

Original Article

Biological heterogeneity of primary cancer-associated fibroblasts determines the breast cancer microenvironment

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Abstract: Cancer-associated fibroblasts are a highly heterogeneous group of cells whose phenotypes and gene alterations are still under deep investigation. As a part of tumor microenvironment, they are the focus of a growing number of studies. Cancer-associated fibroblasts might become a new target of breast cancer therapy, but still more tests and analyses are needed to understand mechanisms and interactions between them and breast cancer cells. The study aimed to isolate cancer associated fibroblasts from breast cancer tissue and to phenotype the isolated cell lines. We focused on various cancer-associated fibroblast characteristic biomarkers and those that might differentiate various cancer-associated fibroblasts' subtypes. Patients with a histological diagnosis of invasive breast cancer (diameter ≤ 15 mm) and qualified for primary surgical treatment were enrolled in the study. Cell lines were isolated from breast cancer biopsy. For the phenotyping, we used flow cytometry, immunofluorescence and RT-qPCR analysis. Based on our study, there was no indication of a clear pattern in the cancer-associated fibroblasts' classification. Results of cancer-associated fibroblasts expression were highly heterogeneous, and specific subtypes were not defined. Moreover, comparing cancer-associated fibroblasts divided into groups based on BC subtypes from which they were isolated also did not allow to notice of any clear pattern of expressions. In the future, a higher number of analyzed cancer-associated fibroblast cell lines should be investigated to find expression schemes.

Keywords: Cancer associated fibroblasts, tumor microenvironment, breast cancer, primary cell lines, oncology

Introduction

Breast cancer (BC) is the most commonly diagnosed cancer in women, with one in nine being diagnosed in her lifetime [1]. Tumors are usually solid matter, forming a compact structure that mimics the character of the tissue. Two types of tissue can be distinguished in the tumor mass: parenchyma, consisting of neoplastic cells and stroma, a heterogeneous population of cells in which cancer cells are suspended [2]. All cancers require a cellular stroma to exchange nutrients and metabolites, which is crucial in developing and progressing of neoplastic disease. Dividing neoplastic cells

interact extensively with the normal tissues around them, stimulating them to create optimal conditions for cancer development [3]. The tumor microenvironment (TME) contains elements of the extracellular matrix and various types of cells, such as fibroblasts-normal (NFs) and cancer-associated (CAFs), endothelial cells, pericytes, macrophages and lymphocytes, connective tissue, and blood vessels [4].

CAFs play a critical role in TME of breast tumors indirectly affecting disease progress, tissue homeostasis, cancer progression, inflammatory and fibrotic conditions and wound healing processes [5, 6]. Their origin is not well-known, but

they are differentiated from various stromal components, and their presence is variable among different cancer types [7, 8]. This kind of fibroblasts differs morphologically and phenotypically compared to their normal counterparts. As a main component of TME, CAFs can promote tumor proliferation, treatment resistance, and immune exclusion by secreting growth factors, inflammatory ligands, and proteins associated with the extracellular matrix [9]. Due to the various features of CAFs, some scientists put efforts to determine different subtypes of CAFs [10-12]. This group of cells is highly heterogeneous. Usually, a categorization of CAFs is based on marker expression, and is related to subtypes of cancer from which CAFs were isolated [13].

BC's clinical symptoms depend on its stage and molecular subtype [14]. The key markers used in the molecular classification of BC subtypes are estrogen receptor alpha (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and the Ki67 proliferation index [15]. Based on the presence or absence of molecular markers, there are four breast cancer subtypes: Luminal A (HR-positive/HER2-negative), Luminal B (HR-positive/HER2-positive), and HER2 (HER2-positive), or triple-negative (HR/HER2-negative) [16]. Usually, BC treatment targets cancer cells instead of components of TME, which drive BC progression. Understanding the molecular basis of BC and TME interactions will increase the number of cured BC patients; more suitable, targeted, and personalized therapies can be designed [17].

Unfortunately, there is a knowledge gap in understanding the expression of genes mediating cellular interactions and paracrine regulatory circuits among different cell types of BC TME and their role in tumorigenesis. The number of studies on CAFs is still insufficient to determine any clear categorization or criteria of CAFs, especially from BC. Isolating and molecular phenotyping of cells comprising non-malignant and cancerous breast tissue will allow us to understand their functions and interactions in TME and design new molecular targets for treating patients.

This study aimed to isolate CAFs from BC tissue and to phenotype the isolated cell lines. We focused on various CAF-characteristic biomarkers and those that might differentiate various

CAF's subtypes. Results of this study will provide data about isolated and characterized CAF cell lines, which could be used to develop new BC models incorporating both CAFs and primary BC cells, and investigate their interactions.

Methods

Patients and tumors

Patients with a histological diagnosis of invasive breast cancer (diameter ≤ 15 mm) and qualified for primary surgical treatment (either breast-conserving surgery or mastectomy) were enrolled in the study. Patients with recurrent breast cancer were excluded from the study. Just after the breast resection, the core needle biopsy (DeltaCut, Pajunk GmbH, Germany) of the tumor in the specimen was performed under ultrasound guidance. The sample was put in a tube with sterile medium and delivered to Radiobiology Department. Ethics approval for the study (no. 283/21) was received from the Bioethics Committee at Poznan University of Medical Sciences. Before the sample collection, we obtained informed consent from each patient after fully explaining all procedures. Patients characteristics and molecular subtypes of tumors were included in **Table 1**.

Pathological review

The morphological and immunocytochemical characteristics of breast cancer tumors were analyzed by hematoxylin- and eosin (H&E) staining with tumor-specific monoclonal antibodies against CK7, CK20, mammaglobin, GCDPF15, ER, PR, and HER2. A pathologist independently reviewed the slide of each breast cancer tissue. Tumors were classified and graded using the WHO 2019 system.

Isolation of CAFs

CAF's were isolated from breast cancer biopsy. The tissue obtained by biopsy was cut into small pieces approx. 1 mm³ in volume, and then placed in 1 mL digestion medium overnight in a humidified incubator containing 5% CO₂ at 37°C. The digestion medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) (Biowest, France), antibiotic agents penicillin/streptomycin at a final concentration of 1% (Merck Millipore Corporation, Germany), 0.14

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Table 1. Patients data and pathology results of breast cancer samples used for culturing CAFs

No	CAF cell line	Age	Side	Grade	ER	PR	HER2	Ki67	BC subtype
1.	CAF17	51	Left	NOS	+	+	+	5%	Luminal HER2+
2.	CAF85	88	Right	NOS	+	+	-	20%	Luminal B
3.	CAF137	77	Left	NHG2	+	+	-	20%	Luminal B
4.	CAF148	64	Left	NST	+	+	-	10%	Luminal A
5.	CAF151	76	Right	NOS	+	+	-	20%	Luminal A
6.	CAF152	62	Right	NOS	+	+	-	30%	Luminal B
7.	CAF153	66	Left	NOS	+	+	-	20%	Luminal B
8.	CAF154	82	Right	NST	+	+	+	15%	Luminal HER2+
9.	CAF155	61	Left	NST	+	+	+	80%	Luminal HER2+
10.	CAF157	68	Right	NHG1	+	+	-	5%	Luminal A
11.	CAF159	74	Left	NOS	+	+	+	20%	Luminal HER2+
12.	CAF161	79	Right	NOS	+	+	-	2%	Luminal A
13.	CAF163	45	Left	NOS	+	+	-	30%	Luminal B
14.	CAF165	77	Left	NOS	+	-	-	15%	Luminal B

mg/mL of hyaluronidase and 1.60 mg/mL collagenase IV. After incubation overnight, the suspension of cells was transferred to a probe containing 2 ml of phosphate buffer saline (PBS) (Biowest, France). The suspension was centrifuged (700 × g, 5 minutes, room temperature (RT)). After that, the supernatant was removed. The cell pellet was suspended in a fresh culture medium and seeded on 3 wells of a 12-well plate (VWR, Germany).

Cell culture

Isolated CAFs were incubated in a humidified incubator containing 5% CO₂. The cell culture medium consisted of DMEM (Biowest, France) supplemented with 10% FBS (Biowest, France) and the addition of antibiotic agents (penicillin/streptomycin at a final concentration of 1%) (Merck Millipore Corporation, Germany). During the first two weeks after the isolation process, cells were cultured with the previously described medium with the addition of epidermal growth factor (EGF) at 10 ng/mL concentration. Cells were passaged when a confluence of cells reached 80-90%. Cultured CAFs with the passage number from 1-6 were used for experiments.

Flow cytometry

CAF cells were harvested, suspended in PBS and washed twice. Cells were incubated for 30 min in 4°C with 5 µL of following antibodies: CD24 (catalog number: #B23133), CD31 (cata-

log number: #B13035), CD44 (catalog number: B37789), CD45 (catalog number: #IM2473U), CD90 (catalog number: #IM1839), CD146 (catalog number: #A07483), CD200 (catalog number: #B43301) (Beckman Coulter, Poland), CD140a (catalog number: 1P-589-T100), CD140b (catalog number: 1P-590-T100), CD29 (catalog number: 1F-219-T100) (EXBIO, Czech Republic). After that, cells were washed once with PBS. Cells stained with the Vimentin antibody (VIM) (catalogue number: 1A-369-C100) were previously fixed and permeabilized with the Fixation/Permeabilization Kit (BD Biosciences, NJ, USA). Next, cells were washed with PBS once and incubated with the antibody for 30 min at 4°C. All stained cells were analyzed by Cytoflex Beckmann Coulter cytometer (Beckman Coulter Life Sciences, ID, USA). Analysis of the obtained results was performed using FlowJo v10 (FlowJo LLC, USA).

Immunofluorescence

CAF cells were seeded on the 8-well chamber slides (VWR, Germany) with a 25000 cells/well density. Cells were washed with PBS, fixed in 4% paraformaldehyde for 20 min at RT and permeabilized with 100% methanol at -20°C for 20 min. Blocking was performed by incubation with 0.2% Triton X-100 and 1% Bovine Serum Albumin (BSA) (VWR, Germany) solution for 30 min at RT. Next, 200 µL of a primary antibody solution (anti-fibroblast activation protein (FAP) produced in rabbit, alpha-smooth muscle actin (α-SMA) produced in mice, interleukin 6 (IL-6)

produced in rabbit, interleukin 8 (IL-8) produced in mouse (all antibodies from Sigma-Aldrich® Solutions, MO, USA)), was added into each chamber and slides were incubated for 2 h in RT. After the incubation, cells were washed thrice with 2% BSA in PBS solution and incubated with 250 µL of secondary antibody solution for 1 h at 37°C in darkness. All slides were washed thrice with 2% BSA in PBS solution and 400 µL of DAPI (catalogue number: SAFSD8417) (VWR, Germany) solution was added. Immunofluorescence was photographed using a Olympus IX83 microscope (Boston Industries, Inc., MA, USA).

RT-qPCR analysis

RNA was isolated using Direct-zol RNA Mini-Prep (Zymoresearch, Irvine, CA, USA). The suspension of 1×10^6 cells in TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) was used. After obtaining 1 µg of total RNA, the reverse transcription using iScript kit (Bio-Rad, Hercules, CA, USA) was performed. The cDNA was amplified in a total volume of 20 µl and diluted 5 times. Next, the expression of genes was analyzed using RT-qPCR: *VIM* (Forward: 5'-GGGAC-CTCTACGAGGAGGAG-3'; Reverse: 5'-CTTTGTC-GTTGGTTAGCTGGT-3'; Probe no. 24), *ACTA2* (α -SMA) (Forward: 5'-CTGTTCCAGCCATCCTTC-AT-3'; Reverse: 5'-TCATGATGCTGTTGTAGGTG-GT-3'; Probe no. 58), *FAP* (Forward: 5'-GGAAG-TGCCTGTTCCAGCAATG-3'; Reverse: 5'-TGTCTG-CCAGTCTTCCCTGAAG-3'; SYBR), *S100a4* (FSP-1) (Forward: 5'-CAGAACTAAAGGAGCTGCTGACC-3'; Reverse: 5'-CTTGAAGTCCACCTCGTTGTC-3'; SYBR). To determine relative expression, human GAPDH gene (Forward: 5'-GTCTCCTCT-GACTTCAACAGCG-3'; Reverse: 5'-ACCACCCTG-TTGCTGTAGCCAA-3'; SYBR) was used as a reference gene. The PCR reaction was conducted in CFX96 Touch Real-Time Detection System (Bio-Rad Hercules, CA, USA) in 10 µl volume.

Statistical analysis

The statistical analysis was performed using PQStat Software v.1.8.2. and Microsoft® Excel® (Microsoft Office Professional Plus 2019). The normality of the observed data distribution was assessed using the Shapiro-Wilk test. The Kruskal-Wallis test and one-way ANOVA were conducted for multiple comparison. The data were deemed significant at $P < 0.05$.

Results

CAF isolation from breast cancer tissue

CAFs, as a part of TME, are an interesting group of cells taking part in tumor-promoting or inhibiting processes. The methods of CAFs isolation differ among various scientific groups [5, 18]. To isolate CAFs, we used a two-step method of isolation: enzymatic and mechanical digestion of BC tissue. After cutting the biopsy material with a scalpel, tissue was placed in the digestion media for 24 hours. The efficiency of the isolation process was almost 95%. We established and phenotyped 14 CAF cell lines from BC specimens. We received a BC biopsy sample from 3 subtypes: 4 luminal A, 6 luminal B and 4 luminal HER2+ types.

CAF primary phenotyping

The flow cytometry analysis was performed for primary characterization and phenotyping of CAF cell lines. We used the CD90, CD31, and CD45 antibodies to confirm the fibroblast phenotype of cells (**Figure 1**). CD90 (Thy-1) is known as a characteristic biomarker of fibroblast from different species and tissues [19, 20]. This protein is also described as a cell surface glycoprotein found on mouse thymocytes, the smallest member of the immunoglobulin superfamily with a molecular weight of 25-35 kDa [21-23]. CD90 is presented on stem cells including mesenchymal stem cells, hematopoietic stem cells, and keratinocyte stem cells, and on various non-lymphoid tissues such as fibroblasts [22, 24, 25]. CD31^{Neg} cells are confirmed as non-endothelial and CD45^{Neg} cells as non-leukocytes. All of the investigated cell lines presented a high level of CD90 expression. CAF cells also showed the CD31^{Neg} and CD45^{Neg} expression profile which was expected. We concluded that obtained cells represent a fibroblast phenotype after double confirmation based on detected positive and negative expression of chosen markers. Moreover, the elongated, spindle-like shape of cells was observed using light microscopy.

Phenotyping of CAF subtypes and characteristic features

The aim of the second part of the study was to phenotype and identify different CAF subtypes. We used 3 different techniques; flow cytometry,

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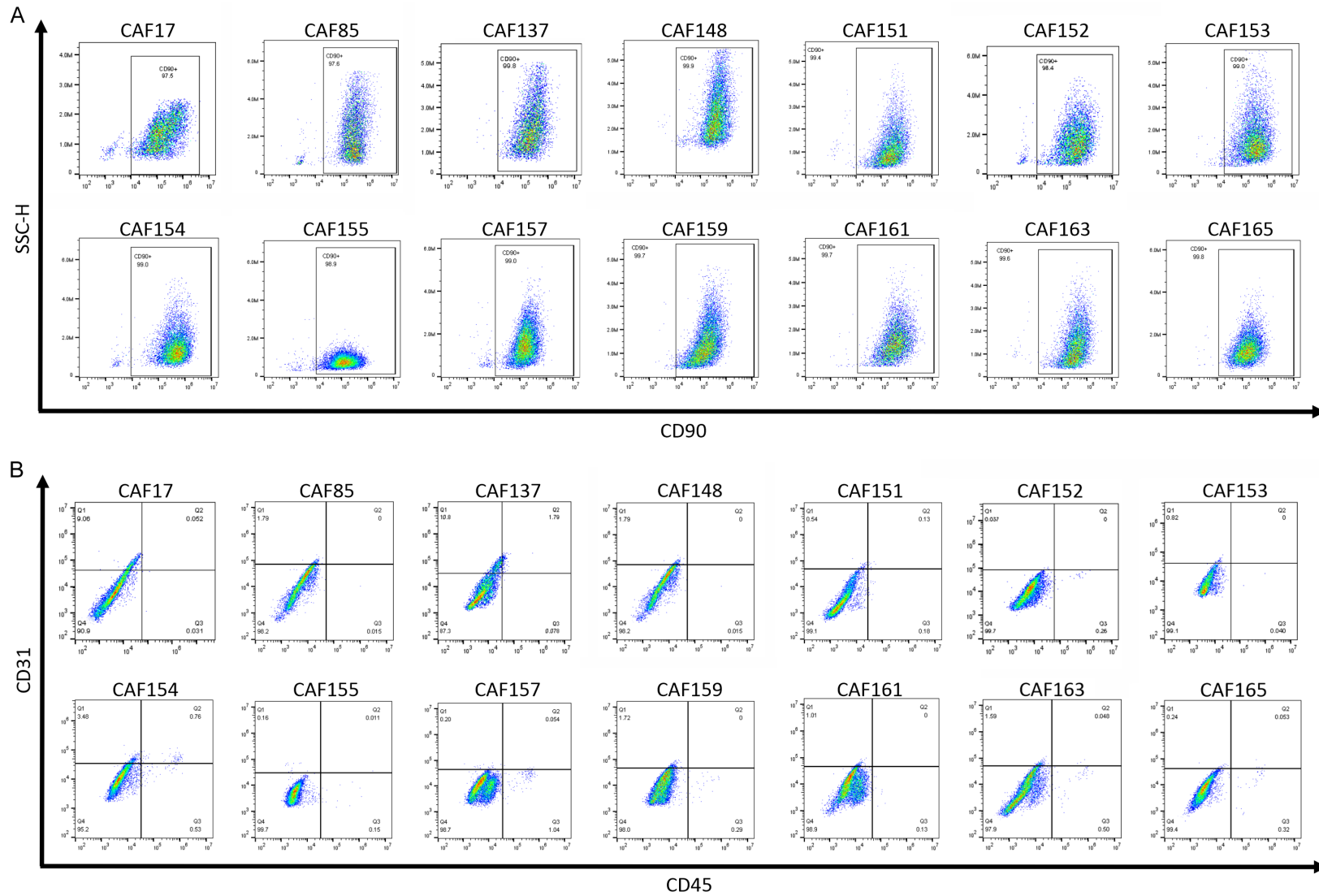


Figure 1. Primary phenotyping of all CAF cell lines. CAFs isolated from patients were characterized using CD90, CD31 and CD45 to confirm their fibroblast phenotype. A: Plots of cells for CD90, a characteristic biomarker of a fibroblasts phenotype. B: Plots of cells for CD31 and CD45. CD31^{Neg} cells are confirmed as non-endothelial and CD45^{Neg} cells as non-leukocytes.

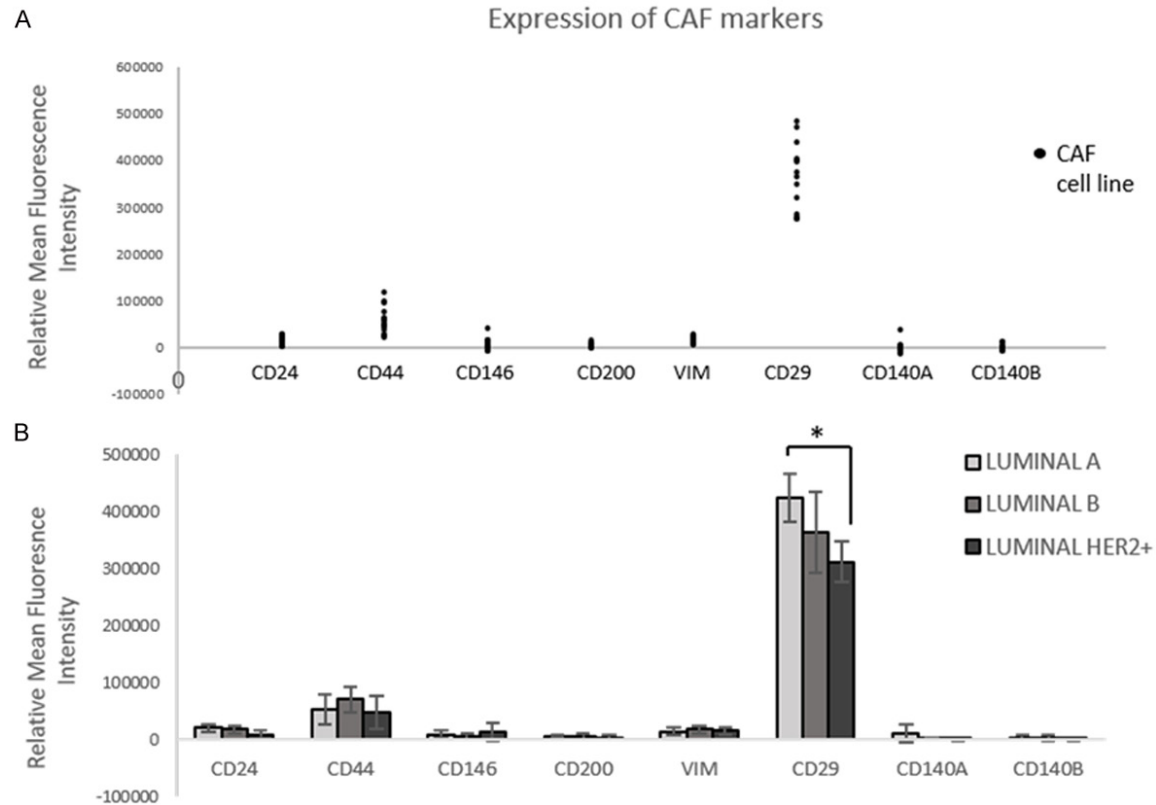


Figure 2. Expression of CAF markers. Various expressions of CAF markers were determined using flow cytometry. We used CD24, CD44, CD146, CD200, VIM, CD29, CD140a and CD140b. A: A plot of relative mean fluorescence intensity and chosen CAF markers. Each black dot symbolized one CAF cell line to show the distribution of established results through one marker. The highest spread of results was observed in CD29, a marker of Integrin beta-1, a cell surface receptor encoded in human by the ITGB1 gene. This integrin form integrin complexes, which function as collagen receptors. B: A plot of relative mean fluorescence intensity and chosen CAF markers considering divided CAFs based on different subtypes of BC. Mean values for each subtype consisted of values of each CAF from the BC subtype group. There was no statistically significant difference between subtypes apart from a pair of luminal A and HER2+ subtypes in CD29.

immunofluorescence, and RT-qPCR. Various expressions of CAF markers were determined using flow cytometry. We used CD24, CD44, CD146, CD200, VIM, CD29, CD140a (PDGFR α), and CD140b (PDGFR β). All established fibroblast cultures were characterized up to the 6th passage for expressing mesenchymal markers, CD44 and CD90. As shown in **Figures 1** and **2**, almost 99% of the cells showed homogeneous expression of CD44 and CD90. The expression of CD24 was analyzed to evaluate the potential contamination of epithelial cells. All the cell cultures were found to be negative for an epithelial phenotype. We identified the established cell cultures as CAF cell lines based on these results.

Some CAF markers were chosen based on the phenotype features detected in CAFs isolated from different types of tumors (**Figure 2A**).

Examples are CD146 and CD200, which expression or lack of it in CAFs is considered an indicator of disease outcome [26]. Interestingly, the presence of those proteins might determine the level of resistance to some therapies [27]. We observed heterogeneity in the level of those markers. Based on the subtype of BC, we revealed a predominating expression for each marker. Analyzing CD146 in CAFs from a luminal A BC subtype, a predominating level of expression was CD146^{Low-medium}, in CAFs from a luminal B BC subtype was CD146^{Neg-low}, and in CAFs from a luminal HER2+ BC subtype the expression was variable. The CD200 expression was more homogenous throughout all CAF cell lines. A predominating level of expression was CD200^{low} or CD200^{negative}.

Vimentin, CD140a (PDGFR α), and CD140b (PDGFR β) are usually detected in all CAFs. Our

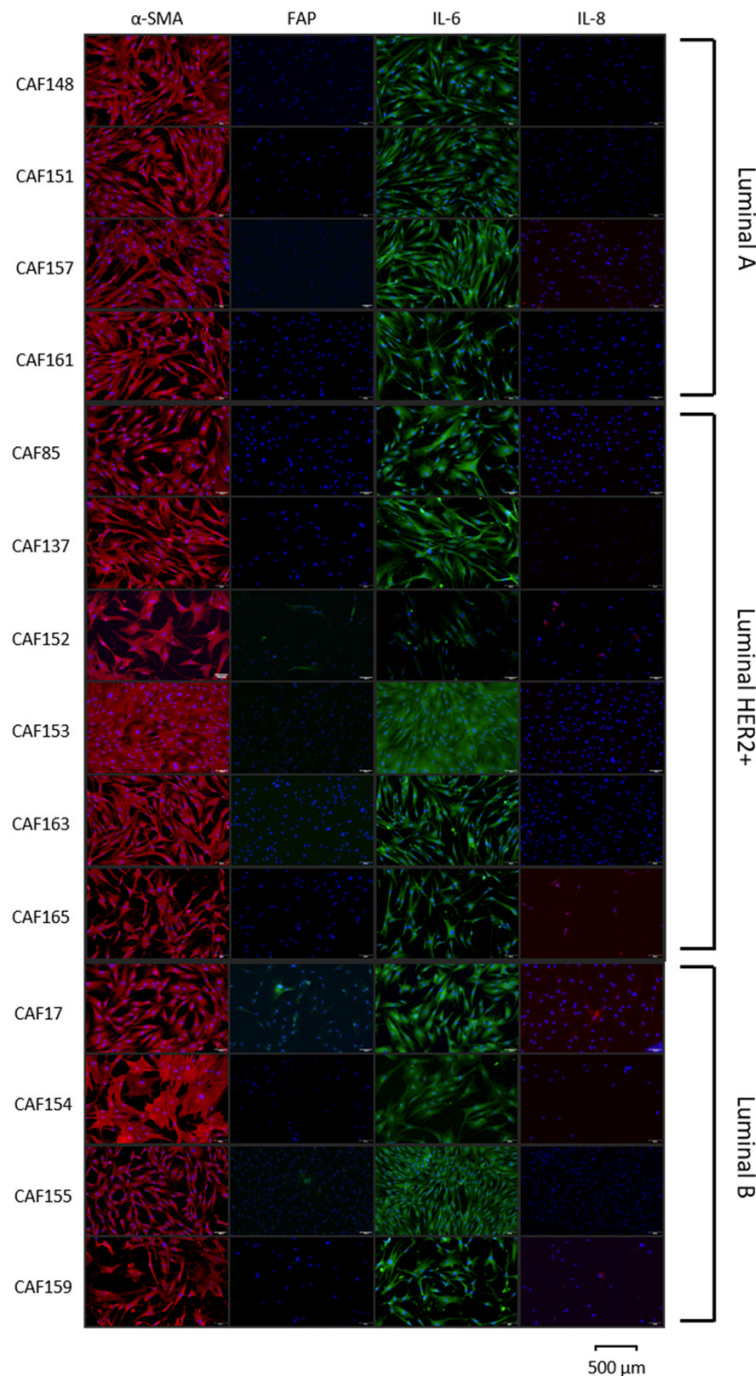


Figure 3. Immunofluorescence phenotyping of CAFs isolated from a luminal A, B, HER2+ BC subtype. CAFs were analyzed for α -SMA, FAP, IL-6 and IL-8. Different antibodies expression levels were observed: α -SMA high, FAP negative to low, IL-6 medium to high, IL-8 negative to low. DAPI dye was used for a nucleus of cells.

results present no statistically significant differences when comparing CAFs isolated from different types of BC. The expression status is similar in all CAF cell lines. The predominating level for VIM was medium, and for CD140A and

CD140B the level was negative or low. We found one statistically significant result regarding CD29 expression. CAFs divided into 3 groups of BC subtype, from which they were isolated, indicated a difference between luminal A and HER2+ subtype in CD29 ($P=0.026$). CAFs from the luminal A BC subtype presented the highest expression level, whereas CAFs from luminal HER2+ BC showed the lowest values (**Figure 2B**).

Our group carried out immunofluorescence staining of all CAF cell lines. We chose four markers for measurement: α -SMA, FAP, IL-6, and IL-8. The analysis showed similar immunofluorescence results for all CAF cell lines. The expression of α -SMA and IL-6 was high in all CAFs, with some cases of medium level of IL-6 in luminal B and HER2+ subtype. Contrastingly, there was no expression of FAP and interleukin 8 in tested cell lines (**Figure 3**).

Gene expression of CAFs varies among different cell lines

We performed the RT-qPCR analysis to determine the expression of VIM, α -SMA, FAP, and FSP-1 genes. As a housekeeping gene, GAPDH gene was used. After obtaining mostly homogenous results from immunofluorescence staining (α -SMA, FAP) and flow cytometry (VIM), we expected similar results from RT-qPCR. Interestingly, observed expression values were quite different. Predominating expres-

sion of α -SMA in CAFs from BC luminal A subtype was on medium level, in contrast to luminal B and HER2+ CAF groups where the level of expression varied (**Figure 4**). Expression of FAP and FSP-1 in CAF cell lines of all BC subtypes

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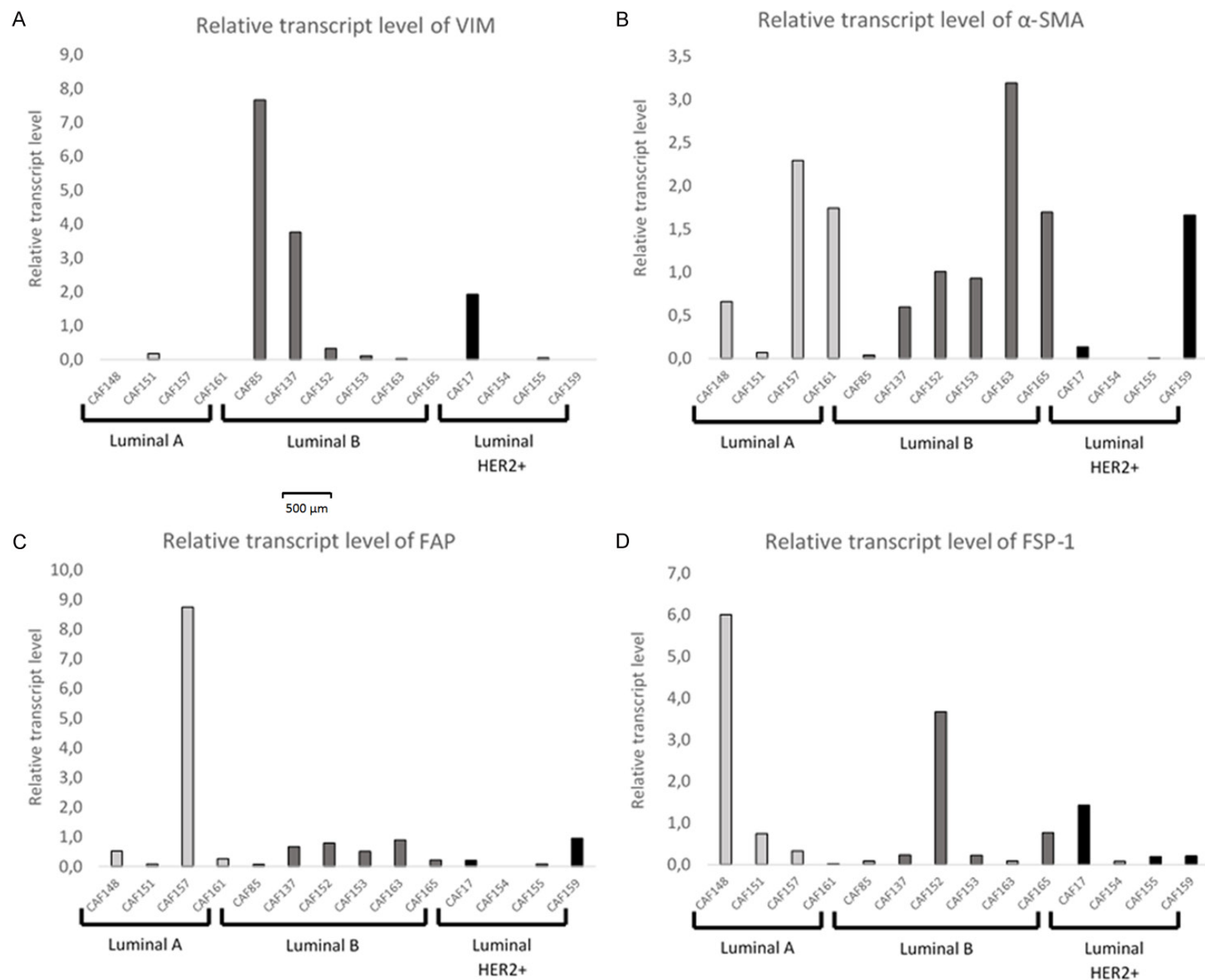


Figure 4. Relative transcript levels of VIM (A), α -SMA (B), FAP (C) and FSP-1 (D). RT-qPCR was performed using all CAF cell lines. CAFs are divided based on different BC subtypes (luminal A, luminal B, luminal HER2+) from which CAFs were isolated. There was no statistically significant difference between subtypes in each gene expression.

were more similar. In the luminal A and B group, predominating FAP and FSP-1 expression was medium, but for the HER2+ group was low. Analyzing vimentin expression, we observed three different expression levels in groups of CAFs. There was no expression of vimentin in the luminal A group, in the luminal B the expression was variable, whereas in luminal HER2+ was low.

Although differences in predominating expression were noticed, they were not high enough to be statistically significant. The summary of all results of CAF cell lines' phenotyping is included in **Table 2**. The obtained results were divided into: negative, low, medium, and high. The predominating expression of each antibody or gene was highlighted as a representative status of expression.

Discussion

CAFs are a highly heterogeneous group of cells whose phenotypes and gene alterations are still under deep investigation. As a part of TME, they are the focus of a growing number of studies. CAFs might become a new target of BC therapy, but they still need more tests and analyses to understand mechanisms and interactions between them and BC cells. This study aimed to isolate CAFs from BC tissue and the phenotype of isolated cell lines. We focused on various CAF-characteristic biomarkers and those which might differentiate CAFs subtypes. We also wanted to observe differences between CAFs isolated from three different BC subtypes: luminal A, luminal B, and luminal HER2+. We divided CAFs into 3 groups based on the subtype of BC from which CAFs were isolated, and the results of those groups were compared.

We isolated 14 CAF cell lines from tissues received from a biopsy of a BC tumour. The overall effectiveness of the isolation process was almost 95%. All analyses were performed up to the 6th passage of cells to maintain similar conditions during culture and tests. The first experiment was based on flow cytometry to verify the fibroblast phenotype of isolated cells. We used CD90, CD45, CD31, CD44 and CD24

as markers for CAF primary phenotyping. The glycoprotein CD90 is a widely expressed surface marker of mesenchymal cells [28], fibroblasts of various organs [29] and myofibroblasts [30]. All tested cell lines indicated high expression of CD90, confirming the fibroblast phenotype of isolated cells. Flow cytometry analysis demonstrated that fibroblastic cells are negative for hematopoietic and endothelial markers CD45 and CD31, defining a homogeneous population of fibroblasts. CD24 was used to confirm the absence of epithelial cells. It is known that fibroblasts associated with TME express CD44 [31]. This adhesion protein takes part in the process connected with cancer cell survival and metastasis [32]. Kinugasa et al. [31] found that CD44 expressed by CAFs might interact with cancer cells to support their survival in hypovascular areas and contribute to the maintenance of cancer stem cell populations. Another group investigated interactions between CAFs and BC cells [32]. They isolated and co-cultured CD44^{High} and CD44^{Neg} CAFs with BC cells and analysed cell survival and drug resistance. Results showed that CD44^{High} CAFs promoted BC cell survival and paclitaxel resistance. Furthermore, CAFs induced inhibition of paclitaxel-induced apoptosis. In our study, the CAF cell lines expressed CD44 at medium to high level.

The lack of selective markers of CAFs is still the concern for many scientific groups, but the new directions in characterizing CAFs and understanding their influence on cancer cells is getting more attention. D'Arcangelo et al. [33] described the CAFs life cycle highlighting that CAF identification biomarkers for different stages of their maturation are not available. Defining them might have therapeutic implications. The absence of specific CAFs' molecular markers has complicated their identification and data comparison between studies. We analysed 6 chosen biomarkers of CAFs: CD146, CD200, VIM, CD29, CD140a, and CD140b. The aim was to investigate biomarkers' expression levels and to compare the results between three CAF groups, which were divided based on BC subtypes from which they were isolated.

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Table 2. Phenotyping of CAF cell lines - summary

BC subtype	CAF cell line	Flow cytometry								Immunofluorescence				RT-qPCR			
		CD24	CD44	CD146	CD200	VIM	CD29	CD140a	CD140b	αSMA	FAP	IL-6	IL8	αSMA	FAP	FSP-1	VIM
Luminal A	CAF148	medium	high	medium	low	low	High	neg	neg	high	neg	high	neg	medium	medium	high	neg
	CAF151	medium	high	low	low	medium	High	low	low	high	neg	high	neg	low	low	medium	low
	CAF157	medium	medium	medium	low	low	High	low	medium	high	neg	high	neg	high	high	medium	neg
	CAF161	high	high	low	low	medium	High	high	neg	high	neg	high	neg	high	medium	neg	neg
	predominating expression	medium	high	low-medium	low	low-medium	High	variable	neg-low	high	neg	high	neg	medium	medium	medium	neg
Luminal B	CAF85	medium	high	neg	low	medium	High	neg	neg	high	neg	high	neg	neg	neg	low	high
	CAF137	medium	high	neg	low	low	High	low	neg	high	neg	high	neg	medium	medium	medium	high
	CAF152	medium	high	medium	low	medium	High	low	medium	high	neg	medium	neg	high	medium	high	medium
	CAF153	medium	high	low	neg	medium	High	neg	neg	high	neg	high	neg	medium	medium	low	low
	CAF163	medium	high	low	low	medium	High	neg	neg	high	neg	high	neg	high	medium	low	neg
	CAF165	low	high	low	neg	low	High	neg	low	high	neg	medium	neg	high	low	medium	neg
	predominating expression	medium	high	neg-low	low	medium	High	neg	neg-low	high	neg	high	neg	variable	medium	medium	variable
Luminal HER2+	CAF17	low	high	high	neg	low	High	neg	neg	high	neg	high	neg	low	low	high	high
	CAF154	medium	high	medium	low	medium	High	neg	neg	high	neg	medium	neg	neg	neg	low	neg
	CAF155	low	medium	neg	neg	medium	High	neg	neg	high	neg	high	neg	neg	low	low	neg
	CAF159	neg	medium	neg	low	medium	High	neg	neg	high	neg	medium	neg	high	high	low	neg
	predominating expression	neg-low	medium-high	variable	neg-low	medium	High	neg	neg	high	neg	medium-high	neg	variable	low	low	low

The table represents results performed by used techniques. The obtained results were divided into: negative, low, medium and high. The predominating expression of each antibody or gene was highlighted as a presentative status of expression. Abbreviations: BC - Breast Cancer, CAF - cancer associated fibroblast, VIM - vimentin, α-SMA - α-Smooth Muscle Actin, FAP - fibroblast activation protein, IL - interleukin, RT-qPCR - real time - quantitative polymerase chain reaction, neg - negative.

In some cases, there was no expression of the CD146 marker, and the level was low or variable when present. Brechbuhl et al. [27] analyzed breast CAFs as a key component altering BC ER expression levels and antiendocrine resistance. They studied patients' tissues to find CD146^{High} and CD146^{Neg} fibroblasts to demonstrate ER+ BC containing those two CAF subtypes. CD146^{Neg} CAFs caused lower ER expression in BC cells, decreased estrogen sensitivity and increased tamoxifen resistance. In contrast, CD146^{High} CAFs did not change any of those parameters. Considering gene expression profiles of tumors with predominantly CD146^{Neg} CAFs, worse patient outcomes were observed. We also analyzed the expression of CD200 to compare its presence between cancer types. There was no available data about this biomarker in BC. Based on the literature, CAFs expressing CD200 appeared in TME of pancreatic cancer [34] or lung cancer [26]. The CD200 is a known membrane glycoprotein which binds to the CD200R1 receptor and initiates tolerance to cells from the myeloid group [35]. In our study, the CD200 expression was highly homogenous in each CAF cell line. The values were low, or CAFs were CD200^{Neg}. MacNeil et al. [34] analyzed CAF subpopulations' expression levels and prognostic values in a cohort of pancreatic ductal adenocarcinoma patients. The group also planned to evaluate the expression and prognostic value of CD200. They observed that CD200 expression was heterogeneous in stromal cells. They concluded that this biomarker did not demonstrate any prognostic value, but they indicated the variable expression pattern of CD200. Another group [26] reported the existence of a subpopulation of patient-specific CAFs which increased the sensitivity of EGFR positive lung cancer to the EGFR-tyrosine kinase inhibitor (EGFR-TKI). They observed that knocking down CD200 expression deprived CAFs of their sensitizing ability, indicating that this protein might be responsible for this effect.

Vimentin is one of the divergent markers defining CAFs in BC [36]. Analyzing flow cytometry results, we noticed a medium protein level of VIM in all CAF lines. In contrast, while considering RT-qPCR analysis, variable results were observed. In most CAF cell lines, there was no VIM expression. The most variable group of CAFs were CAFs isolated from the luminal B BC.

There was no statistically significant differences between groups. The sensitivity of the methods might cause the diverse results of the two techniques. Some authors highlighted the differences between results obtained using various techniques. Quantitative RT-PCR detected low expression levels, whereas flow cytometry is less sensitive [37]. Scientists studied CAFs heterogeneity in TME of Pancreatic Ductal Adenocarcinoma (PDAC), analyzing vimentin expression with association of overall survival in patients [38]. They reviewed 67 PDAC patients, and each primary tumor was analyzed for vimentin. Results presented VIM as an independent predictor of poor survival in PDAC. Another group also explored the expression of typical CAF markers within the cellular subtypes [11]. They revealed that the non-specific mesenchymal marker transcript VIM was expressed by most cell lines. Taking into consideration CAFs influence on BC cells, α -SMA^{High}VIM^{High} CAFs also induced BC stemness via periostin-dependent Wnt signaling [39].

The next CAF biomarker, which we analyzed, was CD29. It is a biomarker of integrin β , generally expressed by CAFs, especially from BC TME [10, 40]. In our results, the CD29 presence on CAF cells' surface was observed, but it differed through CAFs groups of each BC subtype. We noticed a statistically significant difference in CD29 expression between CAFs isolated from luminal A and HER2+ BC subtypes. There is no available data about potential reasons for this difference. Thus, it requires further investigation. The last biomarkers studied using flow cytometry were CD140a (PDGFR α) and CD140b (PDGFR β). We observed a predominating trend in results manifested as a lack of PDGFR α / β expression in CAFs. Kim et al. [41] studied the expression of CAF-related proteins and the implications in breast phyllodes tumor (PT). They noticed that the expression of PDGFR α and PDGFR β in the CAFs raised with increasing histologic grade of PT. Next, they discovered the expression of PDGFR β in the stroma which correlated with shorter overall survival in PT patients, concluding that CAFs are related to breast PT progression. Group conducted by Nishishita et al. investigated the association between PDGFR β CAF markers and vessel markers with clinic pathological factors. In this study, PDGFR β tended to be correlated with high venous invasion. They concluded that

CAFs might have different expression patterns, which is associated with our conclusion about PDGFR α / β expression.

The other part of results involved immunofluorescence staining of CAFs cells. Four antibodies were used: α -SMA, FAP, IL-6, and IL-8 for this test. The α -SMA is the most popular marker for CAF phenotyping [42]. It is a cytoskeletal protein associated with TGF- β production and a highly contractile phenotype [43]. Scientists pointed out that α -SMA expression is observed in different kinds of cells but this biomarker is likely to be the indicator of CAF presence in TME [44]. According to our results, we noticed a high expression of α -SMA in all CAFs. Analyzing RT-qPCR, α -SMA results were more variable. As previously described, the RT-qPCR test is the more sensitive of the techniques we used. α -SMA is expressed by CAFs from different cancer types such as PDAC [45], pancreatic cancer [46], and breast cancer [10]. Myofibroblasts are actively proliferating cells expressing smooth muscle cell markers like α -SMA, enabling them to actively contract wound edges and enhance ECM component synthesis and remodeling to support healing [47]. Moreover, α -SMA^{High} CAFs were found to indirectly affect the immune response via deposition of extra cellular matrix and reconstructed the matrix on which immune cells localize or migrate [48]. Following, the FAP antibody was also tested. Again, we used immunofluorescence and RT-qPCR to check the FAP expression level. FAP is a well-known biomarker expressed at high levels on the cell surface of CAFs. High stromal expression levels of FAP correlate with poor prognosis. This biomarker was independently identified as a surface glycoprotein recognized in activated fibroblasts [49]. In immunofluorescence staining, we did not detect the presence of FAP protein. In contrast, gene expression analysis presented a different outcome. We noticed variable levels of FAP expression with the dominant trend of medium level. This divergence possibly occurred because of insufficient expression of the FAP gene to synthesize an appropriate amount of FAP protein to observe immunofluorescence under microscopy. The expression of protein could also be modified post-translationally, which could affect the protein level. Identifying the fibroblasts and cancer-associated fibroblasts from human cancer tissue using surface markers is difficult, mainly because the mark-

ers used currently are usually not expressed solely by fibroblasts, and the identification of fibroblast-specific surface molecules is still under investigation [50]. One study focused on reducing the pro-tumorigenic activities of CAFs by depleting FAP from fibroblasts growing in a composite environment with epithelial tumor cells [51]. The authors concluded that targeting FAP on CAF suppresses pro-tumorigenic activities and may result in a clinically effective decrease of tumor progression. This correlation was also confirmed in ovarian cancer, where the group found that FAP-positive CAFs are associated with poor patient outcomes [52].

Our group studied the expression of two interleukin-6 and 8. The immunofluorescence analysis indicated high expression of IL-6, but in contrast, there was no expression of IL-8 in CAFs. Many researchers presented a production of IL-6 by TME fibroblasts. Cheteh et al. [53] determined the effect of CAFs on the p53 response to doxorubicin in prostate cancer cells. They discovered that IL-6 produced by CAFs reduces p53 induction and upregulation of the pro-apoptotic p53 target upon treatment with doxorubicin. This study suggested that CAF-derived IL-6 is essential in protecting cancer cells from chemotherapy, and blockage of IL-6 production in CAFs could be a potential therapeutic target. CAFs' tumor-promoting properties were also noticed in gastric cancer [54]. Wu et al. showed that CAFs isolated from gastric cancer produce significant amount of interleukin-6. CAFs enhanced the migration and EMT of gastric cancer cells through the secretion of IL-6 which activates Janus kinase 2/signal transducers and activators of transcription (JAK2/STAT3) pathway in gastric cancer cells. Along with the previous study, the group pointed out that IL-6 targeted therapy could be a complementary approach against gastric cancer by exerting their action on stromal fibroblasts. Another study by Su et al. [12] showed that CD10+ GPR77+ CAFs subtype is related to BC stemness and chemoresistance via sustained secretion of NF- κ B-dependent IL-6 and IL-8. The expression of IL-6 was also a criterion for the categorization of CAF subtypes in PDAC [55]. The inflammatory CAFs (iCAF) were identified by α -SMA^{low}IL-6^{high} expressions and were located more distantly from neoplastic cells, while myofibroblastic CAFs (myCAFs) were determined by α -SMA^{high}IL-6^{low} and located closer

to tumor cells. Another group presented the correlation of IL-6 and IL-8 expression with melanoma cell invasiveness [56]. Our results of phenotyping CAFs in terms of IL-6 and IL-8 as well as studies described above, show how important it is to investigate the IL-6/IL-8 targeted therapies based on the CAFs phenotype.

The last analyzed gene was S100A4 (FSP-1), also called fibroblast specific protein 1. It is mostly expressed on CAFs differentiated from epithelial or endothelial cells [57]. Our analysis mainly indicated heterogeneous FSP-1 expression, but the predominating expression level for all CAF groups was medium. In some cases, there was no expression of this gene. Authors showed that FSP-1 positive CAFs can promote tumor metastasis through secretion of VEGF-A and Tenascin-C [58]. On the other hand, the same CAF type can activate tumor immune response by promoting CD8 positive T cell activation through fusion with dendritic cells [59]. These reports show the controversial nature of this gene, which requires further analysis in CAFs.

The most cited work about CAFs subtypes in BC is by Costa et al. [10]. They determined CAFs as a crucial player in TME. The aim was to characterize four CAF subsets in BC with distinct properties and levels of activation. They performed the analysis of six fibroblast markers (FAP, CD29, α -SMA, S100-A4/FSP1, PDGFR β , and caveolin 1 (CAV1)). In conclusion, these CAF subsets were defined as follows: CAF-S1: CD29^{Med} FAP^{Hi} FSP1^{Low-Hi} α -SMA^{Hi} PDGFR β ^{Med-Hi} CAV1^{Low}; CAF-S2: CD29^{Low} FAP^{Neg} FSP1^{Neg-Low} α -SMA^{Neg} PDGFR β ^{Neg} CAV1^{Neg}; CAF-S3: CD29^{Med} FAP^{Neg} FSP1^{Med-Hi} α -SMA^{Neg-Low} PDGFR β ^{Med} CAV1^{Neg-Low}; CAF-S4: CD29^{Hi} FAP^{Neg} FSP1^{Low-Med} α -SMA^{Hi} PDGFR β ^{Low-Med} CAV1^{Neg-Low}. The group indicated two myofibroblast subsets (CAF-S1 and CAF-S4) which accumulated differently in triple-negative breast cancers. Interestingly, CAF-S1 were able to promote an immunosuppressive environment through a multi-step mechanism. Furthermore, this subtype increases T lymphocyte accumulation and their survival. They revealed that luminal A BC subtype is enriched with CAF-S2 subtype, while HER2+ BC subtypes with CAF-S4. There was no data about CAFs from luminal B BC. They also highlighted that TME of TNBC is more heterogenic due to the presence of CAF-S1 and CAF-S4 subtypes. CAF-S3 cells were associated with juxta-tumor.

Understanding the diverse properties of CAFs throughout the stages of tumor progression and various cancer types remains incomplete. The function of CAFs has remained elusive in light of their heterogeneity and different effects on tumor development [33].

Conclusion

Studies have presented that CAFs include diverse subtypes based on the expression of marker proteins with the capacity to promote or inhibit cancer. Biomarkers of specific CAF subtypes could guide the development of novel genetic and pharmacological approaches to target specific populations [13]. Based on our study, there was no indication of a clear pattern in the CAFs classification. Results of CAFs expression were highly heterogeneous, and specific CAF subtypes were not clearly defined. Moreover, comparing CAF divided into groups based on BC subtypes from which CAFs were isolated also did not allow us to notice any clear pattern of expression. In the future, a higher number of analyzed CAF cell lines should be investigated to find expression patterns. Characterized CAF cell lines, being a part of TME, are crucial for the *in vitro* analysis of interactions between TME and isolated primary BC cell lines. It is essential to find molecular pathways engaged in promoting BC progression.

Although some improvement has been made to establish the functions of phenotypically distinct CAF subtypes, more studies need to be done to demonstrate whether CAF heterogeneity is clinically relevant rather than simply descriptive [13]. A more systematic regularity of the CAF investigation is also essential. However, a standardized nomenclature of CAFs, and the presence of well-characterized CAF populations across different cancer types, is also crucial. The potential clinical success of CAF depends on a deep understanding of their functions and interaction with cancer cells and also how we can change their phenotypic functions to translate this into fibroblast-targeting therapy [42].

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Disclosure of conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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