scientific reports

Check for updates

OPEN

Role of immune-checkpoint LAG3 as a biomarker finding tool in patient-derived organoid cultures of breast cancer

Barbara Carrese[1], Luigi Coppola[1], Giovanni Smaldone[1], Massimiliano D'Aiuto[2], Gennaro Mossetti[3], Antonio Febbraro[4], Andrea Soricelli[1], Marco Salvatore[1] & Vincenza Ciaramella[1]✉

LAG3 plays a regulatory role in immunity and emerged as an inhibitory immune checkpoint molecule comparable to PD-L1 and CTLA-4 and a potential target for enhancing anti-cancer immune responses. We generated 3D cancer cultures as a model to identify novel molecular biomarkers for the selection of patients suitable for α-LAG3 treatment and simultaneously the possibility to perform an early diagnosis due to its higher presence in breast cancer, also to achieve a theragnostic approach. Our data confirm the extreme dysregulation of LAG3 in breast cancer with significantly higher expression in tumor tissue specimens, compared to non-cancerous tissue controls. LAG3 blockade inhibited proliferation of in vitro and ex vivo 3D human organoids and immune micro-environment through both a decrease of PD-L1, TIM-3 and CTLA4 expression and an increased production of several pro-inflammatory cytokines (IFNγ, IL-12, IL-6, IL-1β, TNFα) and EMT markers. These effects trigger a more permissive anti-tumor immune reaction, recruiting immune cells to the tumor sites, boosting the anti-tumor response. LAG3 acts as an immunosuppressive molecule in breast cancer, inhibiting T CD8 + cell proliferation and cytokine production by T cells. We proposed the modulation of a novel checkpoint molecule, such as LAG3, as potential biomarkers associated to a rapid diagnosis.

Keywords Breast cancer, Organoids, LAG3, Biomarkers, Immune micro-environment

Abbreviations

CTL	Cytotoxic CD8 T cells
EMT	Epithelial to mesenchymal transition
LAG3	Lymphocyte activation gene-3
CLL	Lymphocytic leukemia
NK	Natural killer
PDOs	Patient's derived organoids
Treg	Regulatory T
Th	T helper
3D	Three-dimensional
TME	Tumor microenvironment
CTRL	Untreated control

Tumors are a major public health concern and, currently, immunotherapy is the most promising tumor treatment. Indeed, the immune system has an essential role in the surveillance against tumors, but cancer cells develop several strategies to escape immune surveillance[1]. Escaping the surveillance of the immune system, through which proliferation and growth of cancer cells can be successfully achieved, is one of the main mechanisms of development and progression of a tumor[2]. In this context, the immune checkpoints are crucial molecules expressed on the surface of cancer cells or immune cells, particularly on T cells. These molecules can negatively or positively regulate immune responses, as well as mitigate immune injury and prevent the development of

[1]IRCCS SYNLAB SDN, Naples 80143, Italy. [2]Clinica Villa Fiorita, Aversa 81031, Italy. [3]Pathological Anatomy Service, Maria Rosaria Clinic, Pompei, Naples 80045, Italy. [4]Oncology Unit, Casa di Cura Cobellis, Vallo della Lucania, Italy. ✉email: vincenza.ciaramella@synlab.it

autoimmunity to minimize the tissue damage. Several evidence showed that inhibitory or stimulatory immune checkpoint molecules are expressed on a sizeable fraction of tumor types. Furthermore, immune checkpoint molecules regulate not only the epithelial-mesenchymal transition (EMT) and consequently the formation of metastases, but also other processes such as drug resistance, anti-apoptosis, angiogenesis, or increased energy metabolism[3].

Back in 1990, Dr. Frédéric Triebel and colleagues identified Lymphocyte activation gene-3 (LAG3) as a novel lymphocyte activation gene closely related to CD4, resulting in approximately 26% amino acid homology. The authors found a 2-kb mRNA to be selectively transcribed in activated human T and natural killer cells (NK)[4]. LAG3, also named CD223, is the third inhibitory receptors to be targeted in the clinic[5]. Moreover, it is an immune inhibitory checkpoint and is expressed by different T cell subsets such as activated CD4 T helper (Th) and cytotoxic CD8 T cells (CTL), NK cells and regulatory T (Treg) cells[6], as well as stored in the lysosomes, which appear on the surface faster when T cells are activated[7]. The dysregulated expression of LAG3 was reported to be negatively correlated with clinical outcome in a wide variety of cancers, including both hematologic (e.g., chronic lymphocytic leukemia CLL, lymphoma[8] and solid tumor, such as non-small cell lung cancer, colorectal cancer, hepatocellular carcinoma, and muscle invasive bladder cancer. All these tumor conditions show an increased expression of LAG3 correlated with a poor prognosis[9]. However, the prognostic value of LAG3 in breast cancer is controversial because it is associated with breast cancer stage, tumor size, tumor grade, and ER/PR/HER2 status[10]. The discrepancy in breast cancer can be attributed to the various immune microenvironment of breast cancer subtypes. Moreover, LAG3 is considered a potential target of next-generation cancer immunotherapy in human, right next to the well-known PD-1 and CTLA-4, even if, nowadays, its activity and relationship with other immune checkpoint molecules remain poorly understood. Currently, Relatlimab (anti-LAG3) is tested in phase 2–3 double-blind randomized clinical trial, a human IgG4 LAG3–blocking antibody that acts through the binding to LAG3 to restore the function of exhausted T cells[11].

Here, we mainly focus on delineating the role of LAG3 on cancer immune evasion. Furthermore, our aim was to understand the mechanism underlying a possible involvement in the development of breast cancer at an early stage to hypothesize LAG3 as a diagnostic marker.

Methods

Patients' selection

The present study comprised $n = 12$ female patients who underwent breast surgery at the Clinica Villa Fiorita S.p.A (Aversa, Italy), between June 2023 and January 2024. We enrolled only female patients because the incidence of male breast cancer in approximately 1% of all breast cancers worldwide[12]. The study was approved by the Ethics Committee of IRCCS Pascale (Naples, Italy) with reference number 3/19 approved on 29 May 2019. All human samples were collected after obtaining a written informed consensus from any patient and healthy donor, in accordance with the Declaration of Helsinki (Table 1). All below described methods were performed in accordance with guidelines and regulations.

Cell lines and drugs

The human normal (MCF10a) and breast cancer cell lines (MDA-MB-231, T47D, BT474, SKBR3) were provided by IRCCS Synlab SDN Biobank[13] and maintained in DMEM (GIBCO) medium supplemented with 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD) in a humidified atmosphere with 5% CO_2. The identity of all cell lines was confirmed by STR profiling (Promega) on an ad hoc basis prior to perform experiments.

Relatlimab, an anti-LAG3 specific monoclonal antibody (α-LAG3) that binds to LAG3 on T cells (BMS-986016, RELA) was purchased from Selleck Chemicals, Munich, Germany.

Generation of ex vivo 3D cultures from patient samples

We developed a protocol for ex-vivo 3D organoid cultures (patient's derived organoids, PDOs) from breast cancer patient samples[14]. The protocol has been approved by our local Ethics Committee and all patients gave their written informed consent to the use of the tumor sample. All fresh tumor tissue samples were kept on ice and processed in sterile conditions on the day of collection. Tissue fragments were digested in a 37 °C shaker at low to moderate speed (e.g., 200 rpm) for incubation time between 12 and 18 h and cells were separated with serial centrifugation[15,16]. For 3D cultures, cells were seeded in matrigel to preserve three-dimensional structure.

Quantitative real time PCR

Total RNA was extracted using Trizol reagent (Life Technologies). Reverse transcriptase reaction was carried out to convert 1 µg of isolated RNA into cDNA using sensi fast reverse transcriptase (bioline), according to the manufacturer instruction. Expression levels of genes encoding for: IFN-γ, IL-12, IL-1β, TNFα, IL-6, PD-L1, TIM-3, CTLA-4 and LAG3, as well as E-Cadherin and Vimentin, were analyzed using Real time quantitative PCR (RT-qPCR). Gene specific primers were designed by using PRIMER EXPRESS software (Applied Biosystems). Amplifications were done using the SYBR Green PCR Master Mix (Applied Biosystems). The thermal cycling conditions were composed of 50 °C for 2 min (stage 1) followed by a denaturation step at 95 °C for 10 min (stage 2) and then 40 cycles at 95 °C for 15 s and 60 °C for 1 min (stage 3). All samples were run in duplicate, in 25 µL reactions using a quant studio 7 flex (Applied Biosystems) and relative expression of genes was determined by normalizing to Actin, used as internal control gene; to calculate relative gene expression in value it was used the 2-ΔCt or 2-ΔΔCt method. Nonspecific signals caused by primer dimers were excluded by dissociation curve analysis and use of non-template controls.

PDOs patients	12
Breast cancer ($n=6$)	
Sex	
Woman	6
Man	0
Histologic types	
Invasive breast cancer "no special type"	5
Invasive lobular carcinoma	1
Ki67	
Low (0–29%)	3
High (30–100%)	3
Grade	
G1	0
G2	5
G3	1
Malignant tumor size	
(0.1–2 cm)	2
(2–5 cm)	4
Fibroadenoma ($n=6$)	
Sex	
Woman	6
Man	0
Histologic types	
Intracanalicular fibroadenoma	5
Intra- and peri-canalicular fibroadenoma	1
Benign tumor size	
(0.1–2 cm)	2
(2–5 cm)	4

Table 1. Clinical-pathological characteristics of the patients.

Cell viability assay

Cell viability was measured with MTS [3- (4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium] assay. The protocol is based on the reduction of the MTS tetrazolium compound by viable mammalian cells (and cells from other species) to generate a colored formazan dye that is soluble in cell culture media. The formazan dye is quantified by measuring the absorbance at 490 nm. Briefly, cells were seeded at a density of 2000 cells/well in a 96-well microtiter plate in a final volume of 100 µl/well and treated with Relatlimab (α-LAG3) at 10 µg/mL for 5 days. Then, 20 µl/well of MTS reagent was added into each well and incubated for 1 h at 37 °C. Finally, the absorbance of treated and untreated cells was measured using the Victor Nivo Multimode Microplate Reader (PerkinElmer) at 490 nm absorbance.

Immunofluorescence

To characterize PDOs, confocal microscopy analysis was performed. To this aim, images were acquired after three days of formation. Cell nuclei were stained with Hoechst 33,342 (Thermo Scientific™, #H3570) at a final concentration of 1 µg/mL for 1 h at 37 °C. Then, to demonstrate morphology and lymphocyte presence on PDO organoids, CD326 -APC, also known as EpCAM (Epithelial Cell Adhesion Molecule) (Beckman Coulter, #B90408) and PE-Cy™7 Mouse Anti-Human CD45 (BD Pharmingen™, #560915) were utilized, respectively, in a ratio of 1:100 for 1 h at 37 °C. Moreover, to characterize LAG3 expression in tumor and non-cancerous ex vivo PDOs, organoids were incubated with LAG3 (D2G40, Cell Signaling) 1:100 for 1 h at RT and, subsequently, with Alexa Fluor™ 647 donkey α-rabbit IgG (H + L) (Invitrogen #A31573) 1:200 for 1 h at RT in dark condition. Maximal projection images were acquired by confocal microscopy (Mica, Leica Microsystems, Wetzlar, Germany) with a 63x magnification and saved in TIFF format.

Flow cytometry analyses

After 5 days, all patients' PDOs treated, as described before[17], were analyzed using CytoFLEX (Beckman Coulter, Brea, CA, USA). Specifically, PDOs were stained for surface marker detection using the following antibody mixture: CD8-PE, CD4-FITC, CD3-PE-Cyp5, HLA-DR-PB450, EpCAM-APC and CD45-KO. Each antibody was prepared in a 1:10 dilution and mixed throughout. All the antibodies used were purchased from Beckman Coulter. The Kaluza 2.1 software (Beckman Coulter, USA) was used for immune cell characterization. For cytokines evaluation we used the Human Inflammation Panel 1 from LEGENDplexTM (740809, lot. B347171, Biolegend, USA) following the manufacturer instruction. 50uL of tumor and fibroadenoma PDO supernatants

were used for the assay. Cytokines signals were detected using CytoFLEX (Beckman Coulter, Brea, CA, USA) and analyzed using the Biolegend software.

Statistical analysis

Experiments were carried out in triplicate in three independent experiments and results of the assays were expressed as mean ± SD. Statistical analysis was performed using Graphpad Prism software version 9.0 (Graphpad Software Inc., San Diego, CA, USA). For heatmap image data were visualized through the HeatMap plot generated by using the pheatmap R package on CRAN. Data were compared with Ordinary One-way ANOVA statistical test followed by Tukey's test. P values less than 0.05 were considered statistically significant.

Results

Characterization of LAG3 in patient derived organoids of breast cancer

To evaluate the LAG3 expression, we performed analysis of mRNA level by RT-qPCR (Fig. 1) in a panel of 4 breast cancer cell lines, comparing them with MCF10a cell line, a human non-tumorigenic epithelial model. As shown, a high LAG3 expression was observed in all 4 breast cancer cell lines analyzed compared to MCF10a used as control cell line. Also, a high LAG3 expression was observed in a triple-negative breast cancer cell line MDA-MB-231, compared to BT74, T47D, SKBR3 cell lines. For the main characteristics of the selected cell lines with the site of origin, breast cancer subtype, receptor expression, morphological features, common uses and doi see in the supplementary materials (Table S1).

We established in parallel ex vivo 3D breast cancer cultures obtained by surgical samples to estimate the feasibility of both in vitro and ex vivo approaches. The human samples were processed by enzymatic digestion to derive ex vivo PDOs primary cell cultures (Fig. 2); we represent a valid model to study cells and the tumor microenvironment, since they are multicellular organotypic spheroid cultures that preserve the inter-cellular interactions and maintain the genetic diversity of the in vivo tumor.

We were able to establish 12/16 organoid cultures from biopsy specimens with a total of 75% of successful establishment rate, which agrees to literature data[18].

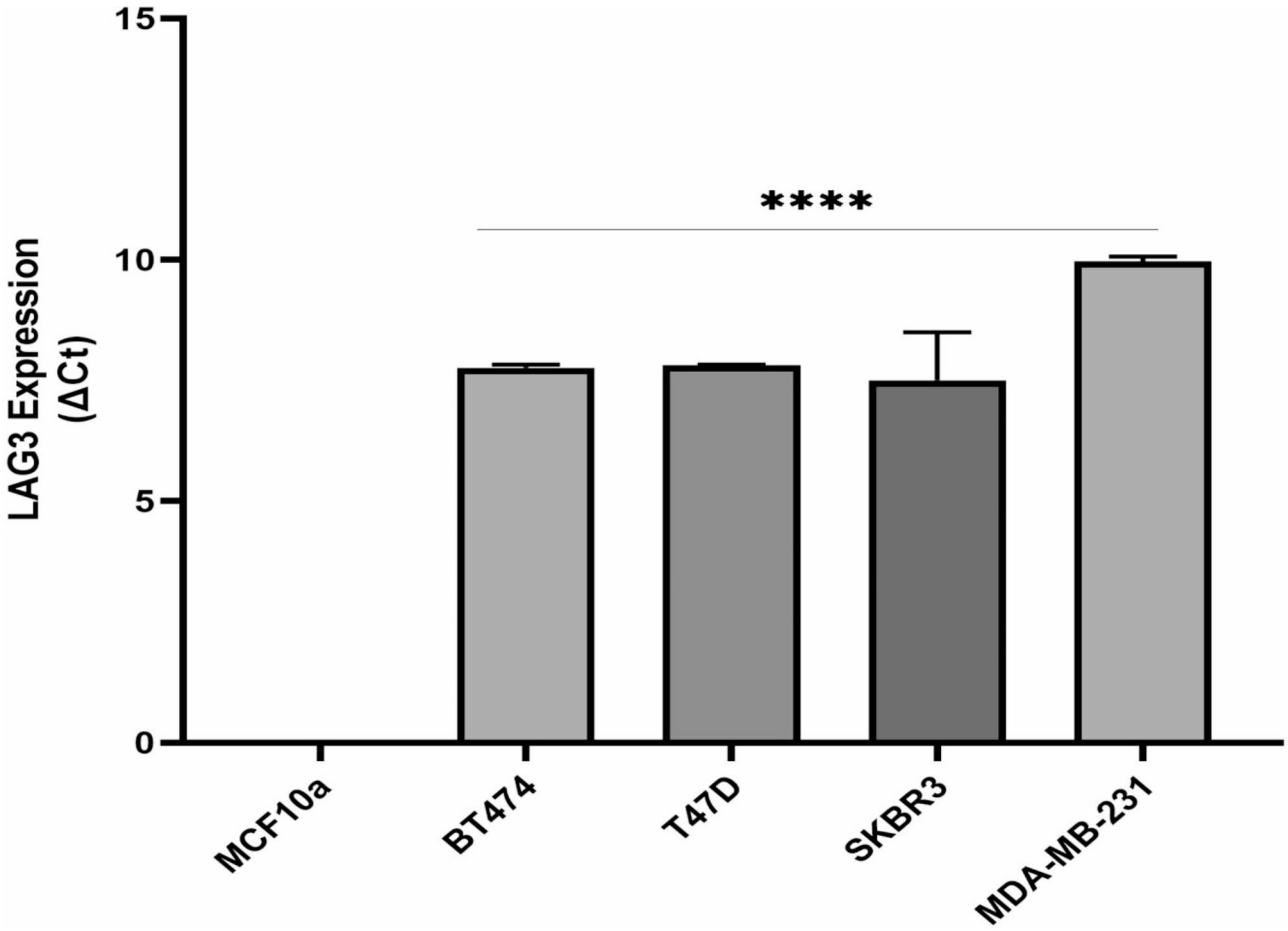

Fig. 1. Real time qPCR analysis of LAG3 in a panel of breast cancer cell lines (BT474, T47D, SKBR3 and MDA-MB-231), comparing them with MCF10a cell line, a human model of epithelial cell line. Results were normalized to β-actin mRNA and analyzed by ΔCt method. One way ANOVA test followed by Tukey's test were used for statistical analysis. ****$p < 0.001$.

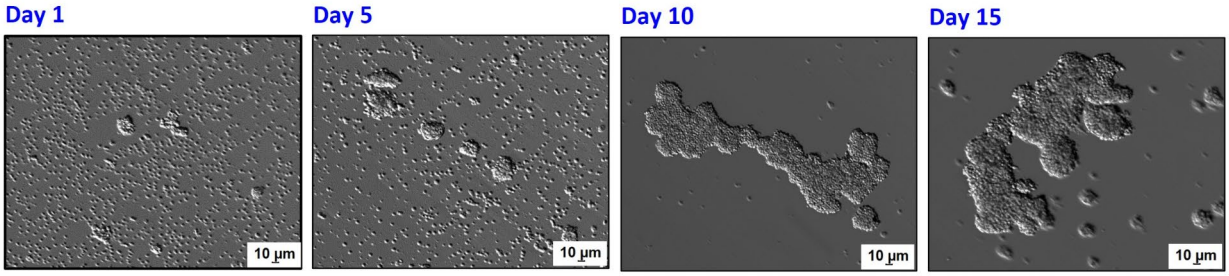

Fig. 2. Representative images of ex vivo PDO culture obtained from breast cancer biopsies are reported. Scale bar: 10 µm.

One week after seeding in matrigel, we confirmed that tumor/immune cell mixture of all samples was preserved after digestion by immunofluorescence microscopy (Fig. 3A). In particular, the presence of immune cells on the PDO microenvironment was observed incubating PDOs with both α-CD45 and α-EpCam and organoids cell nuclei were stained with Hoechst 33,342 (blue signal). We observed a colocalization of α-CD45 (red spots) and α-EpCam (yellow signal). This scenario was evaluated also through flow cytometer analysis to quantify the presence of the immune and tumor component:

we detected the average cell population of 95.63% formed by CD45 + and EpCam+, 57.33 and 38.30 percentages, respectively (Fig. 3B).

Efficacy of LAG3 inhibition on proliferation of in vitro ed ex vivo models

To focus on our clinical model, we used two groups of human PDOs: 6 samples derived from breast cancer (#1-#6) and a non-cancerous group consisting of 6 fibroadenomas (NC01-NC06), as well known as benign breast tumors, used as a control group (Table 1). In the first analysis, we demonstrated a higher significant mRNA LAG3 expression in all tumor PDOs #1–#6 compared to non-cancerous controls NC01-NC06, suggesting the specificity of this marker in tumor tissues (Fig. 4A). This result was confirmed by the localization of LAG3 only in tumor ex vivo PDOs through confocal microscopy analysis (Fig. 4B).

Recent advances in small molecule inhibitors changed the landscape of management of breast cancer patients. Relatlimab, an α-LAG3 monoclonal antibody, is currently one of the most advanced monoclonal antibodies used in clinical trials targeting both solid tumors and hematologic malignancies[19].

To evaluate the effect of LAG3 blockade on the proliferation, we treated both breast cancer cell lines and the seven established ex vivo cultures with Relatlimab at 10 µg/mL for 5 days. Although the magnitude of the response varied between patients, tumor PDOs resulted more sensitive to LAG3 treatment with antiproliferative effect about 50% cell death across all samples (#1–#6), compared to non-cancerous PDOs (Fig. 5A–D). On the contrary, a slight effect was observed on breast cancer cell lines (80% vitality after treatment), probably due to the absence of immune cell component in 2D-model, confirming the appealing of our tumor PDOs model to estimate the effect of anti-LAG3 molecule (Fig. 5C). Interestingly, in Fig. 5B we observed a morphological change of PDOs following treatment with α-LAG3, observing a greater disruption of the tumor PDOs architecture compared to non-cancerous tissue sample.

LAG3 blockade with relatlimab affects tumor microenvironment and epithelial to mesenchymal transition

Typically, LAG3 is expressed on the surface of activated T and NK cells to maintain the homeostasis of the immune response and to prevent the onset of excessive immune reactions. In the tumor microenvironment (TME), the continuous activation of T cells leads to the co-expression of immunosuppressive molecules, such as LAG3, resulting in T cell incapacity and apoptosis, which is the "deceptive mechanism" by which tumors and chronic infectious diseases escape the immune system.

We performed α-LAG3 treatment to explore the modifications in cytokines and other immune checkpoints gene expression by RT-PCR on PDO cultures detecting an increase of relative mRNA expression of IL-12, IFNγ, TNFα, IL-6 and IL-1β (Fig. 6A). All these cytokines can create a favorable microenvironment for inflammatory and immune response and cause the simultaneous decrease of PD-L1, TIM-3, CTLA4 (Fig. 6B), thus indicating a potential role of LAG3 on T cells exhaustion.

To confirm this, we evaluated the expression of 13 inflammatory cytokines from the supernatants of organoids derived from breast cancer patients and fibroadenoma patients. As shown in Supplementary Fig. 1, in fibroadenomas, where LAG3 expression is significantly lower than in tumors (see Fig. 4A) all cytokines analyzed are higher than in tumors, suggesting that the presence of LAG3 may indeed reduce the inflammatory response.

To evaluate EMT associated with drug-induced immunosuppression in the breast cancer tumor microenvironment, we analyzed the expression level of EMT markers on PDOs in presence and in absence of the α-LAG3 treatment (Fig. 6C). We detected an increase in E-cadherin and a decrease in Vimentin mRNAs in the same samples, suggesting that the inhibition of the LAG3 mechanism could modify the phenotypic characteristics of the tumor in favor of epithelial phenotype, less aggressive and invasive.

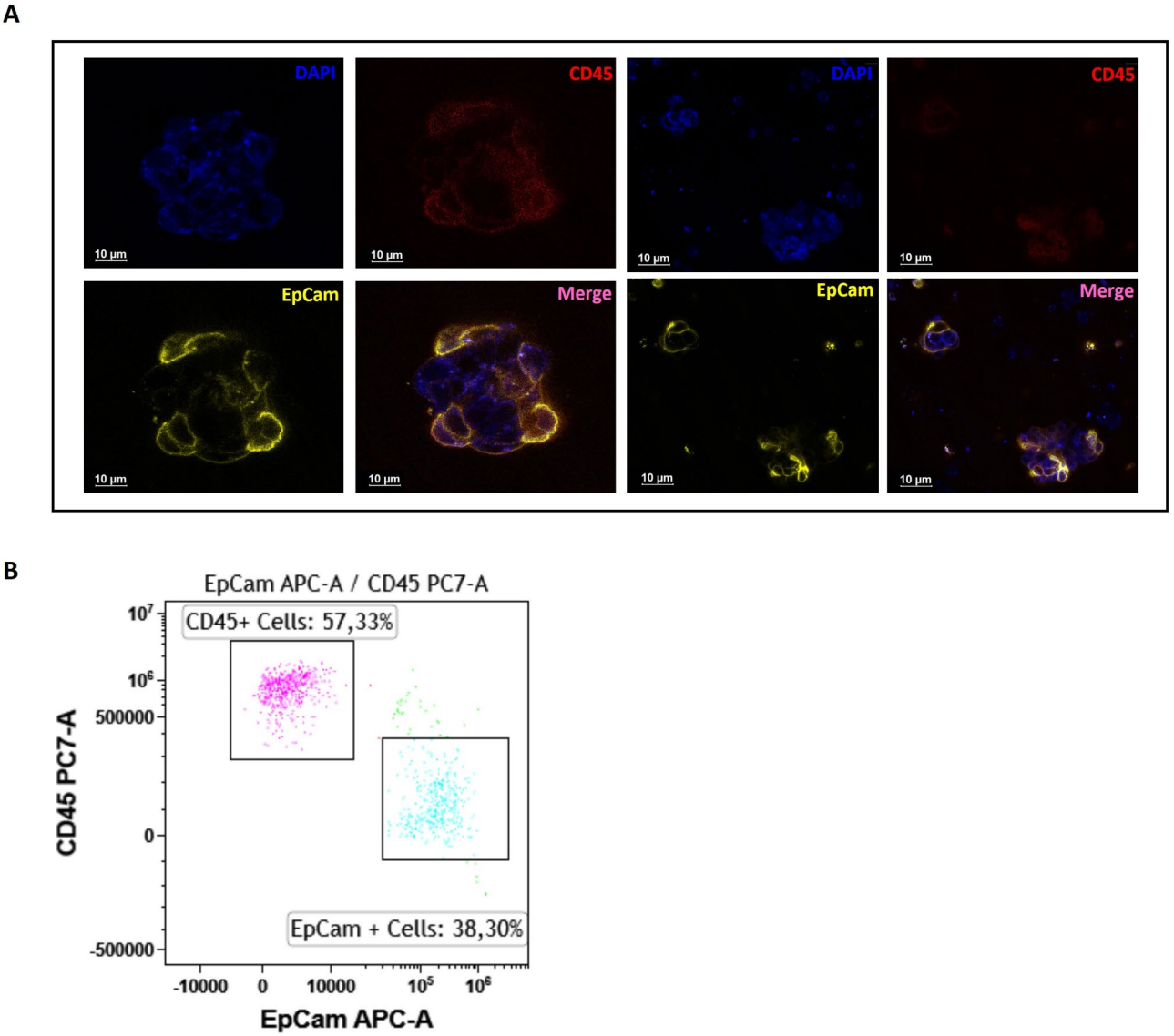

Fig. 3. Characterization of the presence of immune cells on the PDO microenvironment. (**A**) Maximal projection images of PDO incubated with α-CD45 (red spots) and α-EpCam (yellow signal). Cell nuclei were stained with Hoechst 33,342 (blue signal). Scale bar: 10 µm. (**B**) Collected organoids were analyzed by flow cytometric analysis stained with EpCam and CD45. Experiments were conducted on all collected samples, images and plot were representative of one of PDOs cultures.

CD8 + T cells are important for protective immunity against cancer. Exhausted T cells are characterized by a progressive loss of effector functions (cytokine production and killing function) and by the expression of multiple inhibitory receptors such as LAG3. Therefore, we analyzed the lymphocyte population present in both tumor and non-cancerous PDOs before and after 5 days treatment with α-LAG3.

Focusing on the CD8 T cells in PDOs, we detected an increase of 10.43% of CD8 activated cells in treated compared to untreated samples (4.85%) that suggest a significant modulation of CD8 + activated subpopulation as consequence of LAG3 inhibition (Fig. 7). Contrary, in non-cancerous PDOs no difference effect was observed between treated and untreated PDOs (5.85 vs. 6.80%).

Altogether, our results confirm that the presence of LAG3 represents a pro-tumorigenic factor, representing a promising biomarkers for early cancer diagnosis.

Discussion

In the present work, we provide the proof of concept that ex-vivo tumor organoid cultures represent a valid model for choosing T-cell-based therapies, thus representing a significant implementation for translational research and immunotherapy applications. The use of this patient-specific derived model allows several applications for this experimental platform: the study of interactions between tumor cells and T cells, the identification of

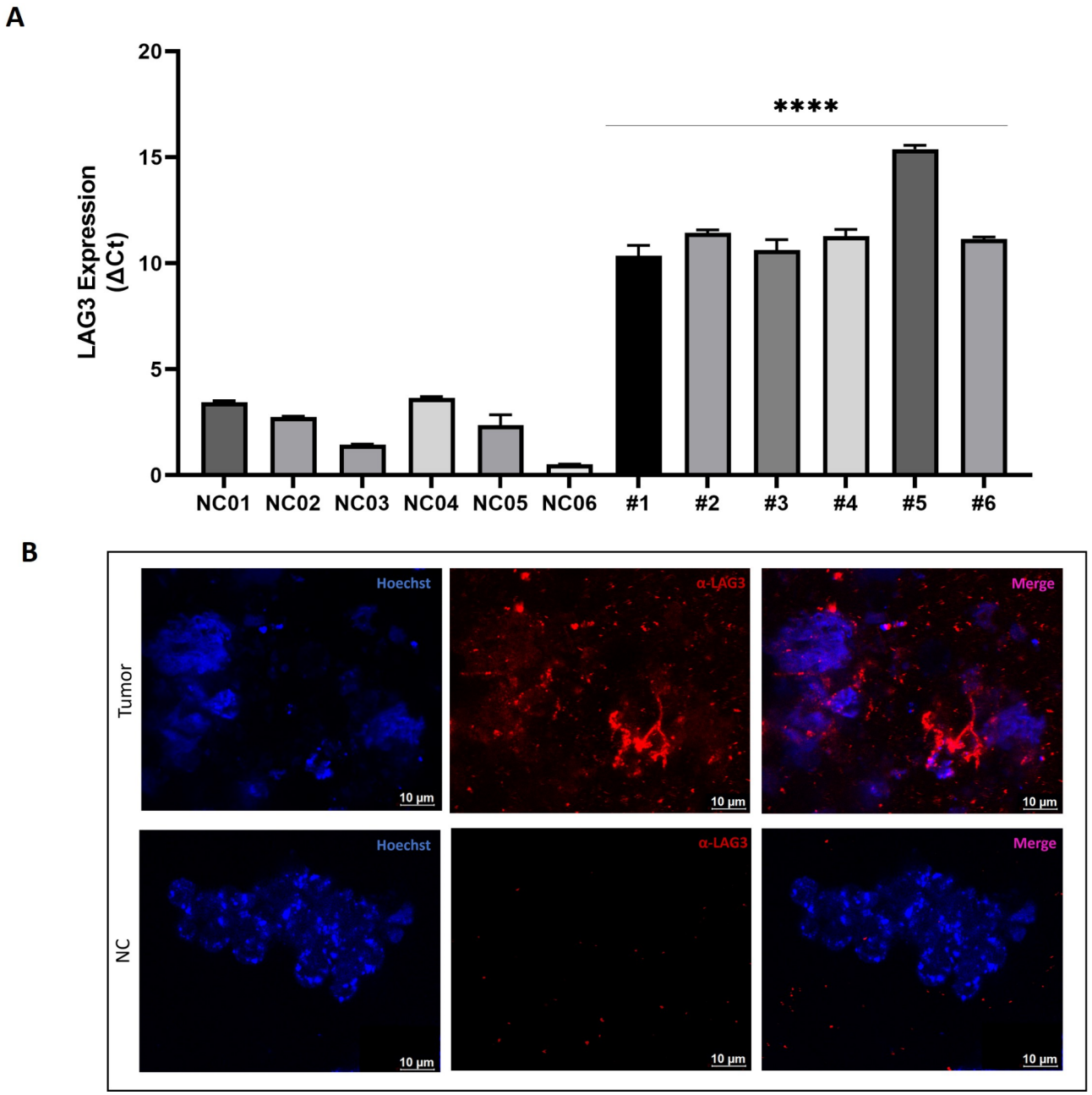

Fig. 4. LAG3 characterization in tumor and non-cancerous PDO. (**A**) Real time qPCR analysis of LAG3 in two groups of PDOs: the first one derived from fibroadenoma samples considered as non-cancerous tissue sample (NC01-NC06) and the second one derived from tumor tissues (#1–#6). Results were normalized to β-actin mRNA and analyzed by ΔCt method. One way ANOVA test followed by Tukey's test were used for statistical analysis. $^{****}p < 0.001$. (**B**) Representative images of LAG3 localization in tumor and non-cancerous ex vivo PDOs. Scale bar: 10 µm.

new molecules involved in response to immunotherapy and the possibility to predict in vivo clinical data in repeatable, simple, and cost-effective manners.

Immuno-checkpoints-based therapies prompted a great breakthrough in the treatment of cancer. Over PD-1/PD-L1 and CTLA-4, LAG3 represent a promising target in cancer immunotherapy since it plays a pivotal role in anti-tumor immunity. Indeed, LAG3 inhibitors entered clinical trials for different cancers, either alone or in combination, and are showing promising efficacy[20].

To further analyze the therapeutic potential of targeting LAG3 in breast cancer we used Relatlimab, first-in-class human IgG4 LAG3–blocking antibody that binds to LAG3 and restores the effector function of exhausted T cells[11,21].

Preliminary we evaluated the presence of immune cells in the PDO microenvironment, and we observed a colocalization of CD45 and EpCam; in particular, $CD45^{+}$ was used as marker to ensure the presence of immune

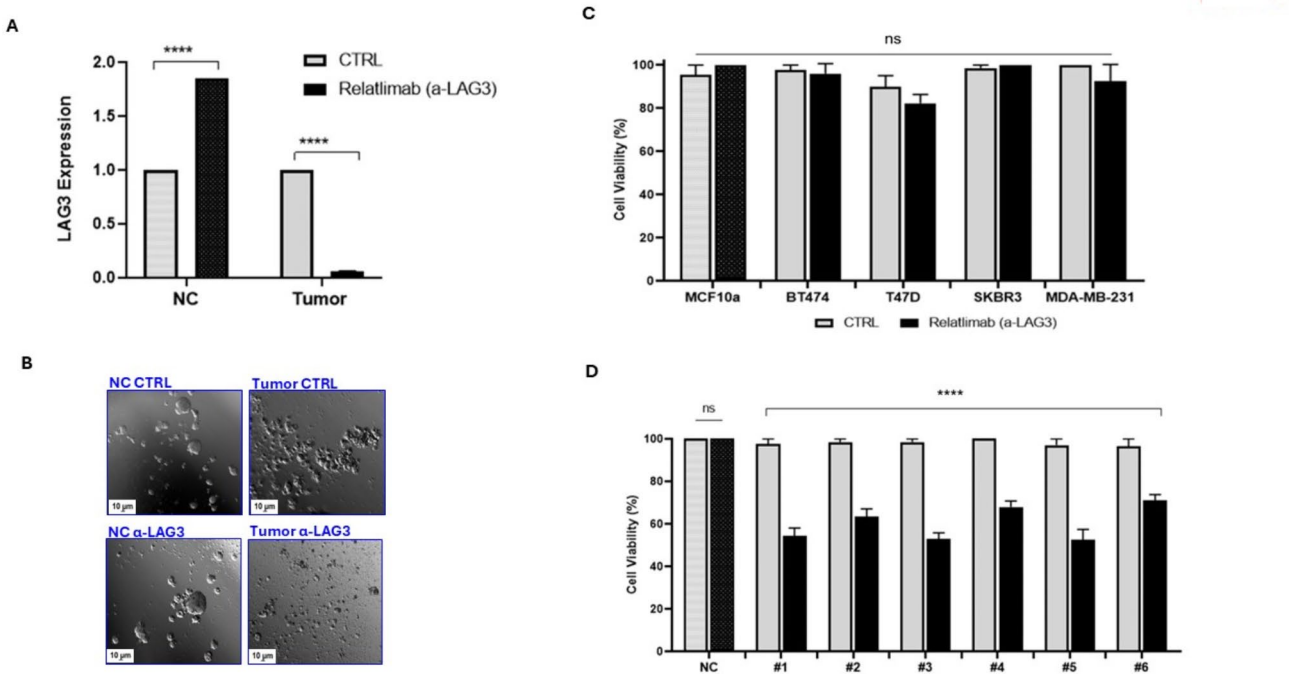

Fig. 5. (**A**) RT- qPCR analysis of LAG3 of both tumor PDOs (tumor) and non-cancerous PDOs (NC) treated with Relatlimab (α-LAG3) at 10 µg/mL for 5 days. (*CTRL* untreated control, **** $p < 0.0001$). (**B**) PDOs image before and after treatment with Relatlimab (*CTRL* untreated control and α-LAG3 = treated, respectively). Scale bar: 10 µm. (**C**) MTS assay of MCF10a, BT474, T47D, SKBR3 and MDA-MB-231 cell lines treated with Relatlimab at 10 µg/mL for 5 days. (*ns* not significant). (**D**) MTS assay of both tumor PDOs (#1–#6) and non-cancerous (NC) PDOs treated with Relatlimab (α-LAG3) at 10 µg/mL for 5 days. (*ns* not significant, **** $p < 0.0001$).

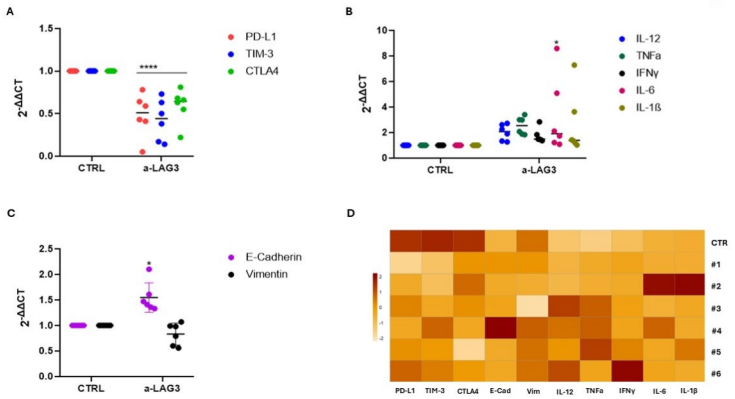

Fig. 6. (**A**) RT-qPCR analysis of cytochines IFNγ, IL-12, IL-1 β, IL-6 and TNFα, (**B**) Immune checkpoint genes as PD-L1, CTLA-4 and TIM-3, and (**C**) Vimentin and E-Cadherin expressed by organoids untreated (CTRL) or treated with Relatlimab at 10 µg/mL for 5 days. Results were normalized to β-actin mRNA and analyzed by ΔCt method. One way ANOVA test followed by Tukey's test were used for statistical analysis (**** $p < 0.001$). (**D**) Graphic representation of all pcr data of tumor treated PDOs (*CTR* untreated control, treated group= #1–#6) on heatmap. R vesion 4.4.1 pheatmap methos.

cells[16], while EpCam represents a diagnostic marker for the detection of carcinoma cells because it is expressed by normal and malignant epithelia[22]. This result was validated by FACS analysis confirming that phenotypic characteristics of the different tumor components are preserved.

In the present study we analyzed LAG3 in breast cell lines observing that all tested cell lines have a high LAG3 expression, associated to inhibitory signals that block the proliferation and activation of T cells, causing their exhaustion. As described in Chronic lymphocytic leukemia patients, also in our experiments we reported that

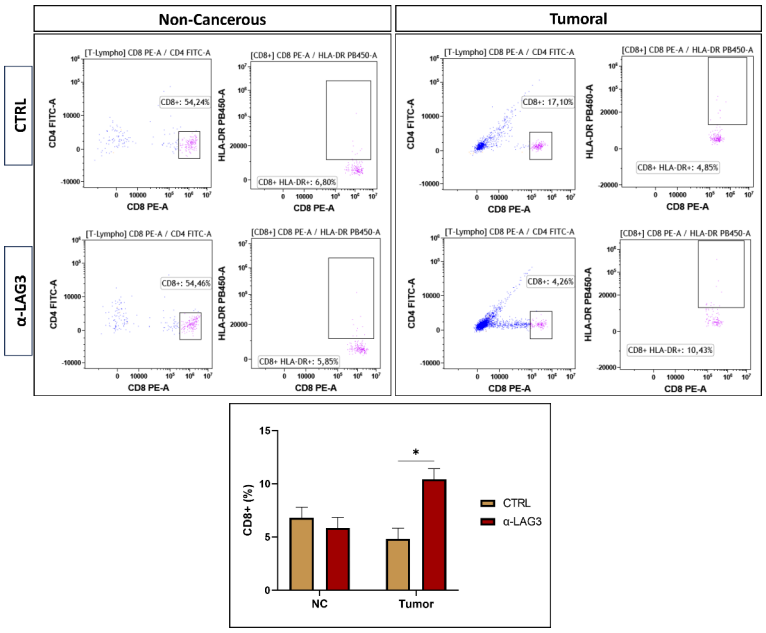

Fig. 7. Dot plots showed CD8 + and CD8 + HLA-DR expressed on the surface of both tumor and non-cancerous PDOs before and after treatment with Relatlimab at 10 µg/mL for 5 days. CD8 + results were better represented by histogram bars. One way ANOVA test followed by Tukey's test were used for statistical analysis. $^{*}p < 0.05$.

the LAG3 blockade is associated to both an increased proliferation of CD4 and CD8 T cells[22] and an enhanced cytokines production like IL-12, IFNγ, TNFα, IL-6 and IL-1β, all cytokines that are able to sustain an immune-reactive and inflammatory micro-environment.

Our results revealed that LAG3 has an immunosuppressive role in breast cancer, since LAG3 sharply inhibited T CD8 + cell proliferation and cytokine production by T cells. Consequently, Relatlimab was able to restore T cell-mediated anti-tumor responses by the increasing of CD8 + T cell proliferation, as well as promoted production cytokines by T cells. Altogether, these data provide new insights into the relevance of LAG3 dysregulation in the clinical outcome of patients with breast cancer, hence suggesting that it may be involved in the suppression of the immune response in this malignancy.

Finally, both the emerging resistance mechanisms associated with these therapies and the profound immunosuppression related to this disease, supports the translation of patient-derived tumor organoids to the clinic as an ex vivo test platform, with the potential to meet the dire need for a means to make more rational treatment decisions for the individual patient.

Nowadays, Relatlimab is widely tested just in clinical trials. Hence, it would be essential to validate the predictive value of organoids, before studies on humans, and LAG3 could be a viable candidate to become a diagnostic marker and predict the fate of a cancer.

Conclusions

LAG3 represents a potential target of next-generation cancer immunotherapy in human and, simultaneously, a biomarker to achieve an early cancer diagnosis. In this context, our results can be of high translational relevance since we generated patient derived organoids as predictive value, prior of human studies, highlighting the key role of LAG3 in the clinical fate of patients affected by breast cancer. In conclusion, our results demonstrate that LAG3 could be a feasible candidate for early cancer detection and to forecast the outcome of a cancer.

Data availability

The biological data used and analyzed during the study are available from the corresponding author on a reasonable request.

Received: 27 May 2024; Accepted: 11 December 2024
Published online: 28 December 2024

References

1. Ribatti, D. The concept of immune surveillance against tumors. The first theories. *Oncotarget* **8** (4), 7175–7180 (2017).
2. Tang, X. Y. et al. Clinical research on the mechanisms underlying immune checkpoints and tumor metastasis. *Front. Oncol.* 11. (2021).
3. Zhang, Y. P. & Zheng, J. K. Functions of immune checkpoint molecules beyond immune evasion. *Regul. Cancer Immune Checkpoints Mol. Cell. Mech. Ther.* **1248**, 201–226 (2020).
4. Triebel, F. et al. LAG-3, A novel lymphocyte-activation gene closely related to CD4. *J. Exp. Med.* **171** (5), 1393–1405 (1990).

5. Andrews, L. P., Marciscano, A. E., Drake, C. G. & Vignali, D. A. A. LAG3 (CD223) as a cancer immunotherapy target. *Immunol. Rev.* **276** (1), 80–96 (2017).
6. Ruffo, E., Wu, R. C., Bruno, T. C., Workman, C. J. & Vignali, D. A. A. Lymphocyte-activation gene 3 (LAG3): The next immune checkpoint receptor. *Semin. Immunol.* 42. (2019).
7. Bae, J., Lee, S. J., Park, C. G., Lee, Y. S. & Chun, T. Trafficking of LAG-3 to the surface on activated T cells via Its cytoplasmic domain and protein kinase C signaling. *J. Immunol.* **193** (6), 3101–3112 (2014).
8. Shapiro, M. et al. Lymphocyte activation gene 3: a novel therapeutic target in chronic lymphocytic leukemia. *Haematologica* **102** (5), 874–882 (2017).
9. Shi, A. P. et al. Immune checkpoint LAG3 and its ligand FGL1 in cancer. *Front. Immunol.* 12. (2022).
10. Liu, Q. et al. Molecular and clinical characterization of LAG3 in breast cancer through 2994 samples. *Front. Immunol.* 12. (2021).
11. Tawbi, H. A. et al. Relatlimab and Nivolumab versus Nivolumab in untreated advanced melanoma. *N. Engl. J. Med.* **386** (1), 24–34 (2022).
12. Gucalp, A. et al. Male breast cancer: a disease distinct from female breast cancer. *Breast Cancer Res. Treat* **173** (1). (2019).
13. Mirabelli, P. et al. SDN biobank: Bioresource of human samples associated with functional and/or morphological bioimaging results for the study of oncological, cardiological, neurological, and metabolic diseases. *Open J. Bioresour.* **4**(1). (2017).
14. Lee, G. Y., Kenny, P. A., Lee, E. H. & Bissell, M. J. Three-dimensional culture models of normal and malignant breast epithelial cells. *Nat. Methods* **4** (4), 359–365 (2007).
15. DeRose, Y. S. et al. Patient-derived models of human breast cancer: protocols for in vitro and in vivo applications in tumor biology and translational medicine. *Curr. protocols Pharmacol.* **60** (1), 14 (2013).
16. Della Corte, C. M. et al. Antitumor activity of dual blockade of PD-L1 and MEK in NSCLC patients derived three-dimensional spheroid cultures. *J. Exp. Clin. Cancer Res.* 38. (2019).
17. Santoro, J. et al. Influence of breast cancer extracellular vesicles on immune cell activation: A pilot study. *Biol. Basel* **12** (12). (2023).
18. Weeber, F. et al. Preserved genetic diversity in organoids cultured from biopsies of human colorectal cancer metastases. *Proc. Natl. Acad. Sci. USA* **112** (43), 13308–13311 (2015).
19. Wang, W. J. et al. Characterization of LAG-3, CTLA-4, and CD8 < SUP>+ TIL density and their joint influence on the prognosis of patients with esophageal squamous cell carcinoma. *Ann. Trans. Med.* **7** (23). (2019).
20. Ascierto, P. et al. Efficacy of BMS-986016, a monoclonal antibody that targets lymphocyte activation gene-3 (LAG-3), in combination with nivolumab in pts with melanoma who progressed during prior anti–PD-1/PD-L1 therapy (mel prior IO) in all-comer and biomarker-enriched populations. *Ann. Oncol.* **28**, v611–v612 (2017).
21. Phillips, A. L. & Reeves, D. J. Nivolumab/Relatlimab: A novel addition to immune checkpoint inhibitor therapy in unresectable or metastatic melanoma. *Ann. Pharmacother.* **57** (6), 738–745 (2023).
22. Keller, L., Werner, S. & Pantel, K. Biology and clinical relevance of EpCAM. *Cell. Stress* **3** (6), 165–180 (2019).

Author contributions

VC designed the study, wrote the manuscript; BC, LC and GS performed in vitro experiments on organoids cultures, did data analysis and made the figures; DM, GM and FA obtained informed consent from patients, collected clinical data, and performed biopsies; SA and MS gave contribution to writing and revising the manuscript and the figures. All authors read and approved the final manuscript.

Funding

This work has been partially supported by Ricerca Corrente.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-83061-8.

Correspondence and requests for materials should be addressed to V.C.