamic assessment of MDSC levels over time and further validation with longer follow up in larger cohorts are needed (European Congress of Lung Cancer, 2021).

HYPOTHESIS:

Preclinical models consistently provide that the Notch signaling pathway (NSP) plays a central role in the tumoral immune response. Decreased NSP activity among the different elements of the adaptive immune response [myeloid cells [(CD, MDSCs, TAM), CD8+ T cells, Treg, immune checkpoints as PD-1] mediates, as an immune escape mechanism in tumor microenvironment. Notch ligands and receptor levels expression in tumor microenvironment (Delta-like decrease expression and Jagged increase expression), especially in Myeloid Suppressor Circulating cells (MDSCs), seems to be key in this sense, mediating an immunosuppressive effect on different populations: CD8+ T cells, Treg expansion, M2 TAM polarization. The analysis of Notch ligands (Delta-like and Jagged) expression levels in Circulating MDSCs and in Peripheral blood mononuclear cells Cells (PBMC) in advanced TNBC patients treated with a ICIs regimen could be a predictive biomarker for anti-PD1/PD-L1 therapy. Our working hypothesis in this project is that high Notch ligand expression levels in MDSCs and/or PBMCby correlates with sensitivity to ICIs in advanced TNBC patients, defined as the achievement of a clinical benefit [Objective response or stable disease lasting more than 24 weeks].

Hypothesis
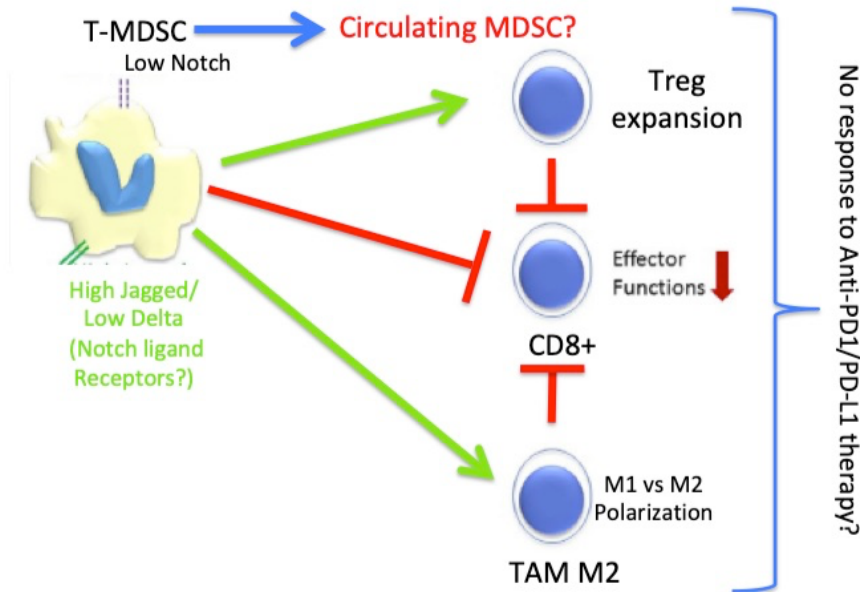
Figure 1: Schematic representation of the reciprocal responses of MDSCs and immune system cells to Notch signaling.
Inhibition of the Notch pathway in MDSCs promotes their accumulation in the tumor microenvironment (MAT). In addition, differential expression of Notch ligands (Jag and Dll) in MDSC promotes the inhibition of effector T-CD8+ cells, the expansion of Treg and the polarization of the macrophage population towards M2 phenotype, generating a more immunosuppressive effect in MAT. Is the same mechanism in circulating MSDC? These patients profile will no response to anti-PD1 treatment?

OBJECTIVES:

Primary Objectives:

- Characterization of Notch ligands (Delta-like and Jagged) and receptor expression levels in Circulating MDSCs in liquid biopsy prior to treatment initiation, at time of first radiologic evaluation and at treatment discontinuation.
- Correlation of Notch ligands (Delta-like and Jagged) and receptors expression levels in Circulating MDSCs at baseline with the objective response rate achieved with a front-line regimen including an ICI (pembrolizumab, atezolizumab, or other) for locally advanced and/or metastatic TNBC.

Secondary Objectives:

- Association of Notch ligands (Delta-like and Jagged) expression levels in Circulating MDSCs with objective response, progression-free survival, overall survival, 12- and 24-month survival rates and duration of response.
- Correlation between Notch ligands expression levels in circulating MDSCs and in tumor stroma.

- Association between levels of Notch ligand expression in circulating MDSCs and PD-L1 expression in circulating MDSCs and tumor tissue.
- Association between Notch ligands expression levels in circulating MDSCs and VEGF, IFN gamma, IL-1B, IL-2, IL-4, IL-6, IL8, IL-10, IL-17, TGF beta TNF alfa, CCL5, Fas/FasL PD-L1 levels in serum.

MATERIAL AND METHODSA-CLINICAL STUDY:

Study design: Prospective exploratory biomarker study.

Phase: Post-authorization study.

Medicines Tested: PD-1/PD-L1immune checkpoint inhibitors (ICIs).

Patient Population: Patients with locally advanced or metastatic Triple Negative Breast Cancer (TNBC) treated with Immune Checkpoint Inhibitors.

Inclusion/exclusion criteria: The subject will be considered included in the study when, complying with the selection criteria, they signed their informed consent form, men or women age ≥ 18 years, abilty to comply with study protocols, and the corresponding data is recorded in the data collection notebook. The subject participating in the study may revoke her consent to the use of her data in the analysis at any time, without expression of cause and without any liability or damage arising for him. The diagnosis of the patient must comply with unresectable, metastatic or recurrent advanced TNBC documented histologically or cytologically. Patients will be excluded from the study if they meet any of the following criteria: central nervous system metastases, uncontrolled tumor related pain, active o history of autoimmune disease o deficiency, concurrent partipation in other interventional study.

Variables: Demographic data and clinical history, complete physical examination, tumor evaluation and response using irRECIST (RECIST v.1.1 adapted to account for the unique tumor response observed with immunotherapeutic agents), complete biochemical studies according to standardized routine clinical practice for all participating centers. Samples to traslational study will be take a 4 timelines:
a) Entry into the study (baseline)
b) Three weeks after the start of treatment
c) Twelve weeks after the start of treatment (coinciding with the first evaluation of the tumor)
d) Tumor progression.

Statistical assumptions: A total of 60 patients treated with standard ICI both in the neoadjuvant or in the first line setting for a Triple Negative breast Cancer will be included in the study over a 3 year period. We estimated that around 60% of patients will achieve responsive criteria (pCR for locally advanced or objective response – SD at 6 months for advanced status).

Evaluations during treatment. Controls during treatment will be carried out according to the usual clinical practice of each center. The causes of treatment interruption or delay will be collected. Treatment with anti-PD-1 / PD-L1 will be maintained until disease progression or unacceptable toxicity or in accordance with the usual clinical practice of each center. Evaluation of response to treatment. A first evaluation of the response to treatment with CT or PET-CT will be performed according to the usual practice of each center between 8 and 12 weeks after the start of anti-PD-1 / PD-L1 treatment.

Study limitations: This part of the project has as its strengths the great experience of the medical team, but we will only have evidence of clinical changes of remission or not of the tumor, and not tacit evidence of the direct implication of the mechanism of action of the drug. Hence the interest of the project section to carry out research at the cellular and molecular level.

Ethics Committee (CEIm): The study will be presented for evaluation to the Research Ethics Committee of the Arnau de Vilanova Hospital, and the incorporation of the patients will be after the approval of the protocol by said committee.

B-MOLECULAR STUDY

The molecular and cellular study involves the collection, processing, temporary storage and shipment of samples from patients with IC from the selected centers to the central laboratory: the laboratory of the Arnau de Vilanova Hospital / FISABIO Biobank where the entire experimental part of the study will be carried out.

1-Blood samples.

Samples of peripheral blood will be obtained according to the usual clinical practice of the center at the following times:

a) Entry into the study (baseline)
b) Three weeks after the start of treatment
c) Twelve weeks after the start of treatment (coinciding with the first evaluation of the tumor)
d) Tumor progression.

From the blood sample, a fraction will be heparinized to obtain mononuclear cells (PBMCs) and MDCS, and the rest will be used to prepare serum for biochemical studies. The samples will be sent to the reference laboratory through a logistics circuit designed for this purpose (already used in previous successful studies), in such a way that the maximum time from the extraction of the blood to its processing will be less than 6 hours, in order to to preserve in material to be analyzed (flow cytometry). The MDSC purification will be carried out at the Valencia University and Arnau de Vilanova Hospital / FISABIO biobank that have state-of-the-art analytical equipment.

1.1 Isolation of mononuclear cells in peripheral blood (PBMC). The isolation of PBMCs will be carried out using the Ficoll-Paque PLUS density gradient centrifugation method. The PBMCs will be collected at the plasma interface, they will be resuspended in buffer for their immunophenotypic analysis.

1.2 Isolation of MDSCs: Flow cytometry and cell sorting. Isolated PBMCs will be used immediately for analysis by flow cytometry and cell sorting (<6 hours, without cryopreservation). PBMCs will be stained for the expression of surface markers using fluorochrome-conjugated anti-human monoclonal antibodies against different molecules (BD Biosciences) MDSCs subpopulations: CD33; CD11b; CD14, CD15; HLA-DR; Lin (CD3/CD4/CD16/CD56/ CD19).

1.3 Notch ligands mRNA expression levels (Delta-like 1, 3 and 4 and Jagged 1 and 2 and PD-1 / PD-L1 in MDSCs. Extraction of total RNA from isolated cell preparations by cytometry is performed using RNAqueous micro kit according to the manufacturer's instructions. The cDNA synthesis will be performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time PCR will be performed using the TaqMan probe system (Applied Biosystem). The mRNA levels will normalize with the Beta-actin mRNA levels.

1.4 Serum determinations by Lumnex multiplex: Circulating concentrations of VEGF, IFN gamma, IL-1B, IL-2, IL-4, IL-6, IL8, IL-10, IL-17, TGF beta TNF alfa, CCL5, Fas/FasL PD-L1, levels in serum and other emerging cytokines will be quantified by Luminex multiplex assays (LuminexR) in serum samples according to the manufacturer's instructions.

2 -Tumor tissue:

- Baseline: Prior to treatment initiation with CPIs (archival tissue or recent biopsy).

- Optional tissue sampling (re-biopsy) has been performed at disease progression.

Determinations in tissue.

2.1 Expression of Notch ligands / receptors will be assessed by gene expression e IHC in TNBCm tumor samples. Tissue samples containing at least 50% tumor cells after microscopic observation will be used for analysis. Two sequential analyzes will be carried out on the available samples before the start of treatment and after.

2.2. Extraction of mRNA and evaluation of the genes expression levels by RT-qPCR of the Notch ligands (Dll-1, 3 and 4 and Jag-1 and 2) and receptors. For RNA extraction, tissue lysing (proteinase K digestion) containing> 50% tumor cells will be performed with Trizol- LS (Invitrogen) according to the manufacturer's instructions. The mRNA samples (1-3 µg) will be processed for reverse transcription following the Superscript III system (Life Technologies). Relative expression levels of Dll-1, Dll-3, Dll-4, Jag-1 and Jag-2 mRNA will be evaluated in tumor tissue using qPCR with TaqMan probes (Applied Biosystems).

2.3 .Determination of the expression by IHC of Notch ligands (Dll-1, 3 and 4 and Jag-1 and 2) in tumor stroma, PD-1 / PD-L1 in tumor cells and stroma,. Tissue samples containing at least 50% tumor cells after microscopic observation will be used for analysis. Two sequential analyzes will be carried out on the available samples before the start of treatment and after. The rabbit polyclonal primary antibodies anti-Jag-1 and 2 (Santa Cruz Biotech.) And anti-Delta-1, 3 and 4 (Rockland Immunoch.) Will be used. The sections will be incubated with the appropriate secondary antibodies (ZSGB-BIO). Diaminobenzidine will be used as the chromogen and the sections will be counterstained with hematoxylin.

2.4 Evaluation of immunohistochemical staining. The stained slides will be independently evaluated by pathologists who will not have knowledge of the clinical information of the patients. The evaluation will be carried out in at least five areas with a magnification of 400x. A final histological score will be obtained for each case by multiplying the staining intensity and percentage scores. The levels of protein expression by IHC will finally be expressed as a function of the histological score as a low expression (histological score <5) or as a high expression (histological score ≥ 5).

2.5 Expression of PD-1 / PD-L1 in tumor cells and stroma. Pre-treatment will be performed on paraffin-preserved tumor specimens using a validated IHC method (Dako). Samples will be graded according to the percentage of tumor cell staining in the membrane at 1%, 5% and 10% in a cell section with a minimum of 100 assessable cells.

STATISTIC ANALYSIS: The correlations of biomarkers and responses with clinical covariates will be investigated. It will be verified if the covariates can improve the prediction and if there is an interaction with the biomarkers. Relevant covariates could be part of the statistical prediction model. Other multivariate techniques (eg, multiple logistic regression, Cox regression, principal component analysis with rotation, or cluster analysis) will be considered to study marker combinations. We will use the R program and GraphPrism version 8.2 (La Holla, Califormia).

STUDY DIFFICULTIES AND LIMITATIONS: The research team has experience in the techniques proposed in the project, so there are no difficulties in carrying it out. In the prospective study, the logistics within the services and hospitals involved (surgery, pathological panatomy anatomy and molecular biology laboratory) should be adjusted to ensure the correct conservation of he samples, so that the surgical pieces are frozen as quickly as possible to prevent RNA degradation. The incidence allows us to guarantee the number of 60 expected patients.

EXPECTED RESULTS AND POTENTIAL IMPACT. The direct benefit of this project is the development of a multidisciplinary research platform on the biology and pathophysiology of Breast cancer. This platform integrates different, clinical, biological and methodological approaches that will be applied to the study of immunotherapy response. The immediate impact is to check specific hypotheses and generate molecular, cellular and genetic analysis tools to explain the pathogenesis of MDSCs in tumor tissues and in blood and help us to identify relevant molecular targets (biomarkers) for the processes investigated.

IMPACT OF SOCIAL AND HEALTH-CARE POTENTIAL: TNBC is one of the top ten leading causes of cancer death worldwide. Immunotherapy has meant a revolution in TNBC treatment, achieving results superior to conventional treatment. Although we will only find a response in 40-50% of patients with unselected aTNBC treated with anti-PD1/PD-L1 regimens, these responses may be significantly longer than conventional therapy. At present there is no reliable biomarker available that allows selected population to respond. Therefore, it is urgent to find and validate more sensitive and specific predictive biomarkers that identifies patients with an advanced TNBC who can benefit from these expensive therapies with non- invasive methods (liquid biopsy).

SPREAD OF RESULTS: The main mode of spread is the publication in journals of high international impact. Dissemination will also be important within the scientific community through conferences and workshops with the participation of groups of basic, clinical and translational research. One of the aspects that we want to take care of is the dissemination of the results, especially those with a significant translational component, including the dissemination between patient associations and health authorities. Finally, the future potential of the work done and results achieved include the pharmaceutical and biotechnology companies interested in using our models and screening systems.

BUDGET

Activity	Costs
MDSCs purification : cell sorting	5.511 €
Gene Expression by qRt-PCR, molecular biology material x 240 samples (expression of genes: PD-L1, Notch Pathway: Dll-1, 3 and 4 and Jag-1 and 2, Notch1, 2, 3 y 4	24.547,53 €
Luminex protein multiplex (VEGF, IFN gamma, IL-1B, IL-2, IL-4, IL8, IL-10, IL-17, TGF beta TNF alfa, CCL5, Fas/FasL PD-L1) x samples	5.000 €
Immunohistochemical studies (expression of proteins: PD-L1, Notch Pathway: Dll-1, 3 and 4 and Jag-1 and 2, Notch1, 2, 3 y 4)	5.000 €
Publication expenses (open access journals)	2.000 €
Sub-total execution costs:	42.058,53 €
Total Budget Requested	**42.058,53 €**
Overheads (20%)	8.411,706€
Total budget requested + 20% overheads	**50.470,236€**

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