Exploring thymic stromal lymphopoietin in the breast cancer microenvironment: A preliminary study

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Abstract. Cancer participates in the immune response by releasing several factors, such as cytokines and chemokines, which can alter the ability of the immune system to identify and eradicate cancer. Notably, the role of thymic stromal lymphopoietin (TSLP) in breast cancer (BC) is currently controversial and unclear. The present study characterized the role of TSLP in BC and its interaction with peripheral blood mononuclear cells, focusing on the $CD14^+CD16^+$ monocyte population via the secretome released by BC cells. The UALCAN and Gene Expression Profiling Interactive Analysis tools were employed to define TSLP expression in BC, and its levels in different BC subtype cell lines were validated using reverse transcription-quantitative PCR and ELISA. In addition, TIMER 2.0 was used to determine the abundance of immune cell infiltration in BC. Subsequently, the effects of BC conditioned medium (CM) and TSLP were investigated on $CD14^+CD16^+$ monocytes via flow cytometry. A Cellular Reactive Oxygen Species (ROS) Assay Kit, Fluo-4 AM assay and ATPlite assay were used to explore the effects of TSLP on monocyte cellular metabolism. The results showed that a reduction in TSLP expression was associated with an unfavorable prognosis in BC. Furthermore, a higher expression of TSLP in CM from a non-tumoral cell line increased the percentage of $CD14^+CD16^+$ monocytes. Finally, it was revealed that TSLP decreased intracellular ATP levels, while increasing intracellular calcium levels and producing ROS in THP-1 cells. Therefore, TSLP may be considered a novel biomarker in the BC microenvironment, where it could regulate cellular metabolism through the expansion of $CD14^+CD16^+$ monocytes.

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Introduction

TSLP is an IL-7-like cytokine that encounters a plethora of roles (1). TSLP receptor (TSLPR) and interleukin 7 receptor-α (IL-7Rα or CD127) form a high-affinity heteromeric complex, which the human TSLP binds to fulfill its physiological functions (2). By forming a ternary complex with its particular receptor, TSLPR, and then with IL-7Rα, TSLP starts signaling (3-5). TSLP is primarily produced by epithelial cells, smooth muscle cells, and fibroblasts in the skin, gut, and lung (6). Furthermore, TSLP can be produced by immune cells, such as monocytes (7), macrophages (8) and mast cells (9). Likewise, immune and non-immune cell subsets TSLPR complex expressing are activated by TSLP. Nevertheless, if we focus on human monocytes of the innate immune system, few studies have been published. For instance, it has been shown that the expression of surface antigens is enhanced by TSLP in $CD14^+$ monocytes/macrophages, in particular increasing CD80 expression (10). Besides, human $CD14^+$ monocytes have exclusively been found to express the TSLPR complex in response to Lipopolysaccharide (LPS) stimulation, also enriching a functionally discrete subset of $CD14^+CD1c^+$ human monocytes (7). Lastly, TSLP has been demonstrated to modulate monocyte metabolism by inducing ROS production (11), but again this process remains unclear and unexplored.

TSLP activated cells lead to various disease models, including cancer (12). Besides, TSLP was found to be expressed in various cancer cell types, including BC (13) that represents the most accidental cancer in industrialized nations, making it one of the most aggressive diseases for women globally (14). The development of BC is influenced by a number of risk variables, including age, gender, and economic development level, aspects connected to hormones, nutrition, genetics, and lifestyle (15). Luminal A, luminal B, $HER2^+$, and triple negative (TNBC) are among the five molecular subtypes of BC that have been identified owing to the analysis of gene expression (16).

In BC, the function of TSLP is still debated and ambiguous. Both a tumor-promoting role and tumor-suppressive role of TSLP was identified. In detail, it has been discovered that TSLP is directly produced by human BC cells through the release of IL-1β by myeloid dendritic cells (DCs), thus causing OX40L expression on DCs *in vitro* (17,18). Inter alia, BC metastases

in the lung expressed TSLP (19,20). Demehri *et al* discovered a tumor-suppressive role for TSLP in murine models of BC carcinogenesis, which is in contrast to the results previously discussed (21). Moreover, it has been found that TSLP expression was absent from most human tumor samples under investigation, indicating a lack of TSLP-TSLPR signaling in BC (22).

Herein, we try to add a new piece on the function of TSLP in BC. Indeed, we investigated TSLP expression in different subtypes of BC. Furthermore, we focused on the effect of TSLP to the immune system to understand which components of innate immunity were altered by the presence of the protein. Finally, our investigation also focused on how TSLP might affect the energy system and metabolism of specific cells of the anticancer immunity.

Materials and methods

In silico study. A user-friendly and interactive online tool called Gene Expression Profiling Interactive Analysis (GEPIA2, http://gepia2.cancer-pku.cn/#index, accessed on 25 June 2024) allows users to analyze RNA sequencing expression data of 9736 tumors and 8587 normal samples from the TCGA and GTEx projects employing a common handling pipeline (23). Utilizing GEPIA2, the tissue-specific expression of TSLP was examined in BC tissues compared to normal tissues, and in BC subtypes ($HER2^+$, luminal A, luminal B, basal-like) in relation to normal tissues. TCGA normal was exclusively used for differential analysis and plotting. Beyond that, the Overall Survival (OS) and Disease Free Survival (DFS) were calculated using the same tool (23). The UALCAN database (https://ualcan.path.uab.edu/, accessed on 25 June 2024) was used as well for assessing TSLP expression in BC subtypes tissues (24,25). Finally, TIMER 2.0 (http://timer.cistrome.org/, accessed on 25 June 2024), a webserver that allows correlation of immune infiltrate abundance and gene expression using deconvolution algorithms (26).

Cell culture. All cell lines (MCF-10A, MCF-7, BT-474, MDA-MB-231, THP-1) were obtained from IRCCS Synlab SDN Biobank. In detail, the BC cell lines were maintained as described below: MCF-7 (luminal A) in Roswell Park Memorial Institute 1640 Medium (RPMI) (Gibco-Thermo-Fisher Waltham, MA, USA) supplemented with 20% heat inactivated (h.i.) fetal bovine serum (FBS), 1% MEM non-essential amino acids, 1 mM sodium pyruvate and 10 μg/ml of human insulin; BT-474 (luminal B) in RPMI 1640 supplemented with 20% h.i. FBS, 1% L-glutamine and 10 μg/ml of human insulin; MDA-MB-231 (TNBC) in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo-Fisher Waltham, MA, USA) supplemented with 10%h.i. FBS and 1% L-glutamine. Lastly, MCF-10, a human epithelial cell line isolated from the mammary gland and used as our control condition, was maintained in Mammary Epithelial Cell Growth Medium bullet kit (MEGM) (Lonza, Basel, Switzerland) supplemented with 10% h.i. FBS and 1% L-glutamine. The THP-1 cell line, monocyte cells isolated from an acute monocytic leukemia patient, was maintained in RPMI 1640 supplemented with 20% h.i. FBS and 1% L-glutamine. All the cell lines were incubated at 37˚C in 5% CO_2 and routinely checked for mycoplasma contamination.

PBMC isolation from whole blood. PBMCs were obtained from five healthy women enrolled from the active protocol 4/21 who have provided written consent to participate in the study (Ethical Committee of IRCCS Pascale of Naples, Italy; approval no. 4/21, 2021). PBMCs were re-covered from venous blood using density gradient centrifugation (Pancoll® density 1,077 g/l, PanBiotech, Aidenbach, Germany) as described previously (27). Briefly, whole blood collected in an EDTA vacutainer was diluted in 5 ml PBS, layered on 3 ml Pancoll and centrifuged at 1,200 x g for 10 min at 4˚C.

BC conditioned media. BC cells were seeded at 10-20% confluence in tissue culture plates. Once the cells reached a confluence of 90%, the cell culture medium was replaced with a serum-free fresh medium. After 24 h, this CM was harvested, filtered (0.20 μm pore size filter), and stored at -20˚C.

ELISA. In a set of experiments, $1x10^6$ cells/ml of BC cell lines were seeded in 24-well plates and grown to confluence for 24 h prior to harvest CM. After treating, the supernatant was collected while the cellular pellets were lysed in Tryton X-100 0.1% (Sigma-Aldrich, Saint Louis, MO, USA). After harvest, both samples were centrifuged at 300 x g at 4˚C and then stored at -80˚C for subsequent determination of extracellular and intracellular mediator content.

TSLP concentrations in supernatant and in cellular lysates of BC cells were measured using commercially available ELISA kit (Catalog No. DY1398-05, R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions. The absorbance was read at 450 nm using an automatic plate reader (Victor Nivo, Perkin Elmer, Waltham, MA, USA). These experiments were repeated five times and data were expressed as pg/ml.

Total RNA extraction and reverse transcription-quantitative (RT-qPCR). Total RNA was extracted by TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Subsequently, it was quantified employing Nanophotometer NP-80 spectrophotometer (Implen, Munich, Germany). 1 μg of total RNA was reverted in cDNA exploiting Xpert cDNA Synthesis SuperMix (Grisp, Porto, Portugal).

qPCR was performed by means of IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on a CFX384 Real-time detection system (Bio-Rad Laboratories, Hercules, CA, USA). GAPDH was used as endogenous controls to normalize Cq (cycle quantification) values applying the $2^{-\Delta Cq}$ formula (28). Each cDNA sample was analyzed in triplicate and the corresponding no-RT mRNA sample was included as a negative control. The following human primers were used in this study: GAPDH forward, 5'-GTCCACTGGCGTCTTCAC-3', reverse, 5'-CTTGAGGCTGTTGTCATACTTC-3'; ACTINB forward, 5'-CAAGAGATGGCCACG-3', reverse, 5'-TCCTTC TGCATCCTG-3'; TSLP forward, 5'-CACCGTCTCTTGTAG CAATCG-3', reverse, 5'-TAGCCTGGGCACCAGATAGC-3'.

Flow cytometric analysis. Fresh PBMC samples obtained as described above were stimulated with BC CM or with TSLP. Specifically, $1x10^5$ PBMCs were pelleted and resuspended with 100 μl BC CM or treated with different concentrations

of TSLP (10, 50, and 100 ng/ml) in 96-well U-bottom plates, and then incubated for 24 h at 37˚C in 5% CO_2. The following monoclonal antibodies from Beckman Coulter (Brea, CA, USA) were used: CD3-PC5 (6607010), CD25-PC7 (A52882), CD14-APC 700 (A99020), CD45-KrO (B36294, CD16-APC 750 (B00845), and Myeloid activation antibody cocktail composed by CD169-PE, HLA-DR-APC and CD64-PB (C63854), CD31-FITC (IM1431U), CD335-PE (IM3711), HLA-DR-ECD (B92438), CD19-PC5 (A07771), CD45-PC7 (IM3548), CD56-APC (IM2474), CD3-APC 700 (B10823), CD9-APC-750 (B13649), CD4-PB (B49197) and CD8-KrO (B00067). Each antibody were purchased by Beckman Coulter (Beckman Coulter, Brea, CA, USA) and were prepared in a 1:10 dilution and mixed throughout. Flow cytometry experiments were conducted using a minimum of 10,000 recorded events using the Cytoflex V2-B4-R2 instrument. At last, data were analyzed through the Kaluza Analysis Software 2.1 (Beckman Coulter, Brea, CA, USA). Doublets and debris (identified based on forward- and side-scatter properties) were excluded from the analysis.

ROS production. To quantitatively assess the reactive oxygen species (ROS) production, the DCFDA/H2DCFDA-Cellular ROS Assay Kit (ab113851) purchased from Abcam (Cam-bridge, UK) was used. In detail, THP-1 cells were seeded in a 96-well plate at the density of $1x10^5$ cells/well in FBS-supplemented medium for 1 h. Then, cells were incubated with TSLP 100 ng/ml in combination with DCFDA (10 μM) for 30 min protected from light, washed with Buffer 1X (provided in the kit), and analyzed on a microplate reader (Victor Nivo, Perkin Elmer, Waltham, MA, USA) with the excitation at 485 nm and the emission at 535 nm according to manufacturer's instructions. ROS production was measured immediately, after 30 min and 1 h. The unlabeled THP-1 cells were analyzed and used as negative controls. Duplicates of the experiments were conducted.

Fluorescent calcium measurement. The calcium-sensitive fluorescent single wavelength dye, Fluo-4 AM (Invitrogen, Thermo-Fisher Waltham, MA, USA) was used to measure the intracellular calcium modulation post TSLP stimulation. THP-1 cells ($1x10^5$ cells in 100 μl/well) were loaded with 10 μM of Fluo-4 AM at 37 C for 30 min, and subsequently treated with TSLP 100 ng/ml for starting time (time zero), 30 min and 1 h. The fluorescent activity of Fluo-4 was acquired using a fluorescence microplate reader (Victor Nivo, Perkin Elmer, Waltham, MA, USA), setting the excitation at 485 nm and the emission at 535 nm, according to manufacturer's instructions.

Intracellular ATP measurement. The ATPlite Luminescence Assay System (Catalog No. 6016943), purchased from PerkinElmer (Waltham, MA, USA), was used to measure the Adenosine TriPhosphate (ATP), according to the manufacturer's instructions. Specifically, to monitor the effects of TSLP, THP-1 cells were plated in 96-well plates (100 μl/well) at the concentration of $1x10^5$, and treated with 100 ng/ml of TSLP. Adding the lysis buffer provided by the kit, the luminescence was measured immediately, after 30 min and 1 h using the luminescence plate, OPTIPLATE (Catalog No. 6005290, PerkinElmer, Waltham, MA, USA), loaded on microplate reader (Victor Nivo, Perkin Elmer, Waltham, MA, USA). The calibration curve was prepared by ATP standard reconstituted in double-distilled water to a concentration of $10x10^6$ pM. The dilution series were drawn up in the range $1x10^6$-1 pM as a base for an ATP-standard curve. Double-distilled water was used as a blank.

Cell Counting Kit-8 assay. To measure cell viability, the Cell Counting Kit-8 (CCK-8) assay (Catalog No. 96992, purchased from Merck KGaA, Darmstadt, Germany) was used. $1x10^3$ THP-1 cells were plated (100 μl/well) in 96-well plates and were treated with TSLP for 24 h at 37˚C in 5% CO_2. After each treatment time, 10 μl of CCK-8 solution was added to each well and incubated for 3 h. The absorbance was read at 450 nm with a microplate reader (Victor Nivo, Perkin Elmer, Waltham, MA, USA). Experiments were performed in triplicates.

Statistical analysis. For the comparison between normal and BC subtype tissues, the GEPIA2 tool used the previously reported statistical method (23) and the UALCAN tool used the previously described statistical method (24,25).

For the prognostic study (OS and DFS), Kaplan-Meier analyses were determined using GEPIA2. GEPIA2 employs the Mantel-Cox test and quartile was applied to determine the expression threshold for splitting the high-expression and low-expression cohorts.

In order to evaluate immune infiltrates, TIMER 2.0 uses partial Spearman's correlation applied on tumor purity to perform the correlation between immune infiltrates estimation value and TSLP expression.

For the *in vitro* study, Graphpad Prism 9 (Graphpad Software, Graphpad Holdings, LLC, CA, USA) was employed for all statistical analysis. Statistical analysis was performed by unpaired two-tailed Mann-Whitney U test when two groups were compared. The non-parametric Kruskal-Wallis test followed by Dunn's test was performed for comparing means in a situation where there are more than two groups. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Evaluation of TSLP expression through an in silico study. To evaluate TSLP expression in BC, we first investigated the gene expression levels of TSLP in BC tissues. As illustrated in Fig. 1A, the expression level of TSLP was significantly lower in BC tissues (red boxplot) compared to the normal tissues (gray boxplot). Given this result, we next analyzed the expression of TSLP in distinct BC patients subtypes. As shown in Fig. 1B, TSLP mRNA was found to be lowly expressed in every BC subtypes compared with normal tissues. These data were obtained through the GEPIA tool.

Furthermore, using the UALCAN tool it emerged that TSLP expression was significantly higher in TNBC in comparison to the Luminal subtypes and $HER2^+$ (Fig. 1C).

Evaluation of prognostic value of TSLP mRNA expression levels in BC patients. Using the 'Survival Map' module of GEPIA, the prognostic significance of TSLP in BC was examined. We studied the survival impact of TSLP in terms of OS and DFS in this manner. Results revealed that lower

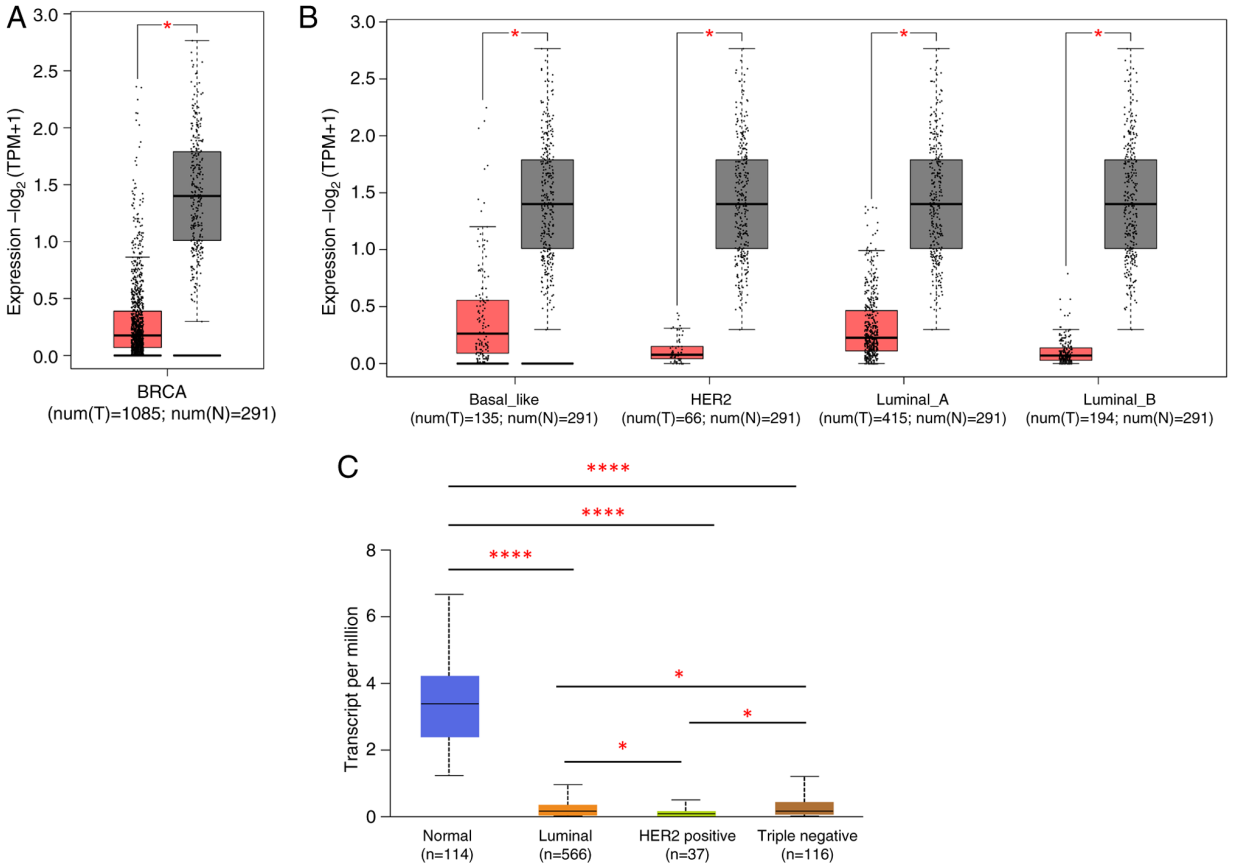

Figure 1. TSLP expression in BC tissues. (A) The expression level of TSLP in normal (gray boxplot) and BC tissues (red boxplot). Differential TSLP gene analysis in BC subtypes compared to normal tissues using the (B) GEPIA and (C) UALCAN tools. *P<0.05; ****P<0.001. TSLP, thymic stromal lymphopoietin; BC, breast cancer; GEPIA, Gene Expression Profiling Interactive Analysis.

TSLP mRNA expression levels in BC patients was associated with a worst prognosis (Fig. 2A). Nevertheless, DFS was not significant (Fig. 2B).

In light of this finding, we proceeded to examine the prognostic impact of TSLP in each BC subgroups. As shown in Fig. 2, the OS and DFS did not change in the TNBC (Fig. 2C), HER2$^+$ (Fig. 2D), and luminal A (Fig. 2E) groups. Instead, it is interesting to note that lower TSLP expression levels in the luminal B group were substantially linked to shorter OS and significantly adverse impacts on DFS (Fig. 2F).

Correlation analysis between TSLP expression and immune cell infiltration. Using the TIMER 2.0, the immune infiltration status was evaluated. In BC, infiltration levels of B Cells was found to be negatively correlated with the TSLP expression. Infiltration level of T cells (CD4$^+$ and CD8$^+$), and macrophages had a positive correlation with the TSLP expression. No significant correlation was observed between neutrophils, dendritic cells and TSLP expression (Fig. 3A).

Next, we evaluated immune infiltration status in each BC subtypes. In basal-like and in HER2$^+$, no significant correlation was observed between all immune cells tested and TSLP expression (Fig. 3B and C). In Luminal, infiltration levels of T cells (CD4$^+$ and CD8$^+$) had a positive correlation with the TSLP expression, whereas no significant correlation was discovered between macrophages, B Cells, neutrophils, Dendritic Cells and TSLP expression (Fig. 3D).

TSLP mRNA expression and concentrations in human BC cell lines. As our analysis revealed that mRNA expression levels of TSLP were reduced in BC tissues compared to control tissues, we examined the mRNA and protein expression level of TSLP in different BC cell lines (Fig. 4).

To this end, cell lines that represent models for luminal A (MCF-7), luminal B (BT-474), and TNBC (MDA-MB-231) BC subtypes, and non-tumorigenic breast epithelial cell line (MCF-10A) used as control, were employed. Having demonstrated *in silico* that the HER2$^+$ subtype had the lowest expression of TSLP, we decided not to perform an *in vitro* study for this tumor type.

As indicated in Fig. 4A and B, TSLP mRNA expression seemed to be higher in BC cell line models in comparison to the control cells, using two different reference genes (GAPDH, Fig. 4A and ACTINB, Fig. 4B). In particular, TSLP expression was significantly higher in luminal B cells compared to the control cells, without finding any significant difference between the various subtypes analyzed.

Interestingly, the scenario completely changed when we evaluated protein expression. As shown in Fig. 4B, intracellular TSLP protein was significantly overexpressed in normal cells (MCF10A) compared to the luminal A and luminal B, (MCF7 and BT474), whereas it was reduced in normaloid cell line respect to the TNBC model (MDA-MB-231). Lastly, TSLP vanished from the tumor cell lines entirely appearing exclusively in the control cell line, according to

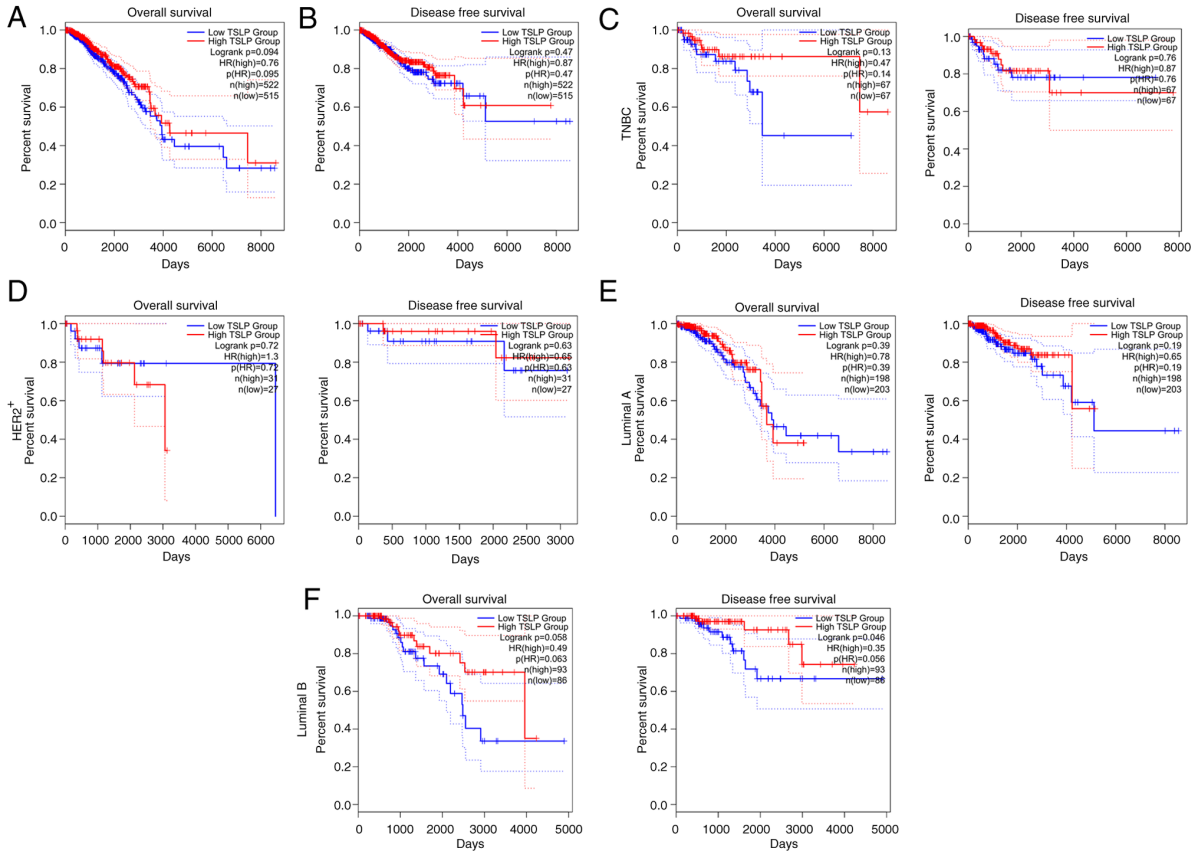

Figure 2. The prognostic impact of TSLP expression level on the Kaplan-Meier curve. The Kaplan-Meier curves calculated in the breast cancer patients population expressed as (A) overall survival and (B) disease free survival. (C) The Kaplan-Meier curves calculated in TNBC subgroup expressed as overall survival (left panel) and disease free survival (right panel). (D) The Kaplan-Meier curves calculated in HER2+ subgroup expressed as overall survival (left panel) and disease free survival (right panel). (E) The Kaplan-Meier curves calculated in Luminal A subgroup expressed as overall survival (left panel) and disease free survival (right panel). (F) The Kaplan-Meier curves calculated in Luminal B subgroup expressed as overall survival (left panel) and disease free survival (right panel). All the curves were determined via GEPIA2 tool. TSLP, thymic stromal lymphopoietin; TNBC, triple-negative breast cancer; GEPIA, Gene Expression Profiling Interactive Analysis.

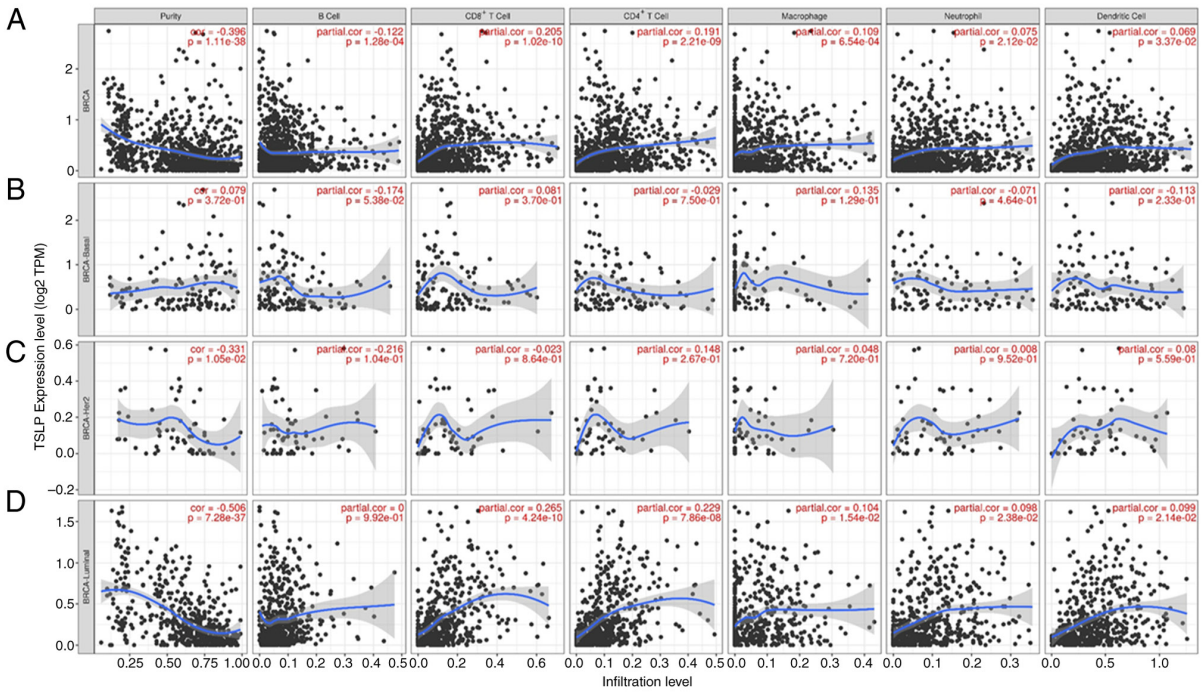

Figure 3. Partial Spearman's correlation between TSLP expression and the abundance of immune cell infiltration (TIMER 2.0) in BC and its subtypes. Association between TSLP expression and infiltration levels of B cell, T cell (CD4+ and CD8+), macrophages, neutrophils and dendritic cells in (A) BC and each subtype: (B) BC-Basal; (C) BC-HER2+; (D) BC-Luminal (P<0.01). TSLP, thymic stromal lymphopoietin; BC, breast cancer.

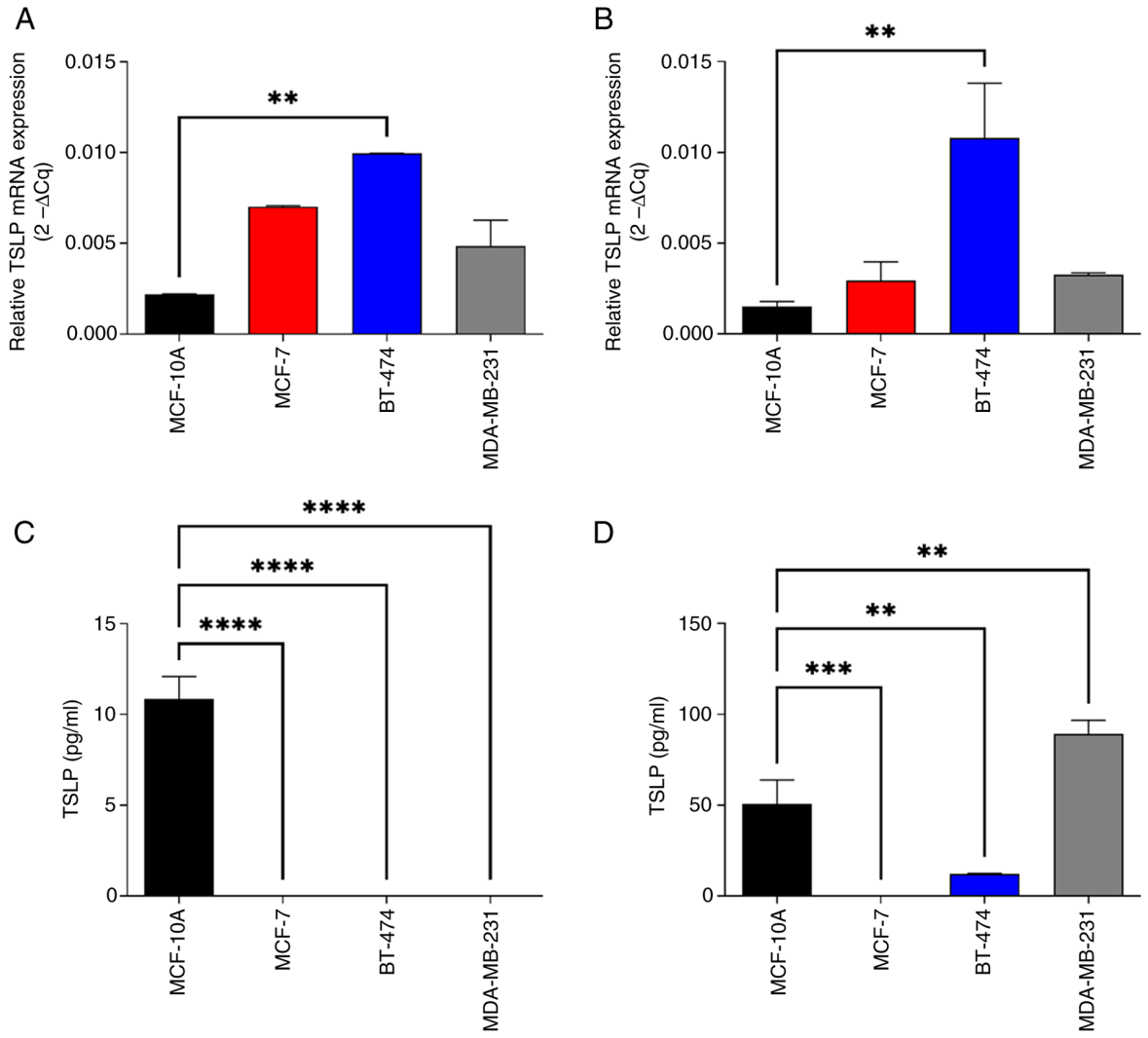

Figure 4. TSLP expression in BC cell lines. (A and B) TSLP mRNA expression, and (C) intracellular and (D) secreted protein levels of TSLP, were detected after 24 h in control cell line (MCF-10A) and in BC subtypes (MCF-7A, BT-474 and MDA-MB-231). TSLP mRNA expression was represented as $2^{-\Delta Cq}$, whereas protein concentration was represented as pg/ml. Data are expressed as mean ± SD of five independent experiments. **P<0.01; ***P<0.001; ****P<0.0001. TSLP, thymic stromal lymphopoietin; BC, breast cancer.

an analysis of the secreted proteins (Fig. 4C), while in the total cell extracts the protein was also present in the other cell lines (Fig. 4D)

BC conditioned media and TSLP affect monocytes CD14⁺CD16⁺ expansion. Subsequently, to investigate the role of TSLP on anti-cancer immunity we treated PBMCs derived from healthy volunteers for 24 h with CM derived from MCF-10A, MCF-7, BT-474 and MDA-MB-231. BC CM cell line models does not affect the percentage of all lymphocytes population (Fig. S1) and classical monocytes CD14⁺ (Figs. S2 and S3). Interestingly, the CM derived from normal cells (MCF-10A) significantly increased the number of CD14⁺CD16⁺ monocytes compared to untreated PBMCs (control) and CM derived from the other BC types used (Figs. 5A and S3). By contrast no effects on activated monocytes CD14⁺CD16⁺HLA-DR⁺ were found (Fig. 5B and C).

To validate these data, the next step was to stimulate PBMCs with increasing concentration of TSLP to verify whether it was able to affect monocytes CD14⁺CD16⁺ expansion. In Fig. 5D, the stimulation with 100 ng/ml of TSLP induced a significant increase of CD14⁺CD16⁺ monocytes proliferation.

Effects of TSLP on cellular metabolism of CD14⁺CD16⁺. Since it has been shown that TSLP increases mitochondrial ROS production causing anti-inflammatory monocytes phenotype (11), we investigated whether TSLP could affect cellular metabolism. To evaluate this mechanism, we used a monocyte cellular model, THP-1 cell line as these cells are positive for CD14 and CD16 markers on the surface (29).

THP-1 cells were treated with best concentration TSLP (100 ng/ml) having an expansion effect on PMBCs for 24 h. Post-treatment, we evaluated ROS production and intracellular calcium signaling via fluorescence, and ATP concentration via luminescence.

In Fig. 6A, time-course experiment revealed that TSLP within 1 h progressively induced the production of ROS in THP-1 cells treated with 10 μM of DCFDA. Considering ROS signaling pathways interact with other biological signaling systems, including calcium, we investigated

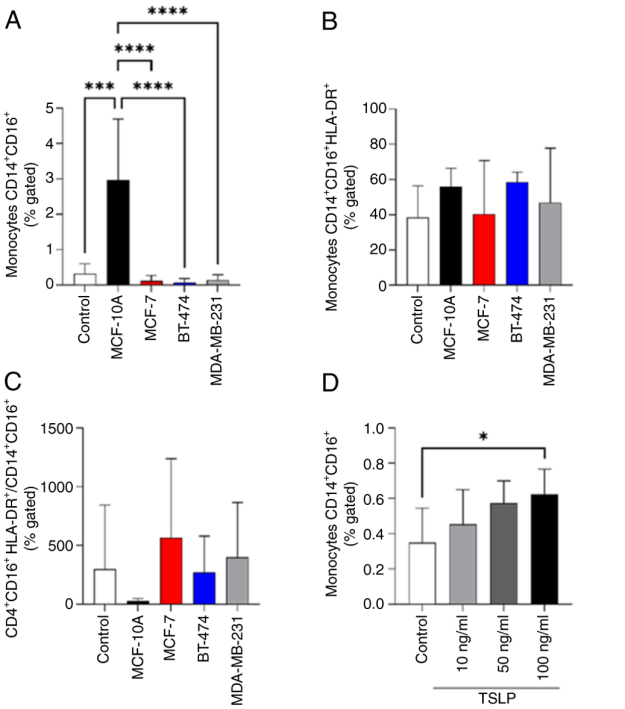

Figure 5. Representative percent gated monocytes $CD14^+CD16^+$ on PBMCs after CM and TSLP treatment. (A-C) $1x10^4$ PBMCs were stimulated with BC CM (MCF-10A, MCF-7, BT-474 and MDA-MB-231) and (D) increased concentrations of TSLP (10, 50 and 100 ng/ml) for 24 h, and subjected to flow cytometry analysis. PMBCs cultured only in complete medium were considered as control. Data are expressed as mean ± SD of five independent experiments. *P<0.05; ***P<0.001; ****P<0.0001. TSLP, thymic stromal lymphopoietin; BC, breast cancer; CM, conditioned medium; PBMCs, peripheral blood mononuclear cells.

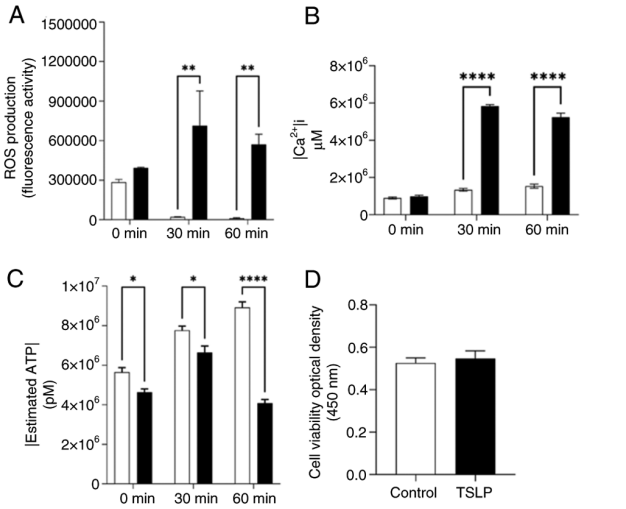

Figure 6. TSLP treatment affects THP-1 metabolism. THP-1 cells were stimulated with 100 ng/ml of TSLP. $1x10^5$ THP-1 cells were used to determine (A) ROS production, (B) intracellular calcium and (C) ATP concentration using (A) 10 μM of DCFDA and (B) 10 μM of Fluo-4 AM, and analyzed immediately, after 30 min and 1 h. (D) $1x10^6$ THP-1 cells were employed to evaluate the effect of TSLP on proliferation. White bars represent the untreated THP-1. Black bars represent the TSLP-treated THP-1. Data are expressed as mean ± SD of five independent experiments. *P<0.05; **P<0.01; ****P<0.0001. TSLP, thymic stromal lymphopoietin; ROS, reactive oxygen species; DCFDA, dichlorodihydrofluorescein.

whether TSLP also had an effect on intracellular calcium. To this end, 10 μM of Fluo 4-AM was used to determine intracellular calcium in TSLP-treated-THP-1 cells. As shown in Fig. 6B, these cells enhanced the intracellular calcium levels compared to the untreated THP-1 in a time-dependent manner.

Next, we studied whether TSLP influenced cellular energy. To this end, an ATPlite assay was performed to measure the intracellular ATP concentration. THP-1 cells treated with TSLP caused a reduction of intracellular ATP levels compared to control cells (Fig. 6C). Finally, to understand whether TSLP caused an effect also on monocytes viability, a CCK-8 assay was conducted. As shown in Fig. 6D, CCK-8 assay highlight that TSLP did not affect THP-1 viability.

Discussion

Over the last ten years, TSLP involvement in a number of cancers has been amply demonstrated. Since its function in BC is still unclear, the role of TSLP continues to be discussed nowadays. In this study, we showed how TSLP could affect the BC microenvironment also playing a defensive function by the immune system.

Using firstly an *in silico* tool, we discovered a lower expression of TSLP in tissues of various classes of BC subtypes compared to healthy tissues, showing also a poor prognosis for these BC patients. Taken together, these findings suggested a tumor-suppressing role of TSLP in BC, as already shown in other cancer types, such as colon (30).

As an immunogenic tumor, BC appears to exhibit a significant association between immune cell infiltration and clinical outcomes (31). In this context, the role of TSLP on immune cells has been the subject of several studies, with contradictory findings. For instance, one study found that TSLP is produced directly by BC cells, promoting a TH2 microenvironment and tumor progression (17).

In our study, the expression of TSLP correlated with the infiltration of different immune cells. Particularly, TSLP expression was associated mainly with T cells ($CD4^+$ and $CD8^+$), B cells and macrophages. In addition, we discovered in Luminal subtype, infiltration level of T cell ($CD4^+$ and $CD8^+$) had a positive correlation with the TSLP expression. Hence, we hypothesized that TSLP might regulate BC tumor immunity through multiple immune cell populations, mainly via adaptative immunity.

To support these results, we set up *in vitro* experiments. Although we found a statistical increase or trend regarding TSLP mRNA in the various BC subtypes compared to normaloid cells, evaluating its levels in BC cell pellets and in their supernatant, we discovered that the expression of TSLP was reduced and even undetectable, respectively. This difference between mRNA and protein levels could be related to posttranscriptional changes that drastically alter TSLP protein levels in BC cells compared with normaloid cells. Future studies will be conducted to try to clarify what the reasons are for this difference between mRNA and TSLP protein levels in BC cells and normaloid cells.

Next, we demonstrated that secretome derived from healthy cells could have a protective role against cancer. As suggested by The Protein Atlas Database, TSLP has a cellular

localization in the Golgi apparatus or vesicles, suggesting its secretion through extracellular vesicles (https://www.proteinatlas.org/ENSG00000145777-TSLP). As a matter of fact, stimulating PBMCs with MCF-10A CM (normaloid cells) increased the percentage of $CD14^+CD16^+$ monocytes population compared to PBMCs with BC CM subtypes.

$CD14^+CD16^+$ monocytes, also called intermediate monocytes or patrolling monocytes, exert phagocytic and anti-inflammatory activities (32,33), exhibiting also enhanced cytotoxic and cytostatic (34). However, their protective value is still not clear.

To determine whether TSLP could be involved in the expansion of $CD14^+CD16^+$ monocytes, we treated PBMCs with different concentrations of TSLP proving that this cytokine affected $CD14^+CD16^+$ monocytes in a dose-dependent manner. Since TSLP induced M2-like effects in THP-1 cells (11), we investigated whether our concentration of TSLP could increase ROS production. The results showed that within 1 h of treatment there is a strong increase in reactive species. ROS are produced from a wide range of sources, including multiple extramitochondrial enzymes and the activity of the mitochondrial respiratory chain (35-37). Notably, calcium has the ability to regulate many of these processes. One may conceptualize ROS and calcium signaling as having a reciprocal interaction, in which ROS can regulate cellular calcium signaling and calcium signaling is required for the production of ROS (37,38).

In this study, we found that TSLP induced an increase in intracellular calcium in THP-1 cells, suggesting that it may be responsible for the significant ROS generation detected. Calcium stimulates the generation of ATP at several levels inside the organelle and is a crucial regulator of mitochondrial function (39). Here we demonstrated a reduction in intracellular ATP levels following stimulation with TSLP. These results can be explained by the fact that as intracellular calcium increases, there is an increased hydrolysis of ATP into ADP (40,41). Reduced respiratory chain activity, which results in decreased ATP levels, and higher AMP stimulate AMPK.

It is already established that TSLP promotes AMPK activation, which modulates mitochondrial biogenesis and stimulates protein signaling associated with mitophagy (11). Our data provide further confirmation of what has already been demonstrated. Due to this energetic and metabolic change of the TSLP-treated cell, we hypothesized that proliferation may also be modulated by this cytokine. After 24 h of treatment, no change in growth was detected, thus modulating exclusively the percentage expansion of $CD14^+CD16^+$ without alternating the cell cycle.

Collectively, these findings provide new insights into the mechanism by which TSLP is involved in BC, and raise the possibility that TSLP acts as anti-tumor mediator by promoting $CD14^+CD16^+$ monocyte expansion, alternating its energetic and metabolic function. In addition, the reduction in TSLP expression could be associated with unfavorable prognosis in breast cancer. High levels of TSLP expression in normal breast tissue compared to low ones in different biological subtypes of breast cancer, lowest in TNBC, could be associated with a key role of this chemokine in cell differentiation or the lack of regulation and dedifferentiation of cancer cells (42). However, further studies are needed to determine other TSLP roles in the BC contest.

In conclusion, we aimed to investigate the role of TSLP in BC with an emphasis on its involvement in immune system activation. Since both the morbidity and mortality rates of BC have significantly increased over the past decades, it is an urgent need to acquire new understandings in mechanisms that affect BC progression. So far, TSLP was found to be lower in BC tissues and in subtypes of BC cell cultures respect to healthy counterparts. In addition, we demonstrated that TSLP could be able to increase the number of $CD14^+CD16^+$ monocytes also by enhancing ROS and Ca^{2+} concentration levels and modulating their energetic metabolism. Hence, the ongoing investigation of the primary molecular processes behind immune-cancer interaction has added a new piece to the understanding of the role TSLP in BC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

GS, MB, AS and SM conceptualized the study. AMG and MB evaluated the accuracy of the data. GS, MB, AMG and SM collected the data and assessed the scientific correctness of the data. MB and SM performed the experiments. GS supervised the work in its entirety. AS, MB, SM, AMG and GS wrote and reviewed the original draft. All authors read and approved the final version of the manuscript. GS, MB, SM and AMG confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The procedures followed in this study, in line with The Declaration of Helsinki, have been approved by the local ethical committees using the active protocol 4/21 (Ethical Committee of IRCCS Pascale Naples, Italy; approval no. 4/21, 2021). Written informed consent to participate was obtained from all subjects involved in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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