Journal Pre-proof

Wnt (canonical and non canonical) pathways in Breast carcinoma with extensive vascular invasion and Inflammatory Breast Carcinoma<!–<ForCover>Remo A, Sina S, Barbi S, Simeone I, Insolda J, Parcesepe P, Giordano G, Cerulo L, Ceccarelli M, Fiorica F, Bonetti A, Pancione M, Manfrin E, Wnt (canonical and non canonical) pathways in Breast carcinoma with extensive vascular invasion and Inflammatory Breast Carcinoma, *Pathology - Research and Practice*, doi: 10.1016/j.prp.2021.153347</ForCover>–>

A. Remo (Conceptualization) (Investigation) (Writing - original draft) (Writing - review and editing) (Project administration), S. Sina, S. Barbi (Methodology), I. Simeone (Methodology), J. Insolda (Methodology), P. Parcesepe (Methodology), G. Giordano, L. Cerulo (Conceptualization) (Investigation) (Methodology), M. Ceccarelli (Conceptualization) (Investigation) (Methodology), F. Fiorica, A. Bonetti, M. Pancione, E. Manfrin (Conceptualization) (Investigation) (Writing - original draft) (Writing - review and editing)

PII:	S0344-0338(21)00008-X
DOI:	https://doi.org/10.1016/j.prp.2021.153347
Reference:	PRP 153347
To appear in:	*Pathology - Research and Practice*
Received Date:	6 November 2020
Revised Date:	12 January 2021
Accepted Date:	15 January 2021

Please cite this article as: { doi: https://doi.org/

Wnt (canonical and non canonical) pathways in Breast carcinoma with extensive vascular invasion and Inflammatory Breast Carcinoma

Running title: **Wnt pathways and neoplastic vascular invasion**

Remo A.[1], Sina S.[1], Barbi S.[2], Simeone I.[3,4], Insolda J.[1], Parcesepe P.[2], Giordano G.[5], Cerulo L.[4,6], Ceccarelli M.[4,6], Fiorica F.[7], Bonetti A.[8], Pancione M.[4], Manfrin E.[2]

[1] Pathology Unit, ULSS9 "Scaligera"and Breast Unit (Eusoma's certification n°1030/00), Verona, Italy,

[2] Department of Pathology and Diagnosis, University of Verona, Verona, Italy

[3] Center for Genomic Science of IIT@SEMM – Istituto Italiano di Tecnologia, Milan, Italy

[4] Department of Science and Technology, University of Sannio, Benevento, Italy,

[5] U.O.C. Oncologia Medica, Ospedali Riuniti Azienda Ospedaliera Universitaria, 71122 Foggia, Italy.

[6] Bioinformatics Laboratory, BIOGEM,Ariano Irpino,Avellino, Italy.

[7] Radiotherapy Unit, ULSS9 "Scaligera"and Breast Unit (Eusoma's certification n°1030/00), Verona, Italy,

[8] Oncology Unit, ULSS9 "Scaligera"and Breast Unit (Eusoma's certification n°1030/00), Verona, Italy,

Corresponding author: Andrea Remo M.D. remino76@yahoo.it
Pathology Unit and Breast Unit (Eusoma's certification n°1030/00)
ULSS 9 "Scaligera"
ph. +39-0442622313 fax +390442622323

Erminia Manfrin M.D erminia.manfrin@univr.it
Department of Pathology and Diagnosis,
University of Verona,
Verona, Italy
Ph +39-0458124810 fax +39-0458027136

ABSTRACT

Background: Breast carcinoma with extensive peritumoral vascular invasion (ePVI-BC) is a cancer with massive vascular invasion (>10) detected in more than one slide. This neoplasm shows clinic-pathological affinity with inflammatory breast carcinoma (IBC). In this paper we evaluate their biological relationship through the study of surrogate markers (β-catenin and NFAT5) of Canonical (cWnt) and non-canonical (nWnt) Wnt pathways activation.

Methods: By immunoistochemistry, we investigate β-catenin and NFAT5 in 39 IBC, 74 ePVI-BC and 84 control cases (CG-BC).

Results: cWnt was activated in 100% of ePVI-BC, in 64% of IBC and 10% of CG-BC. nWnt was activated in 20% of ePVI-BC, 50% of IBC and 1% of CG-BC. The prognosis of carcinomas with nWnt activated was poor similar to IBC. The statistical analysis evidences as both the pathways are synergistic in malignant progression and survival time. β-catenin show an important association with prognostic factors and NFAT5 shows a relevant prognostic role on OS ($p=1.5*10^{-6}$) and DFS ($P=1,2*10^{-4}$). nWnt is associated with a worse prognosis independently of cWnt. cWnt is associated

with adverse prognosis (DFS p=0.0469; OS p=0.004891) but its prognostic role is indifferent in carcinoma with nWnt activated.

Conclusions: Canonical Wnt pathway is involved in malignant progression with dominant role for vascular invasion whereas non canonical Wnt pathway plays an important role on survival time including the capacity to identify carcinomas with IBC-like prognosis. Furthermore ePVI may represent a "prodromal form of IBC" as demonstrated by its clinicopathological and biological similarity with IBC.

Introduction

Breast carcinoma with extensive peritumoral vascular invasion (ePVI-BC) has been defined as neoplasm with presence of neoplastic emboli in the lumen of peritumoral lymphovascular spaces, evaluable in more than one slide and visible in more than 10 vascular spaces [1]. This neoplasm shows clinic-pathological affinity with Inflammatory breast carcinoma (IBC) sharing the poor prognosis [1,2]. IBC is considered the most aggressive form of primary breast cancer and recognized as T4d in neoplastic staging [2]. The clinical diagnostic aspect of IBC includes inflammatory signs *mastitis-like* thought due to the presence of numerous neoplastic dermal lymphatic emboli, that alone define "occult" inflammatory breast carcinoma [3].

Recently, system biology approach revealed the activation of canonical (cWnt) and non canonical (nWnt) pathways in IBC. This was confirmed *in vivo* by an aberrant expression of β-catenin (cWnt) and NFAT5 (nWnt) surrogate markers [4]. The activation of Wnt signaling is often associated with cell fate determination and epithelial-mesenchymal transition (EMT) whereby glandular epithelial cells undergo a genotypic and phenotypic switch from an epithelial cell to an elongated spindle cell [5] as demostrated in metaplastic [5,6] and triple negative mesenchymal-like breast carcinoma [7]. In this paper we evaluate NFAT5 and β-catenin in ePVI-BC and IBC and their prognostic role.

Material and method

Patients

In this study we enrolled three cohorts of patients affected by breast carcinoma diagnosed between January 1992 and December 2006 at the G.B. Rossi Hospital in Verona, Italy. The study groups were already enrolled according the declaration of Helsinki in previous studies published [1,4] and for the new patients a specific informant consensus was produced. Data on the patient's medical history, surgery, pathologic evaluation, and results of staging procedures (bone scan, chest film, and upper abdominal ultrasound examination) were evaluated. The specimens removed by surgical biopsy or surgery were retrospectively reviewed by two breast pathologists (E.M. and A.R.). Pathologic assessment included the primary tumor size, histologic type, histologic grade, and axillary lymph nodes status and immunoistochemical profile consisting in Estrogen and Progesteron receptors, proliferative index (ki67) and her-2 status [2]. The clinical files were examined for treatment received by patients as systemic adjuvant therapy and surgical treatment. The study groups including: i) inflammatory breast carcinoma (IBC) ii) Breast carcinoma with extensive peritumoral vascular neoplastic invasion (ePVI-BC) iii) Control group (CG-BC).

IBC (39 cases) Clinical and/or histopathologic criteria were adopted to include the patients in the IBC group. The histopathologic diagnosis of IBC was made when neoplastic emboli were observed within dermal lymphatic spaces. Clinical evidence of IBC consisted of diffuse erythema, peau d'orange, edema, warmth, tenderness, breast enlargement, and diffuse induration of the breast on palpation [1,2].

ePVI-BC (74 cases). The presence of neoplastic emboli in the lumen of peritumoral lymphovascular spaces, evaluable in more than one tumor block on H&E-stained slides, poised at more than one high power microscopic field away from the boundaries of the main tumor and visible in more than 10 vascular spaces was consistent with a diagnosis of ePVI-BC as previously reported [1].

CG-BC (84cases) A casual subset of patients affected by primary BC without histologic criteria of IBC and ePVI-BC represented the control group-BC. Patients included in the control group were

casually recruited among the first 10 consecutive breast cancer cases diagnosed each year between 1992 and 2006 [1].

Tissue microarray and immunoistochemistry

The tissue microarrays (TMAs) were constructed from the archival tissue blocks of IBC, ePVI-BC and CG-BC samples available in sufficient amount for TMA construction according instruction as previously reported [4]. Microarray sections were then reviewed to ensure that the sections from each case were morphologically similar to those of the corresponding whole tissue section and represented cancerous or normal epithelial cells. Further 3 μm-thick sections were then cut from each of the master blocks and mounted on super frost plus slides, baked at 60°C for 60 min, deparaffinized, and rehydrated through graded alcohol rinses for immunohistochemical (IHC) analyses. The presence and distribution of tissue polypeptide antigen was visualized by incubation with the specific primary antibody using Leica Bond-Max autostainer system (Milan, Italy).

All immunohistochemical staining were interpreted regardless of staining intensity by three independent investigators (P.P., A.R. and E.M.) blinded to clinical data and laboratory results. The percentage of positive cancer cells, identified by immunoreactivity for each marker, was estimated in triplicate tissue cores. At least three different representative blocks of each case were evaluated to ensure that the staining was homogeneous in the whole tumor. For each tissue section, we also evaluated the expression pattern. For NFAT5, the expression pattern was so recognized: i) absent expression (neg), ii) cytoplasmic (C), iii) nuclear/cytoplasmic (N/C) and iv) nuclear (N)(Fig.1A,B,C,D). The activation of nWnt is considered when nuclear pattern expression is present, as reported in Literature [4]. For this reason we consider NFAT5 as positive for the patterns N and N/C and negative for the pattern C and neg. For β-catenin, the expression pattern was so classified: i) absent expression (neg.), ii) membranous (M), iii) membranous/cytoplasmic (M/C) and iv) nuclear (N); (Fig.1E,F,G,H). The presence of nuclear and membranous/cytoplasmic pattern expression of β-catenin is considered the hallmark of cWnt pathway activation [8]. For this reason we consider β-

catenin as positive for the patterns N and N/C and negative for the pattern M and neg. Normal breast tissue cells adjacent to neoplastic cells served as positive internal controls. The characteristics of primary antibodies and the corresponding experimental conditions are reported previously [4].

Statistical Analysis

The Fisher exact and c2 tests were used to assess the association between categorical and ordinal variables in the three different groups of invasive cancer (IBC, ePVI-BC, and CG-BC). Disease-free survival (DFS) was defined as the length of time from the date of surgery and any relapse, the appearance of a second primary cancer, death, or the date of last follow-up visit. Overall survival (OS) was determined as the time of surgery to the date of death from any cause, or the date of the last follow-up visit. Survival plots according to age were drawn using the Kaplan-Meier method. All the calculations were performed using the R statistical software. A *P* value less than 0.05 was considered statistically significant.

Results

Clinical pathological features

Among the variables analyzed statistical differences were found between IBC, ePVI-Bc and CG-BC. No statistically differences emerged from the clinicopathologic comparison between ePVI-BC and IBC, except for the distribution of patients in different pathologic node (pN) categories and the age of patients at the onset of disease. The patients affected by ePVI-BC had mean age at diagnosis younger than IBC and CG-BC while the prognosis preserved a biological behavior intermediate between IBC and CG-BC. The clinical-pathological features characterizing study groups have been reported previously [1].

NFAT5 and β-catenin expression

β-catenin is positive in 100% of ePVI and in 64% of IBC and 10% of CG-BC. The rate of NFAT5 positive increase from 1% of CG-BC to 50% of IBC. In ePVI-BC NFAT5 was positive in 20% (Tab.I).

The different expression between the three study groups is statistically relevant (NFAT5 p-value<10^{-6}; β-catenin p-value <10^{-6};)(Tab.II,III). The co-expression of both markers has been revealed in 100% ePVI-BC, 81% IBC, and 11% of CG-BC (p-value <10^{-6})(Tab.I,IV). The expression data are summarized in Tab.I

The correlation analysis between the clinical-pathological features and the markers showed statistical associations between β-catenin and mean age at diagnosis, pT, pN, grading, size, ki67, her-2, Estrogen/Progesteron receptor status (Tab.II) and between NFAT5 and mean age at diagnosis, pT, pN, Estrogen and Progesteron receptor status (Tab.III). β-catenin showed more important association with prognostic factors (Tab.II) than NFAT5 (Tab.III) suggesting a more important role in tumorigenesis and malignant progression. A synergy between both pathways is revealed by statistical analysis evident in Tab.IV.

Overall Survival and Disease Free Survival

The Kaplan-meier representations of Disease Free Survival (DFS) and Overall Survival (OS) for NFAT5 and β-catenin show their prognostic impact (Fig.2,Tab.II,III). Breast carcinoma with NFAT5 negative has better DFS (p-value <0.000012) and OS (p-value <10^{-6})(Tab.III) as well as β-catenin negative (DFS, p-value 0.00469; OS p-value 0.004891)(Tab.II). A synergy between both pathways is revealed by statistical analysis reported in Fig.2 and Tab.IV (DFS, p-value <10^{-6}; OS p-value <10^{-6}).

Discussion

Inflammatory breast carcinoma (IBC) is a rare entity of breast cancer characterized by angioinvasivness and poor prognosis [1,2]. The IBC shows significant expression levels of multiple angiogenetic genes [9] showing an interesting clinic-pathological affinity with carcinoma with extensive vascular invasion (ePVI-BC) [1]. Recently, a significant association between β-catenin/NFAT5 expression and IBC phenotype [4] was reported suggesting an involvement of the

canonical (cWnt) and non canonical Wnt (nWnt) pathways in its tumorigenesis. The role of these pathways in ePVI-BC is still unknown.

cWnt plays a key role in the process called epitelial-mesenchimal transition (EMT) [5]. This process includes loss of adherence junction, apical-basal polarity, acquisition of mesenchymal phenotype, gain of motility and invasion. Historically, IBC is a notable exception to the association between the loss of adherence junctions (as E-cadherin) and acquisition of EMT phenotype [10]. The persistent expression of E-cadherin represents one of the immunoistochemical IBC profile [11] causing the "passive dissemination" of tumor emboli [10]. Recent studies demonstrated that breast cancer cells undergoing to EMT acquire characteristics of "tumor-initianting" or cancer stem cells (CSC) [12]. The maintenance of CSC contributes to cancer progression and is sustained by activation of cWnt [12]. In IBC cWnt is activated in 64% of cases compared to 11% of CG-BC (p-value $<10^{-6}$) probably due to the presence of CSC as reported [13]. In ePVI-BC all cases (100%) show an activation of cWnt suggesting its potential role in determinating neoplastic vascular invasion (Tab.I). Wnt genes are also inversely correlated with the expression of ER, PgR and Her-2 proteins (through an increasing of CSC) [12] as found in triple-negative mesenchymal-like [7] and metaplastic breast carcinomas [5,6,14]. Current series confirm this inverse association (ER p=0.017181; PgR p=0.015895; Her-2 p=0.018473)(tab.II) regardless the study group.

nWnt pathway includes NFAT5 as showed by Bayerlova et al. [15] through bioinformatic model applied to breast cancer expression data. This cascade can result in the activation of small GTPase, as RhoC GTPase (upregulated in IBC and basal-like breast cell line) [16]. The NFAT5 activation stimulates the expression of NF-kB and various pro-inflammatory cytokines as IL-1, IL8 and TNF-α [17] and its transactivation activity is induced by p38alpha and beta [18]. In breast cancer cells, the stress (osmotic) induces VEGF-A, through NFAT5, that synergies with pro-inflammatory IL-17 inducing cancer progression and metastasis [19,20,21]. The vasculogenic mimicry of IBC, usually driven primarly by VEGF [13], support the role of NFAT5 in its pathogenesis. The activation of nWnt

is more frequent in IBC than in CG-BC (49% vs 1%)(p-value< 10^{-6})(Tab.I) and in ePVI-BC represents the 20% of carcinomas showing poor prognosis similar to IBC.

The difference of Wnt activation (at least one pathway activated) between IBC (81%), ePVI-BC (100%)(Tab.IV) and CG-BC (11%) is statistically relevant (p< 10^{-6}). Study in vitro has supposed an inhibitor role for NFAT5 in the regulation of cWnt signals [22] but 36% of breast carcinomas with cWnt activated show nWnt activation and 80% of nWnt activated carcinomas have also cWnt activated. The statistical analysis highlights as the action of both pathways is synergistic in tumorigenesis, malignant progression and survival time (Tab.IV). The OS and DFS evidence NFAT5 as effective survival prognostic factor (p=1,2*$10^{-4;}$ DFS)(p=1.5*10^{-6} O^S). NFAT5 cascade (nWnt) makes worse the prognosis regardless the cWnt status (Fig.2). The β-catenin determines adverse prognosis on survival (Tab.II,IV)(p=0,004891; OS)(p=0,0469; DFS) but its role is insignificant in carcinomas with nWnt activated (Fig.2).

The activation of WNT pathways supports the biological similarity between IBC and ePVI-BC. For this reason, ePVI-BC could be proposed as "*prodromal form of IBC*" in which the clinical diagnostic criteria are not still manifested cause of an early diagnosis (patient younger), as previously reported [1].

In conclusion, canonical Wnt pathway (β-catenin dependent) is involved in breast tumorigenesis and malignant progression with a dominant role in identifying neoplastic angioinvasivness. Non canonical Wnt (NFAT5-related) plays an important prognostic role on survival time (DFS,OS) assuming the capacity to recognize breast carcinoma with IBC-like prognosis. Our data suggest as ePVI-BC may represent a "prodromal form of IBC" as suggested by its clinical, pathological and biological similarity with IBC.

Authors Statement:
Remo A. conceptualization, investigation, writing - original draft and writing, review and editing, Project administration
Sina S. . Image interpretation
Barbi S. Statistical support - Methodology
Simeone I. Statistical support – Methodology, Image interpretation
Insolda J. Technical support for IHC - Methodology
Parcesepe P. Technical support for IHC - Methodology
Giordano G. patients' management

Cerulo L. Conceptualization, Investigation, Statistical support - Methodology
Ceccarelli M. Conceptualization, Investigation, Statistical support - Methodology
Fiorica F. patients' management
Bonetti A. patients' management
Pancione M. Image interpretation - review and editing
Manfrin E. conceptualization, investigation, writing - original draft and writing, review and editing.

Declaration of Interest:
The Authors declare no conflict of interest

References

[1] Manfrin E, Remo A, Pancione M, Cannizzaro C, Falsirollo F, Pollini GP, Pellini F, Molino A, Brunelli M, Vendraminelli R, Ceccarelli M, Pagnotta SM, Simeone I, Bonetti F. Comparison between invasive breast cancer with extensive peritumoral vascular invasion and inflammatory breast carcinoma: a clinicopathologic study of 161 cases. Am J Clin Pathol. Sep;142(3); (2014): 299-306.

[2] Lakhani S.R., Ellis I.O., Schnitt S.J., Tan P.H., Van der Vijver. WHO classification of tumor of the breast. Lyon France: IARC Press; (2012).

[3] Charafe-Jauffret E., Mrad K., Labidi S.I., Hamida A.B., Romdhane K.B., Abdallah M.B., Ginestier C., Esterni B., Birnbaum D., Ayed F.B., Xerri L., Viens P., Mezlini A., Jacquemier J. Inflammatory breast cancers in Tunisia andFrance show similar immunphenotypes. The Breast 16, (2007): 352-358.

[4] Remo A, Simeone I, Pancione M, Parcesepe P, Finetti P, Cerulo L, Bensmail H, Birnbaum D, Van Laere SJ, Colantuoni V, Bonetti F, Bertucci F, Manfrin E, Ceccarelli M. Systems biology analysis reveals NFAT5 as a novel biomarker and master regulator of inflammatory breast cancer. J Transl Med. May 1;13; (2015):138.

[5] Hayes MJ, Thomas D, Emmons A, Giordano TJ, Kleer CG. Genetic changes of Wnt pathway genes are common events in metaplastic carcinomas of the breast. Clin Cancer Res. Jul 1;14;(2008) (13): 4038-44.

[6] Ng CKY., Piscuoglio S., Geyer FC., Burke KA., Pareja F., Eberle CA., Lim RS., Natraian R., Riaz N., Mariani O., Norton L., Vincent-Salomon A., Wen YH., Weigelt B., Reis-Filho Js. The landscape of somatic Genetic alteration in Metaplastic Breast Clin Cancer Res. Jul 15; 23(14); (2017): 3859-3870.

[7] Le du F., Echkardt B.L., Lim B., Litton J.K., Moulder S., Meric-Bernstam F., Gonzalez-Angulo A.M., Ueno N.T. Is the future of personalized therapy in triple negative breast cancer on molecular subtypes? Oncotarget 6(15): (2015); 12890-12908.

[8] Khalil S., Tan G.A., Giri D.D., Zhou X.K, Howe L.R. Activation status of Wnt/ß-catenin signaling in normal and neoplastic breast tissues: relationship to HER2/neu expression in human and mouse. PLoS One. 7(3); (2012):e33421.

[9] Van der Auwera I1, Van Laere SJ, Van den Eynden GG, Benoy I, van Dam P, Colpaert CG, Fox SB, Turley H, Harris AL, Van Marck EA, Vermeulen PB, Dirix LY. Increased angiogenesis and lymphangiogenesis in inflammatory

versus noninflammatory breast cancer by real-time reverse transcriptase-PCR gene expression quantification. Clin Cancer Res. Dec 1;10(23); (2004): 7965-71.

[10] Rodriguez FJ, Lewis-Tuffin LJ, Anastasiadis PZ. E-cadherin's dark side: possible role in tumor progression. Biochim Biophys Acta. Aug;1826(1); (2012): 23-31.

[11] Kleer CG, van Golen KL, Braun T, Merajver SD. Persistent E-cadherin expression in inflammatory breast cancer. Mod Pathol. May 14(5); (2001): 458-64.

[12]Yu QC., Verheyen EM., Zeng AY. Mammary Development and Breast Cancer: A Wnt Perspective Cancers. Cancers (Basel). Jul 13;8(7); (2016): 65.

[13] Robertson FM, Bondy M, Yang W, Yamauchi H, Wiggins S, Kamrudin S, Krishnamurthy S, Le-Petross H, Bidaut L, Player AN, Barsky SH, Woodward WA, Buchholz T, Lucci A, Ueno N, Cristofanilli M. Inflammatory breast cancer: the disease, the biology, the treatment. CA Cancer J Clin. Mar-Apr; 61(2); (2011): 134.

[14] Sulaiman A., Sulaiman B., Khouri L, McGarry S., Nessim C., Arnaout A., Li X., Addison C., Dimitroulakos J., Wang L. Both bulk and cancer stem cell subpopulations in triple-negative breast cancer are susceptible to Wnt, HDAC, and ERα coinhibition. FEBS Lett. Dec; 590 (24); (2016): 4606-4616.

[15] Bayerlová M, Klemm F, Kramer F, Pukrop T, Beißbarth T, Bleckmann A. Newly Constructed Network Models of Different WNT Signaling Cascades Applied to Breast Cancer Expression Data. Plos One. Dec 3;10(12); (2015): e0144014.

[16] Wu M, Wu ZF, Merajver SD. Rho proteins and cell-matrix interactions in cancer. Cells Tissues Organs. 185(1-3) (2007): 100-3.

[17] Li C, Liu M, Bo L, Liu W, Liu Q, Chen X, Xu D, Li Z, Jin F. NFAT5 participates in seawater inhalation-induced acute lung injury via modulation of NF-κB activity. Mol Med Rep. Dec;14(6) (2016): 5033-5040.

[18] Libert S, Willermain F, Weber C, Bryla A, Salik D, Gregoire F, Bolaky N, Caspers L, Perret J, Delporte C..Involvement of TonEBP/NFAT5 in osmoadaptative response of human retinal pigmented epithelial cells to hyperosmolar stress. Mol Vis. Jan 31;22; (2016):100-15

[19] Aramburu J, Drews-Elger K, Estrada-Gelonch A, Minguillón J, Morancho B, Santiago V, López-Rodríguez C. Regulation of the hypertonic stress response and other cellular functions by the Rel-like transcription factor NFAT5. Biochem Pharmacol. Nov 30;72(11); (2006):1597-604.

[20]Amara S, Alotaibi D, Tiriveedhi V. NFAT5/STAT3 interaction mediates synergism of high salt with IL-17 towards induction of VEGF-A expression in breast cancer cells. Oncol Lett Aug;12(2); (2016): 933-943.

[21] Guo K., Jin F. NFAT5 promotes proliferation and migration of lung adenocarcinoma cells in prt through regulating AQP5 expression. Bioch Biophys Res Comm; 465; (2015): 644-649.

[22] Wang Q, Zhou Y, Rychahou P, Liu C, Weiss HL, Evers BM. NFAT5 represses canonical Wnt signaling via inhibition of β-catenin acetylation and participates in regulating intestinal cell differentiation. Cell Death Dis. Jun 13;4: (2013): e671.

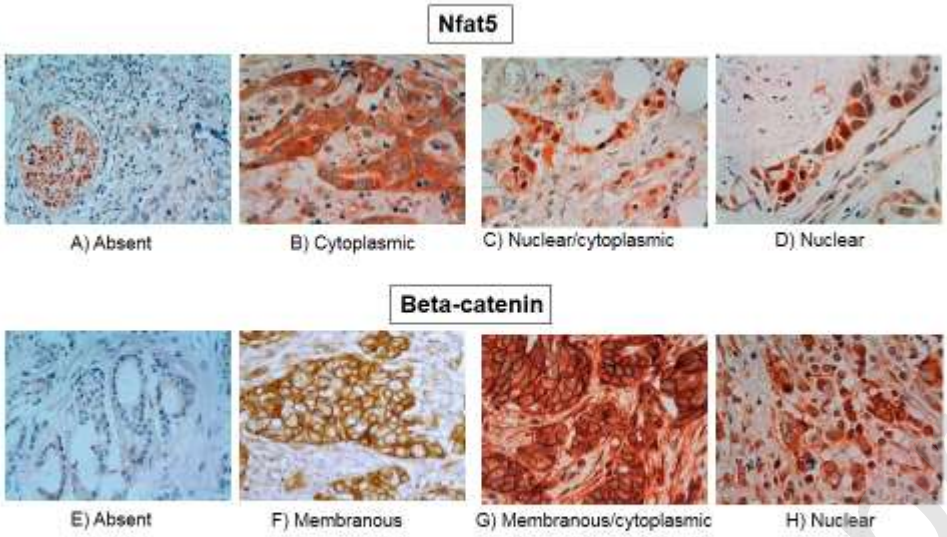

Fig.1 Expression Patterns for NFAT5 and β-catenin. NFAT5: A) absent expression (neg); B) cytoplasmic (C); C) nuclear/cytoplasmic (N/C); D) nuclear (N). NFAT5 was considered positive for patterns N and N/C 4. β-catenin: E) absent expression (neg); F) membranous (M); G) membranous/cytoplasmic (M/C); H) nuclear (N). β-catenin was considered positive for patterns M/C and N 8.

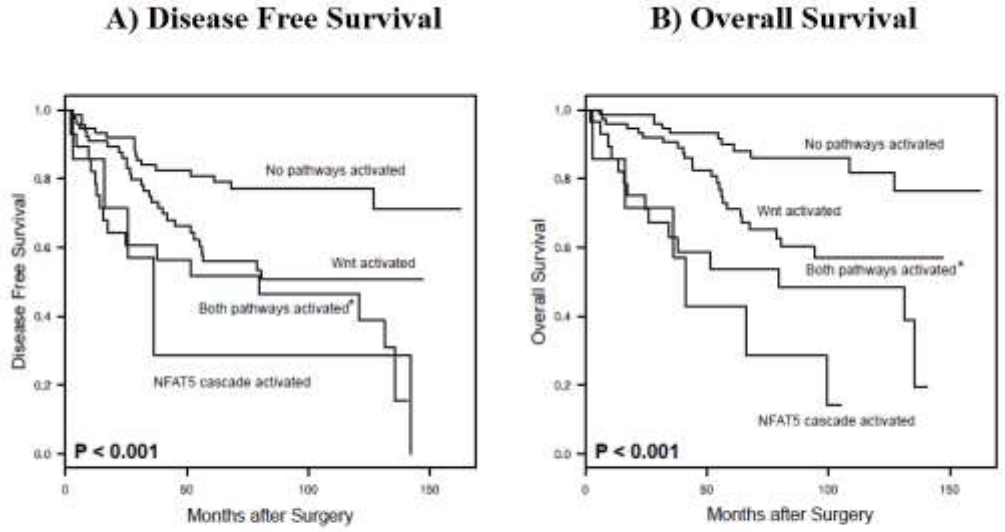

Fig.2 Kaplan-Meier representation of Disease free (DFS) and Overall Survival (OS) rates by pathway activation. The stratification of breast cancer for activation of both pathways evidences as NFAT5 cascade makes worse the prognosis of patient regardless canonical Wnt. The beta-catenin determines an adverse prognosis but its prognostic role is insignificant for OS and DFS in carcinoma with NFAT5 activated. The activation at least one of pathways (*) stratifies with statistical difference (p<10-6) in IBC (81%), ePVI-BC (100%) and CG-BC (11%).

Table I. Rate of marker expression stratified by study groups. The grey zone identifies the cases in which almost one pathway is activated.

A. Markers Expression in IBC		**Pathways activity = 81%**			
Beta-catenin/NFAT5	Nuclear	Nuclear/cytoplasmic	Cytoplasmic	Absent	Total
Nuclear	1 (3%)	2 (5%)	1 (3%)	1 (3%)	5 (13%)
Membranous/Cytoplasmic	1 (3%)	9 (23%)	3(8%)	7 (18%)	20 (51%)
Membranous	3 (8%)	3 (8%)	4 (10%)	2 (5%)	12 (31%)
Absent	0 (0%)	0 (0%)	1 (3%)	1 (3%)	2 (5%)
Total	5 (13%)	14 (36%)	9 (23%)	11 (28%)	39

B. Markers Expression in ePVI-BC		**Pathways activity = 100%**			
Beta-catenina/ NFAT5	Nuclear	Nuclear/cytoplasmic	Cytoplasmic	Absent	Total
Nuclear	0 (%)	0 (%)	4 (5%)	2 (3%)	6 (8%)
Membranous/Cytoplasmic	4 (5%)	11 (15%)	21 (28%)	32 (44%)	68 (92%)
Membranous	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Absent	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Total	4 (5%)	11 (15%)	25 (34%)	34 (46%)	74

C. Markers Expression in CG-BC		**Pathways activity = 11%**			
Beta-catenina/NFAT5	Nuclear	Nuclear/cytoplasmic	Cytoplasmic	Absent	Total
Nuclear	0 (%)	0 (%)	1 (1%)	0 (%)	1 (1%)
Membranous/Cytoplasmic	0 (%)	0 (%)	2 (2%)	6 (7%)	8 (10%)
Membranous	0 (%)	0 (%)	12 (14%)	46 (56%)	58 (69%)
Absent	0 (%)	1 (1%)	1 (1%)	15 (18%)	17 (20%)
Total	0 (%)	1 (1%)	16 (19%)	67 (80%)	84

Table II. Correlation analysis between clinical-pathological variables and activation of canonical ("β-catenin dependent") Wnt pathway.

TABLE II

Variables		**Beta-catenin**		**OR (95% CI)**	**P-value**
		Negative	**Positive**		
Study Groups	*IBC*	14 (15.6%)	25 (23.1%)	1.0	**< 10^{-6}**
	ePVI-Bc	0 (0%)	74 (68.5%)	Inf	
	CG-BC	76 (84.4%)	9 (8.3%)	0.1 (0 - 0.2)	
Age	*median (IQR)*	59.5 (50.7 - 69.5)	51.9 (40.9 - 63.5)		**0.000683**
pT	*T1*	58 (68.2%)	38 (35.5%)	1.0	**0.000088**
	T2	14 (16.5%)	38 (35.5%)	4.1 (2 - 8.9)	
	T3	1 (1.2%)	5 (4.7%)	7.6 (1.2 - 149.3)	
	T4	12 (14.1%)	26 (24.3%)	3.3 (1.5 - 7.5)	
pN	*N0*	51 (68.9%)	19 (19.6%)	1.0	**< 10^{-6}**
	N1	12 (16.2%)	35 (36.1%)	7.8 (3.5 - 18.8)	
	N2	8 (10.8%)	23 (23.7%)	7.7 (3.1 - 21.3)	
	N3	3 (4.1%)	20 (20.6%)	17.9 (5.4 - 82.4)	
pM	*M0*	3 (3.6%)	4 (3.9%)	1.0	0.923545
	M1	80 (96.4%)	99 (96.1%)	0.9 (0.2 - 4.3)	
Grading	*G1*	17 (21%)	3 (2.9%)	1.0	**< 10^{-6}**
	G2	42 (51.9%)	28 (26.7%)	3.8 (1.1 - 17.3)	
	G3	22 (27.2%)	74 (70.5%)	19.1 (5.8 - 87.2)	
Size	*median (IQR)*	15 (10 - 22)	22 (18 - 30)		**0.000002**
Ki67	*High*	15 (17.6%)	28 (26.4%)	1.0	**0.018406**

	Low	55 (64.7%)	47 (44.3%)	0.5 (0.2 - 0.9)	
	Medium	15 (17.6%)	31 (29.2%)	1.1 (0.5 - 2.7)	
Her2	*0*	54 (66.7%)	55 (51.9%)	1.0	**0.018473**
	1+	11 (13.6%)	8 (7.5%)	0.7 (0.3 - 1.9)	
	2+	4 (4.9%)	12 (11.3%)	2.9 (1 - 11.1)	
	3+	12 (14.8%)	31 (29.2%)	2.5 (1.2 - 5.6)	
Er	*Negative*	13 (15.3%)	31 (29.8%)	1.0	**0.017181**
	Positive	72 (84.7%)	73 (70.2%)	0.4 (0.2 - 0.9)	
PgR	*Negative*	18 (22.8%)	41 (39.4%)	1.0	**0.015895**
	Positive	61 (77.2%)	63 (60.6%)	0.5 (0.2 - 0.9)	
DFS	*1 year*	0.94 (0.89 - 0.99)	0.88 (0.82 - 0.95)		**0.00469**
	2 years	0.90 (0.84 - 0.97)	0.79 (0.72 - 0.88)		
	5 years	0.76 (0.68 - 0.86)	0.55 (0.45 - 0.67)		
OS	*1 year*	0.98 (0.94 - 1.00)	0.93 (0.88 - 0.98)		**0.004891**
	2 years	0.96 (0.93 - 1.00)	0.87 (0.81 - 0.94)		
	5 years	0.86 (0.78 - 0.94)	0.66 (0.57 - 0.77)		

Table III. Correlation analysis between clinical-pathological variables and activation of non canonical (Nfat5-related) Wnt pathway.

TABLE III

Variables		**NFAT5**		**OR (95% CI)**	**P-value**
		Negative	**Positive**		
Study Groups	*IBC*	20 (12.3%)	19 (54.3%)	1.0	**$< 10^{-6}$**
	ePVI-BC	59 (36.4%)	15 (42.9%)	0.3 (0.1 - 0.6)	
	CG-BC	83 (51.2%)	1 (2.9%)	0 (0 - 0.1)	
Age	*median (IQR)*	54.6 (46.2 - 64.6)	59.1 (44.9 - 70.2)		0.462395
pT	*T1*	87 (55.8%)	9 (25.7%)	1.0	**$< 10^{-6}$**
	T2	46 (29.5%)	6 (17.1%)	1.3 (0.4 - 3.7)	
	T3	5 (3.2%)	0 (0%)	0.0	
	T4	18 (11.5%)	20 (57.1%)	10.7 (4.3 - 28.6)	
pN	*N0*	63 (44.7%)	6 (20.7%)	1.0	**0.017861**
	N1	39 (27.7%)	8 (27.6%)	2.2 (0.7 - 7)	
	N2	20 (14.2%)	11 (37.9%)	5.8 (1.9 - 18.7)	
	N3	19 (13.5%)	4 (13.8%)	2.2 (0.5 - 8.6)	

pM	*M0*	4 (2.6%)	3 (8.8%)	1.0	0.126141
	M1	147 (97.4%)	31 (91.2%)	0.3 (0.1 - 1.5)	
Grading	*G1*	19 (12.6%)	1 (2.9%)	1.0	0.056152
	G2	59 (39.1%)	10 (29.4%)	3.2 (0.6 - 61)	
	G3	73 (48.3%)	23 (67.6%)	6 (1.1 - 110.5)	
Size	*median (IQR)*	18 (12 - 25)	20 (15 - 30)		0.176039
Ki67	*High*	37 (23.9%)	6 (17.1%)	1.0	0.623387
	Low	82 (52.9%)	19 (54.3%)	1.4 (0.6 - 4.2)	
	Medium	36 (23.2%)	10 (28.6%)	1.7 (0.6 - 5.5)	
Her2	*0*	88 (58.3%)	21 (60%)	1.0	0.817897
	1+	16 (10.6%)	2 (5.7%)	0.5 (0.1 - 2)	
	2+	13 (8.6%)	3 (8.6%)	1 (0.2 - 3.3)	
	3+	34 (22.5%)	9 (25.7%)	1.1 (0.4 - 2.6)	
Er	*Negative*	31 (19.7%)	13 (41.9%)	1.0	**0.01134**
	Positive	126 (80.3%)	18 (58.1%)	0.3 (0.2 - 0.8)	
Pgr	*Negative*	37 (24.7%)	22 (68.8%)	1.0	**0.000003**
	Positive	113 (75.3%)	10 (31.3%)	0.1 (0.1 - 0.3)	
DFS	*1 year*	0.93 (0.89 - 0.97)	0.83 (0.71 - 0.96)		**0.000012**
	2 years	0.89 (0.84 - 0.95)	0.66 (0.52 - 0.83)		
	5 years	0.69 (0.62 - 0.78)	0.47 (0.32 - 0.67)		
OS	*1 year*	0.93 (0.89 - 0.97)	0.83 (0.71 - 0.96)		**$< 10^{-6}$**
	2 years	0.89 (0.84 - 0.95)	0.66 (0.52 - 0.83)		
	5 years	0.69 (0.62 - 0.78)	0.47 (0.32 - 0.67)		

Table IV Correlation analysis between clinical-pathological variables and activation of almost one of both pathway.

TABLE IV

Variables		**Beta-catenin and/or NFAT5**		**OR (95% CI)**	**P-value**
		Negative	**Positive**		
Study Groups	*IBC*	8 (9.8%)	31 (27%)	1.0	**$< 10^{-6}$**
	ePVI-Bc	0 (0%)	74 (64.3%)	Inf	
	CG-BC	74 (90.2%)	10 (8.7%)	0 (0 - 0.1)	
Age	*median (IQR)*	59.5 (51.4 - 69.5)	52.2 (41 - 64)		**0.001484**
pT	*T1*	57 (74%)	39 (34.2%)	1.0	**$< 10^{-6}$**
	T2	14 (18.2%)	38 (33.3%)	4 (1.9 - 8.5)	
	T3	0 (0%)	5 (4.4%)	Inf	

	T4	6 (7.8%)	32 (28.1%)	7.8 (3.2 - 22.3)	
pN	*N0*	48 (70.6%)	21 (20.6%)	1.0	**< 10^{-6}**
	N1	12 (17.6%)	35 (34.3%)	6.7 (3 - 15.8)	
	N2	5 (7.4%)	26 (25.5%)	11.9 (4.3 - 39.1)	
	N3	3 (4.4%)	20 (19.6%)	15.2 (4.6 - 69.8)	
pM	*M0*	2 (2.7%)	5 (4.5%)	1.0	0.501959
	M1	73 (97.3%)	105 (95.5%)	0.6 (0.1 - 2.7)	
Grading	*G1*	17 (23.3%)	3 (2.7%)	1.0	**< 10^{-6}**
	G2	39 (53.4%)	30 (26.8%)	4.4 (1.3 - 19.9)	
	G3	17 (23.3%)	79 (70.5%)	26.3 (7.8 - 122.2)	
Size	*median (IQR)*	15 (10 - 20)	22 (15 - 30)		**< 10^{-6}**
Ki67	*High*	14 (18.2%)	29 (25.7%)	1.0	**0.023702**
	Low	50 (64.9%)	51 (45.1%)	0.5 (0.2 - 1)	
	Medium	13 (16.9%)	33 (29.2%)	1.2 (0.5 - 3.1)	
Her2	*0*	50 (68.5%)	59 (52.2%)	1.0	**0.007476**
	1+	10 (13.7%)	8 (7.1%)	0.7 (0.2 - 1.8)	
	2+	4 (5.5%)	12 (10.6%)	2.5 (0.8 - 9.5)	
	3+	9 (12.3%)	34 (30.1%)	3.2 (1.5 - 7.7)	
Er	*Negative*	11 (14.3%)	33 (29.7%)	1.0	**0.011873**
	Positive	66 (85.7%)	78 (70.3%)	0.4 (0.2 - 0.8)	
PgR	*Negative*	14 (19.7%)	45 (40.5%)	1.0	**0.002804**
	Positive	57 (80.3%)	66 (59.5%)	0.4 (0.2 - 0.7)	
DFS	*1 year*	0.95 (0.90 - 1.00)	0.88 (0.82 - 0.95)		**< 10^{-6}**
	2 years	0.92 (0.86 - 0.98)	0.79 (0.71 - 0.87)		
	5 years	0.81 (0.72 - 0.90)	0.53 (0.43 - 0.64)		
OS	*1 year*	0.99 (0.96 - 1.00)	0.93 (0.88 - 0.98)		**< 10^{-8}**
	2 years	0.99 (0.96 - 1.00)	0.86 (0.80 - 0.93)		
	5 years	0.90 (0.83 - 0.97)	0.65 (0.56 - 0.75)		