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Biomarker Study of the Antitumoral Activity of Denosumab in the

Pre-Operative Setting of Early Breast Cancer.

(*D-BIOMARK*)

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Title	Biomarker Study of the Antitumoral Activity of Denosumab in the Pre-Operative Setting of Early Breast Cancer. (D-BIOMARK).
Study Phase	0
Indication	Women with initial diagnosis of breast cancer candidate to curative surgery as first therapeutic approach.
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1. Abstract

D-Biomark is a randomized window trial designed to evaluate the antiproliferative, proapoptotic and immunomodulatory effects of single-agent denosumab in early breast cancer. A total of 60 patients were enrolled and 58 evaluated, all HER2 negative, 10 of them being 10 TNBC, 27 premenopausal and 31 postmenopausal patients. Patients were randomized (2:1), with the experimental group receiving two subcutaneous doses of denosumab (120 mg) after diagnosis, separated by 7 days, and the control group receiving no treatment. An initial biopsy was obtained at the time of diagnosis (biopsy A). Two to three weeks after inclusion, at the time of surgical excision, a second biopsy of the surgical sample (biopsy B) was obtained. Analyses was performed comparing paired samples. Efficiency of denosumab was confirmed by the drop in soluble RANKL levels. The primary endpoints were not met, as denosumab did not reduced tumor cell proliferation, and don't increase apoptosis.

No associations between RANK/RANKL expression by immunohistochemistry and soluble RANKL and the response variables were found.

2. Introduction

The receptor activator of nuclear factor κB, known as RANK and its ligand (RANKL), members of the tumor necrosis factor (TNF) superfamily, have emerged as potential therapeutic targets in cancer. [1,2]

RANK is a type I transmembrane protein, which when bound to RANKL leads to activation of downstream signaling pathways, such as the canonical and non-canonical NF-κB, MAPK, and PI3K-AKT pathways related to tumor proliferation.[3] The RANK/RANKL axis plays a crucial role in bone remodeling through activation of osteoclasts. RANK signaling regulates mammary gland development and mammary cell fate. RANKL is the main mediator of the proliferative and protumorigenic role of progesterone [2–4] Preclinical data have shown that RANK-overexpressing mice are more susceptible to mammary tumorigenesis, and conversely, pharmacological or genetic inhibition of the pathway prevents or attenuates tumor appearance in the breast. RANKL inhibition reduces tumor cell proliferation in preneoplastic lesions. and reduces tumor cell survival in adenocarcinomas. Blockade of the RANK pathway also reduces the incidence of lung metastases and leads to differentiation of tumor cells. [3–5]

Preclinical data generated in the laboratory of Dr Gonzalez Suarez and others demonstrate that the RANK pathway has a dual role in breast tumorigenesis, with intrinsic effects on tumor cells[3,5] and extrinsic through the regulation of the immune response.[6] In MMTV-PyMT mouse models of luminal breast cancer, deletion of RANK exclusively in the tumor cells was found to produce an enhanced antitumor immune response, with an increase in infiltrating CD8+ T lymphocytes and a reduction in immunosuppressive myeloid cells.[6]

From a clinical point of view, the RANK pathway has been widely studied as a regulator of bone resorption, leading to the development of Denosumab, a highly specific immunoglobulin 2 (IgG2) type monoclonal antibody, which binds with great affinity to RANKL and neutralizes its activity. OPG is a natural negative regulator of RANK pathway that acts as a decoy receptor and prevents the binding of RANKL to RANK. Denosumab is approved for the prevention of skeletal events in patients with bone metastases, for the treatment of unresectable giant cell tumors of bone, and for the treatment of osteoporosis [2,7]

RANK expression in human mammary adenocarcinomas is associated with an aggressive tumor phenotype: hormone receptor-negative tumors (estrogen and progesterone receptors, ER, PR), with a high histological grade (GH) and high proliferative index. RANK protein expression (detected by immunohistochemistry) is more common in ER-negative breast adenocarcinomas, but it is also expressed in a subset of ER-positive luminal tumors. Recent results support that RANK protein expression in tumor cells is an independent marker of poor survival in postmenopausal patients with non-metastatic breast tumors (regardless of ER expression, histological grade, clinical stage, and tumor size).[8,9]

Based on the preclinical results that demonstrate the anti-proliferative, pro-apoptotic, and pro-differentiation capacity of RANK pathway inhibition, several clinical trials with denosumab have been conducted in breast cancer. In the adjuvant setting, the results of the prospective, randomized, double-blind clinical trial ABCSG-18 (NCT00556374) with 3425 postmenopausal women with early breast cancer and adjuvant treatment with aromatase inhibitors, showed that adjuvant denosumab at a dose of 60 mg two times a year, significantly delayed time to first fracture, increased bone mineral density, and in a recent update showed a disease-free survival benefit at 8-year follow-up. Highlighting the data presented in 2022 where it was confirmed, by censoring the cases due to late crossover and use of antiresorptive agents, the benefit in terms of progression-free survival, bone metastasis-free survival and overall survival of denosumab as adjuvant therapy, thus recommending its routine use in postmenopausal women with hormone receptor-positive breast cancer.[10–12] In contrast, in the D-CARE clinical trial (NCT01077154) with 4509 pre- and postmenopausal women, no changes in bone metastasis free survival were observed, and disease free survival between the groups with or without denosumab after 5-year follow-up was similar.[13]

At neoadjuvant context in the GeparX study, the addition of denosumab to neoadjuvant chemotherapy did not increase the pathologic complete response (pCR) rate in early breast cancer, even in tumors with high RANK expression by immunohistochemistry (IHC).[14–16]

The D-BEYOND a window of opportunity trial, evaluated the biologic effect of denosumab in premenopausal women diagnosed with early breast cancer. It included 27 patients, all tumors expressing hormone receptors except one which was a case of triple negative neoplasia. All patients received two subcutaneous injections of denosumab (120 mg/dose) separated by one week, prior to breast surgery. The baseline biopsies were compared with the surgical sample. The study did not achieve its primary objective, since a reduction in cell proliferation parameters (Ki 67) or an increase in cell apoptosis was not observed. However, a brief course of denosumab induced an increase in the inflammatory infiltrate measured by TILs, especially CD8+ T lymphocytes. A decrease in infiltration by regulatory T cells was also observed in some patients. High serum RANKL (sRANKL) concentrations and increased expression of the RANK metagen (expression of genes that reflect activation of the RANK pathway in tumor cells) prior to treatment were associated with a better immunomodulatory response to denosumab. However, this trial showed limitations

such as the limited number of participants, not having a control arm and only including young premenopausal patients. [6,17].

With all these data, the D-BIOMARK study was developed with the scientific interest of finding biomarkers of response to denosumab in clinical practice. The schedule is similar to that of D-BEYOND trial but with a larger number of participants, the inclusion of pre and postmenopausal women, and triple negative tumors, and with the benefit of including a control group to validate all the response variables. This phase 0 trial tried to determine if a short course of denosumab affects tumor cell proliferation and survival and the discovery of possible biomarkers of response to this treatment.

3. Hypothesis

It was hypothesized that Denosumab, through RANKL inhibition, would reduce tumor cell proliferation and survival in early breast cancer.

4. Objectives:

A. Primary objective

- Demonstrate the antiproliferative and/or proapoptotic activity of denosumab in early breast cancer.

Endpoints to evaluate the primary objective:

Changes in the percentage of tumor cells expressing Ki67 and/or cleaved caspase 3 between Biopsy A (pre-treatment) and Biopsy B (post-treatment).

B. Secondary objectives

- Correlate the antiproliferative/proapoptotic activity of denosumab with the expression of RANK, RANKL (protein expression)
- Characterize the differences in the antiproliferative/proapoptotic activity of denosumab and modified RANK/RANKL ratio (mRNA) by Modified ratio of Rank/RankL: MR={log(RANK) -1.2} / log(RANKL).
- Characterize the differences in the antiproliferative/proapoptotic activity of denosumab between the different phenotypes of breast cancer.
- Characterize the differences in the antiproliferative/proapoptotic activity of denosumab between pre- and postmenopausal patients.

Endpoints to evaluate the secondary objectives:

Correlate changes in Ki67 with RANKL, RANK expression (mRNA and protein).

Correlate changes in Ki67 with the expression of estrogen and progesterone receptors.

- The safety of denosumab and the performance of biopsies according to the criteria of the CTCAE v4.

5. Study Design

This was a Phase 0 clinical trial or a biomarker study. Patients with early breast cancer (Stages I and II) candidates to tumor excision as first therapeutic approach were randomized (in a 1:2) to receive two doses of denosumab prior to surgery, control patients did not receive any treatment. An initial biopsy was obtained at the time of diagnosis (biopsy A). Two doses of subcutaneous denosumab (120 mg) were administered following diagnosis separated by 7 days. After two to three weeks, at the time of surgical excision, a second biopsy (tumor punch) was obtained from the surgical specimen (biopsy B). The objectives of this trial were tested through the comparison between both biopsies.

5.1.1 Sample Size: 60 patients

5.1.2 Summary of key Subject Eligibility Criteria

- Women (older than 18 years) diagnosed with breast cancer in early, curable, stage (I or II) candidate to surgery as first therapeutic approach.
- HER-2 negative.
- No previous systemic treatment for any malignancy.
- Low risk of ONJ or Hypocalcemia:
- Adequate Serum calcium or albumin-adjusted serum calcium ≥ 2.0 mmol/L (8.0 mg/dL) and ≤ 2.9 mmol/L (11.5 mg/dL)
- No prior history or current evidence of osteonecrosis of the jaw
- No Active dental or jaw condition which requires oral surgery, including tooth extraction. No planned invasive dental procedures
- If subjects are pre-menopausal they should be willing to use highly effective methods of contraception (per institutional standard) during treatment and for 6 months after the end of treatment

5.2 Procedures

Informed consent, tumor Biopsy, denosumab administration, biomarker determination, concomitant medication and adverse events were registered.

Evaluation of tumor cellularity was centrally assessed from FFPE tissue and frozen human tumor tissue on the hematoxylin and eosin-stained tissue sections. For patients with multiple samples, the sample with the highest tumor content was chosen. Serial FFPE tissue sections (4 μm) were immunohistochemically stained for Cleaved-Caspase3 (Cell Signaling) and ki67. The quantification of Cleaved-Caspase-3 was done using QuPath® software (platform for bioimage analysis), and Ki67 the fixation conditions, processing, antibodies used, and evaluated results were performed according to international recommendations.[18,19]

The evaluation of conventional BC markers, including estrogen receptor (ER), progesterone receptor (PR), HER2, and Ki-67, was centrally performed in the pathology department of the Hospital de Bellvitge. The

status of ER and PR was defined according to the guidelines of the American Society of Clinical Oncology and the College of American Pathologists (ASCO-CAP).[20]

Tissue sections (4 μm) from FFPE tissues of human primary breast tissue were used to assess RANK and RANKL. For each patient, representative unstained slides of the primary tumor were shipped to NeoGenomics Laboratories (California, USA) for immunohistochemical staining of RANK (N1H8, Amgen), RL (M366, Amgen), blinded to clinical information. The percentage of stained cells and their intensity (0, negative; 1+, weak; 2+, moderate; and 3+, strong) in the tumor cells were reported by Neogenomics. An H-score was calculated using the following formula: H = (% of cells of weak intensity × 1) + (% of cells with moderate staining × 2) + (% of cells of strong staining × 3). The maximum possible H-score is 300, corresponding to 100% of cells with strong intensity. In addition, we independently evaluated RANK and RANKL H scores in tumor cells but also in the stroma at the laboratory of Dr Gonzalez-Suarez.

RNA was extracted from O.C.T.™ samples at baseline and at the time of surgery. Prior to extraction, a section of the sample was stained with H&E where the percentage of tumor content was assessed. At least 30% tumor cellularity was considered necessary to perform the RNA extraction. Total RNA was isolated from tumor pieces using Maxwell® RSC simplyRNA Tissue Kit (AS1340 Promega), following the manufacturer's instructions.

5.3 Statistical analyses

Those variables containing more than 30% of zeros values were recoded as binary (zero vs greater than zero). Pre vs post values were compared using a paired t-test or McNemar test for numeric and binary variables, respectively. Independent samples t-test was used to compare post-pre difference between groups, while two sample McNemar test for binary variables. To assess whether the effect of treatment depended on a third variable, the interaction term of this variable and treatment was tested in a logistic or a linear model on numeric or binary response variable, respectively.

To compare baseline variables and possible response variables the Mann–Whitney U and Fisher's exact tests were used for continuous and categorical variables, respectively. All correlations were measured using the Spearman's non-parametric rho coefficient. A Logistic regression analysis was performed to define the odds ratio of developing a response variable. All reported P-values were two-tailed. All analyses were performed using R version 4.1.3 (available at www.r-project.org), GraphPad Prism version 5 and IBM SPSS Statistics version 25 (IBM Corp, Armonk, NY USA).

6. Results

6.1 Description of the group.

In May 2021, the recruitment of patients within the D-Biomark clinical trial (NCT03691311) was completed, 60 patients were included as planned, however, only 58 patients were evaluable because one patient received neoadjuvant letrozole during the pandemic period for SarsCov2 due to a delay in the scheduled date of surgery, and another patient only received 1 dose of denosumab due to withdrawal of consent. Table 1 shows the clinicopathological characteristics of the population at the time of inclusion.

The analysis does not show statistically significant differences between both groups except for the percentage of patients with tumors with low Ki67 <15%, which are higher in the experimental group 43.24% compared to the control 9.52%; we also noted a higher percentage of lobular carcinomas in the experimental group 32.4% vs 14.3%, although this difference is not statistically significant. A case (a lobular carcinoma) with a clinical stage IIIA was included: due to a mistake in the measure of the initial mammography, it was cataloged as cT2, however, when reviewing the images in our center, it was reassessed as cT3, which changes stage IIIA. Equally a surgical approach was decided as the 1st therapeutic maneuver and she was randomized to the experimental arm. Of the total number of patients with triple negative neoplasms recruited, it should be noted that 3 of them are apocrine tumors, 2 included in the group treated with denosumab and 1 in the control group. Apocrine tumors have a more indolent behavior, with less tumor aggressiveness with respect to typical triple negative breast carcinomas. Therefore, the data presented in the TNBC subgroup of patients should be taken with caution.

Table 1: Clinicopathological characteristics of the 58 evaluable patients, at the time of inclusion.

		Experimental	**Control**	**p-value**
Patients (n=58)		37	21	
Age (range)		57.0 (37-88)	55.4 (40-80)	
Subrogate Molecular Sub Type* N= (%)	TNBC N= (%)	6 (16.22%)	4 (19.05%)	0.71
	Luminal A-like (Ki67 <20)	19 (51.35%)	8 (38.09%)	
	Luminal B-like (Ki67≥20)	12 (32.43%)	9 (42.86%)	
Menopausal status N= (%)	Pre	17 (45.9%)	10 (47.6%)	1.0
	Post	20 (54.1%)	11 (52.4%)	
Histological grade N= (%)	G1	13 (35.1%)	4 (19.0%)	0.43
	G2	20 (54.1%)	14 (66.7%)	
	G3	4 (10.8%)	3 (14.3%)	
Ki 67 N= (%)	<15	16 (43.24%)	2 (9.52%)	**0.01**
	15-30	12 (32.43%)	14 (66.67%)	
	>30	9 (24.33%)	5 (23.81%)	
Histological Subtype N= (%)	DIC	21 (56.8%)	15 (71.4%)	0.31
	LIC	12 (32.4%)	3 (14.3%)	
	Others	4 (10.8%)	3 (14.3%)	
Clinical Stage N= (%)	IA	26 (70.3%)	17 (81.0%)	0.74
	IB	0 (0%)	0 (0%)	
	IIA	8 (21.6%)	4 (19.0%)	

	IIB	2 (5.4%)	0 (0%)	
	IIIA	1 (2.70%)	0 (0%)	

BC subtypes were defined according to the St Gallen 2015 Consensus Meetings using immunohistochemical surrogates as follows: Luminal A: ER and/or PR(+), HER2(−), Ki67 < 20%; Luminal B: ER and/or PR(+), HER2(−), Ki67 ≥ 20; Basal: ER(−), PR(−), and HER2(−), irrespective of Ki-67 score

6.2 Side Effects

In relation to the side effects, 1 case of osteonecrosis of the jaw classified as grade 3 was reported: a patient included in the experimental arm who received 2 doses of denosumab on March 10 and 17, 2021. From April 19, 2021 she presented discomfort and pain in the jaw and was visited by Maxillofacial surgery. Despite initially classified as gingivitis, in February 2022 the diagnosis of mandibular osteonecrosis of the right quadrant 44-47 was confirmed. She required surgical intervention on April 5, 2022 with pathology results that confirm the diagnosis. This event was consider related to the treatment (denosumab); however, the patient is an active smoker like possible triggering risk factor. The rest of the reported toxicities were grade 1 like discomfort at the injection site in 3 cases (8.11%).

6.3. Denosumab was associated with systemic drop of free RANKL but not with a reduction in tumor cell proliferation (Ki67) or survival (Cleaved Caspase-3).

A blockade of the RANK-RANKL pathway was confirmed by the drop in free serum RANKL (sRANKL) measured by ELISA in the experimental group treated with denosumab (p <0.001 T-Test for samples paired), but not in the control group without treatment (p 0.246) (Figure 2-A, left). However, the levels of the bone marker, TRACP5b (n=37) did not show significant changes pre vs post in both groups (Figure 2A, right). To confirm these results, a second bone marker CTX (n=39) was evaluated. Again, no changes after denosumab were found (data not shown). It is unclear whether the lack of changes is due to technical limitations (for CTX many samples were below the detection limit of the kit), or due to the biology/ kinetics of bone markers.

Mean values of sRANKL or Trap5b values did not differ according to the menopausal status defined clinically as more than 1 year with amenorrhea, or ≥ 60 years old (using serum from the same day of collection). In contrast, higher levels of OPG were detected in postmenopausal women (p 0.01) Figure 2-B.
In premenopausal patients, no associations between progesterone levels and sRANKL, or RANKL expression in tumor cells were found (Fig 2-C).

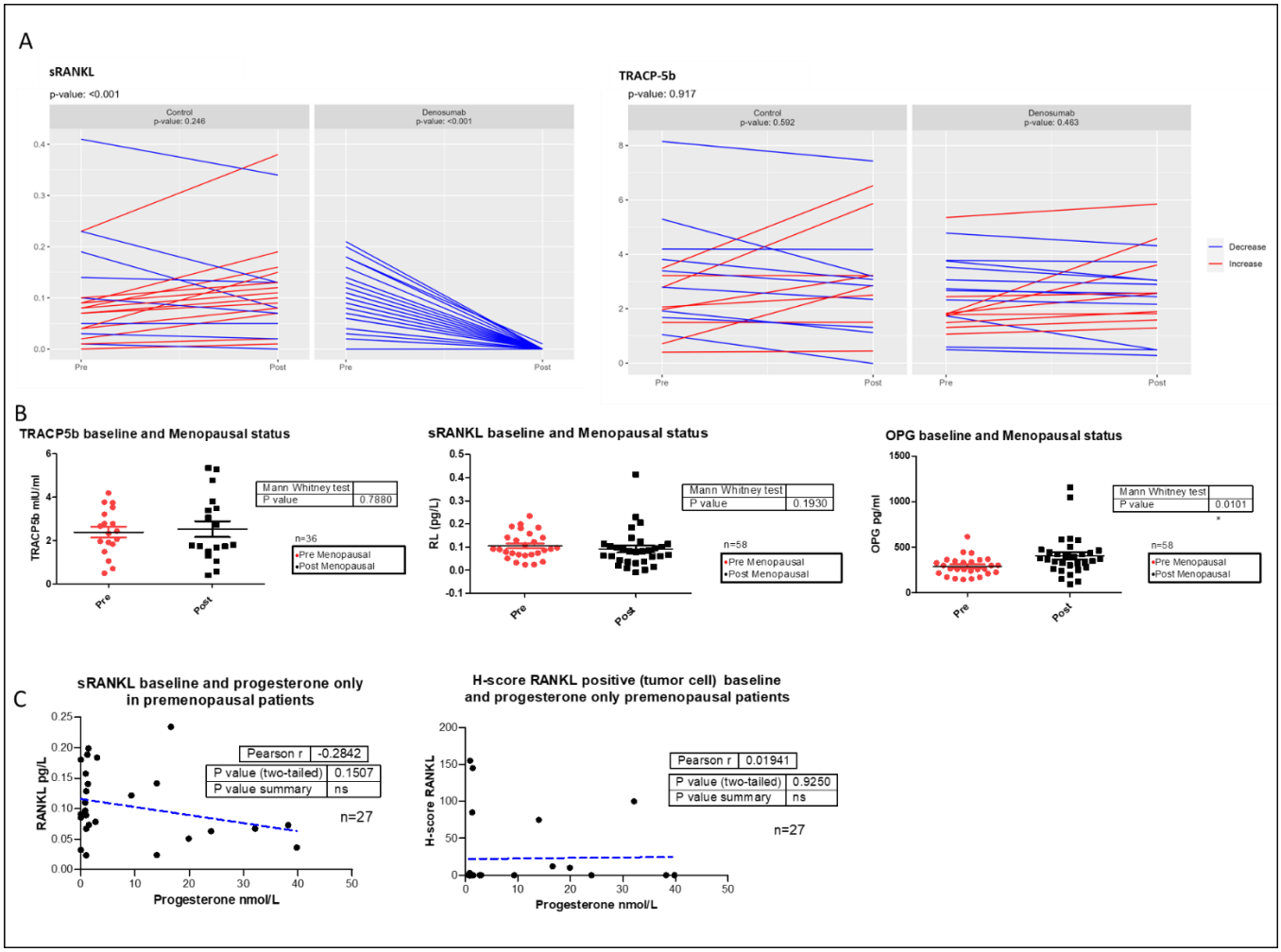

Figure 2**. A. Serum RANKL (sRANKL) and TRACP-5b levels at diagnosis and surgery in both treatment arms (control and Experimental group).** Denosumab was associated with reduction in the levels of free RANKL, but the levels of the bone remodeling marker Trap5b did not show changes in any group**. B. Serum sRANKL, TRACP5b, OPG and menopausal status.** sRANKL and TRACP5b levels at baseline were comparable between pre-and postmenopausal women, defined clinically as more than 1 year with amenorrhea, or ≥ 60 years old**.** Statistically significant higher levels of OPG were detected in postmenopausal women. C. **sRANKL and H-score RANKL (tumor) and progesterone at baseline in premenopausal patients.** No correlation was found between progesterone values and RANKL serum level or tumor expression of RANKL.

There was a very good correlation between the RANK and RANKL protein expression analyses determined in our laboratory and that outsourced to the company Neogenomics (Figure 3). RANK and RANKL protein expression in the stroma were also evaluated at Dr Gonzalez Suarez laboratory.

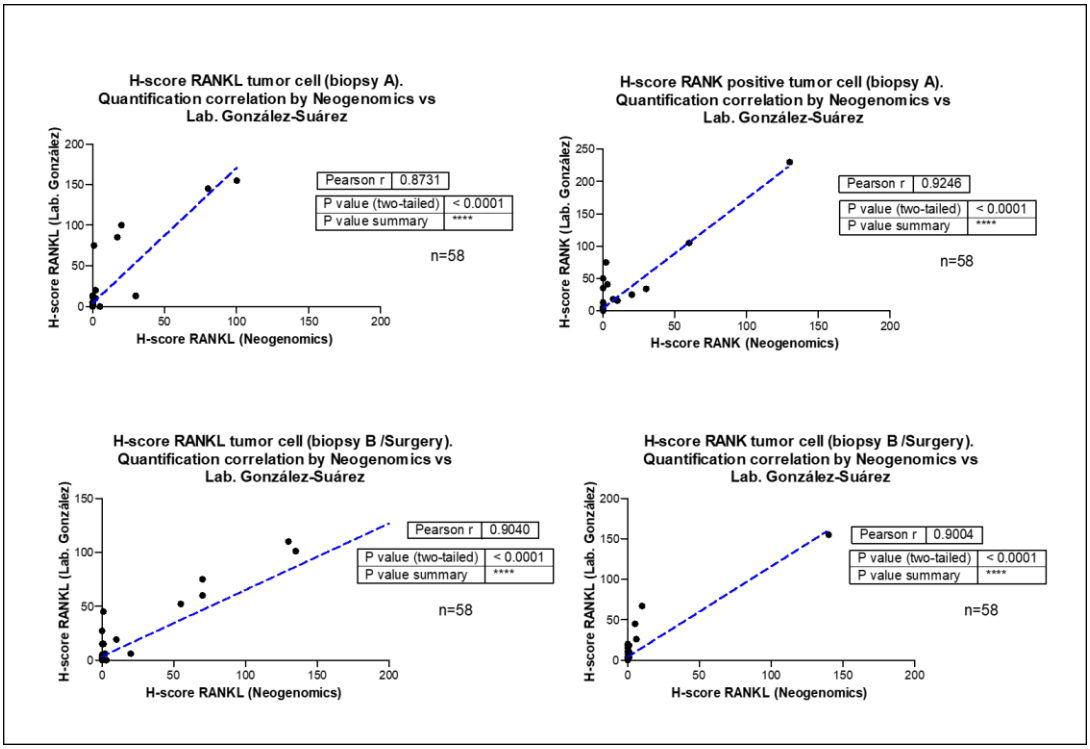

Figure 3**. RANK and RANKL tumor cell (H-score) Correlation between quantifications performed by the Negenomics laboratory (sample outsourcing) vs in-house results from Dr. González-Suárez's laboratory.**

The primary endpoints of the project were not achieved. Denosumab did not reduce tumor cell proliferation or survival among paired biopsies. Quantifications were performed blindly by two independent pathologists specialized in breast cancer. The percentage of tumor cells that express Ki67 increased in the experimental arm, but also in the control arm. The mean Ki67 increased by almost 5 percentage points when comparing the surgical samples with the baseline biopsy in both the experimental and control groups, so these changes are not explained by denosumab treatment (Figure 4). The pathologists justify this increase (slight, although statistically significant) by a greater field count in the surgical piece compared to the baseline biopsy. Quantification of Cleaved-Caspase 3 staining was done using QPath and positivity was reported by H-score. The score is obtained by the formula: 3 x percentage of strongly staining nuclei + 2 x percentage of moderately staining nuclei + percentage of weakly staining nuclei, giving a range from 0 to 300. Denosumab did not increase tumor cell apoptosis. There seems to be a greater increase in apoptosis in the controls. Possibly this is because at baseline some samples from the experimental group show very high levels of cleaved-caspase 3 (Figure 5).

No reduction in Ki67 or increase in Cleaved-Caspase 3 were observed after denosumab when tumor samples were classified according to hormonal receptor expression, histologic type, or menopausal status (Figure 4 and 5) or when the analyses was performed only in tumors expressing RANK or RANKL at baseline Figure 6.

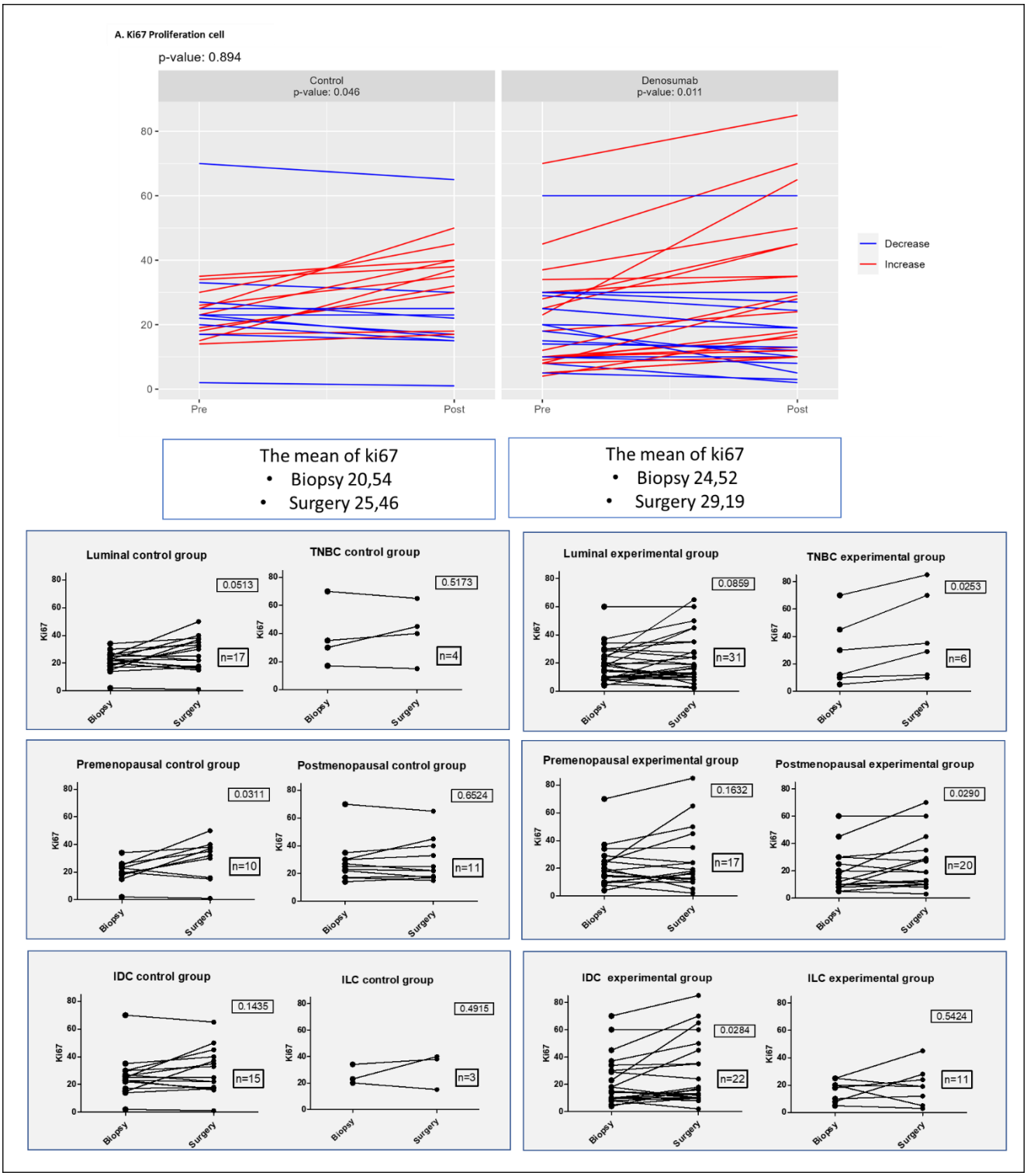

Figure 4. **Denosumab does not change tumor cell proliferation (measured by the % of Ki 67+ cells).** An increase in the % of Ki67 is evidenced by the T-test analysis for paired samples in the experimental group and in the control group. IDC: infiltrating ductal carcinoma; ILC: infiltrating lobular carcinoma

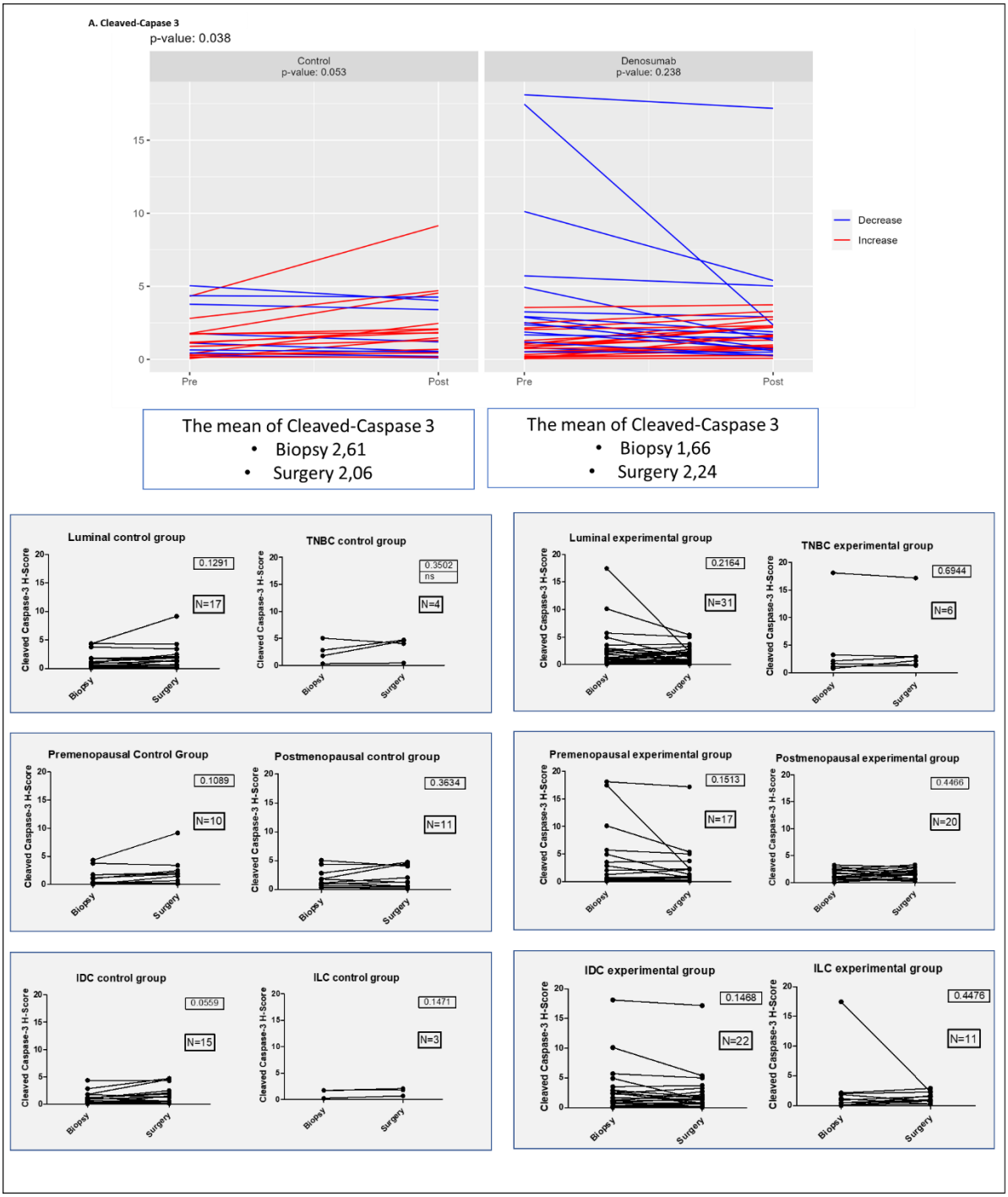

Figure 5. Denosumab does not increase tumor Cell apoptosis (measure by Cleaved-caspase 3, Qpath). There are no statistically significant changes in relation to apoptosis in the experimental group or in the control. No changes are detected in the analysis by subgroup by the T-test analysis for paired samples. IDC: infiltrating ductal carcinoma; ILC: infiltrating lobular carcinoma

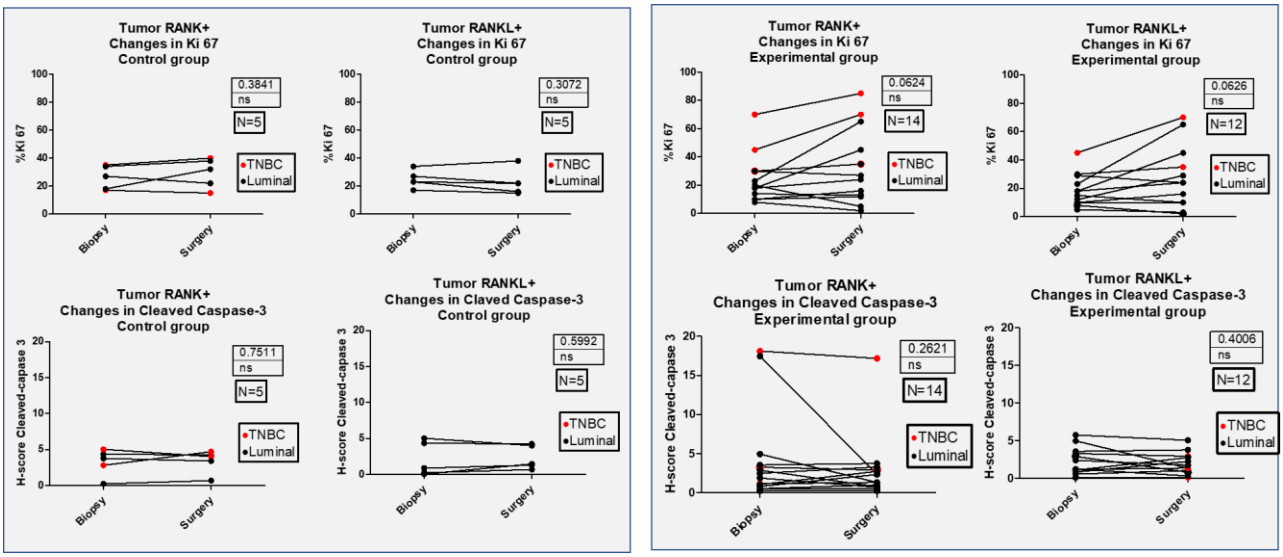

Figure 6. **Denosumab does not increase tumor Cell apoptosis (measure by Cleaved-caspase 3, Qpath) and does not reduce tumor cell proliferation (measured by the % of Ki 67+ cells) in those tumors with immunohistochemical expression of RANK or RANKL quantified by H-score**. No changes are detected in the analysis by subgroup by the T-test analysis for paired samples. RANK +: tumors with RANK immunohistochemical expression ≥1%; RANKL+: tumors with RANKL immunohistochemical expression ≥1%; IDC: infiltrating ductal carcinoma; ILC: infiltrating lobular carcinoma

6.4 Response variables and r interaction variables

Trying to elucidate possible interaction variables that could be related to changes in Ki 67 and Cleaved caspase-3 the following were tested: PRE MR={log(RANK) - 1.2} / log(RANKL), Surrogate molecular subtype, H-RANK expression baseline and H-RANKL in tumor cells and stroma cells, sRANKL at baseline (serum RANKL tested by ELISA), Menopausal status, estradiol, progesterone and follitropin values, tumor type and histological grade. Only follitropin and RANKL expression in tumor cells were detected as interacting variables for Ki67, again follitropin for Cleaved-Caspase 3, and histological grade for OPG. Table 4

Table 4. Response variables detected in the whole patient cohort number of patients and possible interaction variables.

RESPONSE VARIABLE	CONTROL	UP/ DOWN	EXPERIMENTAL	UP/ DOWN	CONTROL VS EXPERIMENTAL	INTERACTION VARIABLE	P VALUE
KI 67	**0,0462**	UP	**0,0105**	UP	0,8942	Follitropin	**0,01427996**
						Tumor cell RANKL H-score	**0,01165744**
CLEAVED-CASPASE 3	0,0527	UP	0,2381	DOWN	**0,0381**	Follitropin	**0,02514243**
sRANKL	**1,0000**	UP	**0,0000**	DOWN	**0,0541**	None with statistical significance	
OPG	**0,0411**	DOWN	0,0705	UP	**0,0124**	Histological grade	**0,00242329**
TRACP5b	0,5921	UP	0,4628	UP	0,9168	None with statistical significance	

7. Conclusion

Denosumab did not affect tumor cell survival nor cell proliferation in early breast cancer.

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