APRIL and BAFF increase breast cancer cell stemness

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Abstract

Background: Recent advances in cancer immunology revealed immune-related properties of the cancer cell as a promising new therapeutic target. The two TNF superfamily members, APRIL and BAFF even though were primarily studied for their role in lymphocyte maturation, their presence in a number of normal and cancer solid tumors including breast cancer revealed an association with tumor growth and aggressiveness. **Methods**: In the present work, we have explored the role of APRIL and BAFF in breast tumor development, progression and metastasis as well as resistance to therapy. We studied their effect on the epithelial to mesenchymal transition and migration of breast cancer cells, and their action on the important sub-population of cancer stem cells identified by autofluorescence and ALDH activity. Their action on an number of pluripotency genes was examined and breast cancer stem cell ability to form mammospheres was also utilized. The receptor and the signaling pathway involved were also investigated as well as the role of steroid hormones in their action. **Results:** Our findings show that in breast cancer both APRIL and BAFF increase epithelial to mesenchymal transition and migratory capacity, as well as cancer stem cell numbers, by inducing pluripotency genes such as KLF4 and NANOG. These effects were mediated by their common receptor BCMA and the JNK signaling pathway. Interestingly, androgens enhance APRIL transcription and subsequently its pluripotency effect. **Conclusions:** All these data support the significant role of APRIL and BAFF in breast cancer disease progression and provide evidence for a new possible mechanism of therapy resistance, especially in aromatase inhibitors-treated patients, were local androgen is increased.

Keywords

APRIL, BAFF, TNF members, pluripotency, EMT, cancer stem cells, therapy resistance, aromatase inhibitors

Background

A major recent advancement in cancer therapeutics relay on the discovery of cancer-immune cell interaction in the tumor microenvironment [1, 2], which led to the initiation of trials and the recent establishment of the first cancer immune therapies [3, 4]. However, in the majority of cases, these therapies target immune cells (resident or infiltrating the tumor stroma) [5], leading to an immune checkpoint blockade [6], while the notion of immune-related properties of the cancer cell *per se* and its possible regulation as a possible therapeutic target is less well defined and begins to emerge only very recently [7-9].

A great number of immune-related molecules has been identified and targeted in cancer immunotherapy. Among them, TNF superfamily members (including TNF, FAS and TRAIL and their receptors) [5], have been actively investigated and targeted immune therapies in a number of malignancies. The TNF superfamily, that includes 19 different ligands and 29 receptors, control cell survival and differentiation and play an important role in the growth, organization and homeostasis of different tissues by modulating major signaling pathways [10]. However, an exhaustive analysis of the expression and secretion of TNFSF and TNFRSF members in neoplasia has not been made until now. Our group has focused on two, relative recent addition of this superfamily, namely APRIL (A PRoliferation Inducing Ligand) and BAFF (B-cell Activating Factor of the TNF Family). These two ligands, act via their cognate receptors B-Cell Maturation Antigen (BCMA) and Transmembrane Activator and CAML Interactor (TACI) which are responsive to both ligands while BAFF-Receptor (BAFF-R) is a specific receptor for BAFF. They had initially been reported to have a central role in lymphocyte maturation; however, they have been also identified as significant players in several other conditions including neoplasia [11]. Indeed, BAFF

and APRIL have been detected in a number of solid tumors [12], activating significant kinase signaling pathways, such as p38, JNK, NFκB and inducing, in the majority of tumors, cell survival and growth. Previous work by our group has shown their presence in a number of normal and cancer solid tumors including breast cancer [13-16]. More specifically, we were the first to report APRIL and BAFF expression in breast tissue and that BAFF is constantly expressed in tumors, while APRIL is related to tumor grade [15]. Recently, higher APRIL expression was shown in human triple negative carcinomas and APRIL was reported to induce cell proliferation both *in vitro* and *in vivo*, suggesting an association of APRIL signaling pathways with tumor aggressiveness [17].

An important aspect in tumor development, progression and metastasis as well as resistance to therapy is the presence and activity of cancer stem cells, an important sub-population with unlimited proliferative potential, an ability to initiate and/or reform a tumor and an enhanced resistance to therapy [18]. Therefore, in the present work, we examined the action of APRIL and BAFF on breast cancer stem cells, in order to further decipher their role in regulating the ability of breast cancer cells to survive and escape therapeutic approaches. Our results clearly show that both APRIL and BAFF promote breast cancer cell stemness acting via their common receptor BCMA. This effect was found to be, at least in the case of APRIL, enhanced by androgens which also induced APRIL expression. Interestingly, increased androgens levels could be achieved locally, by the use of aromatase inhibitors. Indeed, when we used a GEO-deposited study letrozole (aromatase inhibitor) treated patients we found that letrozole treated patients had higher APRIL levels after treatment. All these evidences also support an autocrine effect of these immunomodulatory molecules in

breast cancer cells and provide a new mechanism for cancer disease progression as well as hormonal therapy resistance.

Methods

Cell cultures and chemicals

The T47D breast cancer cell line and MDA-MB-231 were purchased from DSMZ (Braunschweig, Germany), and were cultured in RPMI supplemented with 10% fetal bovine serum (FBS), at 37 °C, 5% CO_2. All media were purchased from Invitrogen (Carlsband, USA) and all chemicals from Sigma (St. Louis, MO), unless otherwise stated.

RT-PCR

RT-PCR was performed as described previously [19]. Positive controls were run in parallel with samples included in the study. We used adipose-derived mesenchymal stem cells as positive controls of BAFF and APRIL [13], isolated human lymphocytes as positive controls for BAFFR and TACI, and HepG2 cells for BCMA[19]. The primers used for the study were as follows: **BAFF** forward: 5′-TTC TAG GGC ACT TCC CCT TT-3′, reverse: 5′-CTC AAG ACT GCT TGC AAC TGA-3′; **APRIL** forward: 5′-TCT CCT TTT CCG GGA TCT CT-3′, reverse: 5′-CCA GAA TGG GGA AGG GTA TC-3′; **BAFFR** forward: 5′-AGG ACG CCC CAG AGC C-3′, reverse: 5′-AGT GTC TGT GCT TCT GCA GG-3′; **TACI** forward: 5′-AGT GAA CCT TCC ACC AGA GC-3′, reverse: 5′-CTC TTC TTG AGG AAG CAG GC-3′; **BCMA** forward: 5′-GTC AGC GTT ATT GTA ATG CAA GTG T-3′, reverse: 5′-TCT TTT CCA GGT CAA TGT TAG CC-3′; **18S** RNA forward: 5′-ATG GTC AAC CCC ACC GTG T-3′, reverse: 5′-TTC TGC TGT CTT TGG AAC TTT GTC-3′. All primers

were selected from qPrimer Depot (qPrimerDepot, http://primerdepot.nci.nih.gov) and synthesized by VBC Biotech (Vienna, Austria).

Real Time PCR

Total RNA was isolated with the Nucleospin RNA II isolation kit (Macherey-Nagel EURL, Fr). Absence of DNA was verified by PCR for G3PDH. One μg of RNA was subjected to ABI High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR with SYBR Green was performed with DyNAmo SYBR Green qPCR Kit (Finnzymes, Oy, Finland), using the StepOnePlus™ System (Applied Biosystems), at 95°C for 3 min followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 60 sec. PCR reactions were performed using the following primer pairs (synthesized by Eurofins Genomics): **c-MYC**: forward CACCGAGTCGTAGTCGAGGT and reverse TTTCGGGTAGTGGAAAACCA **SOX-2**: forward AGG AGC CTT CCT TTT CCA GA and reverse CGACGAGAGCTCCTACCAAC, **ALDH1A1**: forward TCCTCCTCAGTTGCAGGATT and reverse GCACGCCAGACTTACCTGTC, **KLF4**: forward GTCAGTTCATCTGAGCGGG and reverse AGAGTTCCCATCTCAAGGCA. The number of cycles used was optimized to fall within the linear range of PCR amplification. Changes were normalized according to cyclophilin A expression (forward: ATG GTC AAC CCC ACC GTG T and reverse: TTC TGC TGT CTT TGG AAC TTT GTC).

Detection of cancer stem cells

Autofluorescence based detection: Cells after treatment with APRIL or BAFF (100ng/ml) for 4 days were detached by trypsin-EDTA from the culture plate and

centrifuged (800g 10min). The pellet was re-suspended in PBS+2% FBS at a cell concentration of $1x10^6$ cells/ml. They were analyzed by flow cytometry (Attune® Acoustic Focusing Cytometer, Applied Biosystems) at a cell population of at least 20000 at 488 (580/30)/488(530/40 (BL2-A/BL1-A). Dot Blot Diagram taking into advantage the finding of Miranda-Lorenzo et al. 2014 [20] that cancer stem cells exhibit a higher level of autofluorescence.

***Aldehyde Dehydrogenase activity based detection*:** Stem cells, that have the characteristic to express high levels of the enzyme aldehyde dehydrogenase (ALDH) were detected by the use of ALDEFLUOR™ kit (Stem Cell Technologies Inc., Vancouver, Canada). According to the manufacturer's instructions, a cell suspension of $1x10^6$ cells/ml assay buffer, untreated or after treatment with APRIL or BAFF (100ng/ml) for 4 days, was incubated (45 min, at 37°C) with the ALDEFLUOR™ Reagent which is a fluorescent substrate for ALDH with (control sample) or without (test sample) the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB). The fluorescent reaction product, that is retained within the cells and is proportional to the activity of ALDH, was measured by flow cytometry (Attune® Acoustic Focusing Cytometer, Applied Biosystems). Data acquisition was performed using identical instrument settings for each test and control sample on a population of 20000 cells in a SSC-H/BL1-H Dot Blot Diagram.

Mammosphere formation

Mammosphere formation was assayed as previously described[21]. For primary mammosphere generation, cells (70-80% confluent) were detached by trypsinization, centrifuged at 580 x g for 2 min, re-suspended in 2 ml of ice-cold PBS and passed several times through a 25 G syringe needle. Then cells were seeded in 6-well plates,

coated with 1ml/well of 1.2% poly-(2- hydroxyethyl methacrylate (pHEMA) solution in absolute ethanol, for 48h at 40°C (600 cells/cm^2). Cells were incubated in the presence of APRIL or BAFF (100ng/ml), testosterone-BSA (10^{-6}M) or vehicle, in a humidified atmosphere at 37°C and 5 % CO2, for 7-12 days, without disturbing the plates or replenishing the medium. The number of mammospheres (primary generation, defined as a cellular mass of at least 10 cells more than 50μm in size) was counted, with a Leica DMIRE2 inverted microscope, at 40X magnification. For the generation of secondary mammospheres, primary mammospheres were collected, washed twice with 1ml PBS, centrifuged at 115g for 5 minutes and trypsinized with 300 μl of 0.5 % trypsin/0.2 % EDTA at 37°C for 2 min. After trypsin neutralization with 1ml of serum-containing medium and centrifugation at 580 g for 5 min, single cells from were resuspended in 200 μl of ice-cold PBS and seeded in pHEMA coated 6-well plates (600 cells/cm^2). Cells were incubated for 7 additional days and secondary mammospheres were counted as described above.

Immunocytochemistry

For the detection of immunoreactive proteins in breast cancer cells, treated or not (control) with APRIL or BAFF (100ng/ml) for 4 days, cells were fixed with 2% paraformaldehyde. Afterwards, they were incubated at room temperature for one hour with 3% BSA in TBS, and then overnight at 4 °C with the primary antibody (c-MYC 1/600, ALDH1A1 1/800, SOX-2 1/100 all from Santa-Cruz, and NANOG 1/100 from eBiosciences). For the detection of antibody binding, the UltraVision LP Detection System: HRP Polymer Quanto (Thermo Scientific, Cheshire, UK) was used, with 3'3-Diaminobenzidine (DAB) as chromogen. Stained cells were lightly counterstained in Harris hematoxylin for 10 s, hydrated and mounted in Permount (Fisher Scientific, Fair Lawn, NJ). Internal controls for specificity of immunostaining included

replacement of primary antibody with non-specific serum (negative). Slides were evaluated for the presence and the intensity of staining (quantified using ImageJ).

EMT transition

The EMT status of cells was determined by measuring the ratios of vimentin/keratins expression levels, using image analysis after laser scanning microscopy [22] and FACS analysis. Cells in cytospin preparations (used for microscopy) and in suspension (used for microscopy and FACS) were immunostained with primary antibodies for keratins 8, 18, 19 (mouse monoclonal antibodies, A45-B/B3, R002A, Micro- met AG, Munich, Germany) and vimentin (polyclonal antibody, Santa Cruz Biotechnology, sc-7558) and anti-mouse and anti-rabbit secondary antibodies labeled with Alexa 488 (green staining, Invitrogen) and CF555 (red staining, Biotium) dyes respectively. Untreated and treated cells from cytospin preparations and immunostained suspended cells attached on alcian blue coated coveslips were analysed by confocal (Leica SP) microscopy. To prevent any signal interference (green and red) generated by the different emission spectra, the detection of each one of the markers was performed by sequential laser confocal scan. Fixed confocal settings were used for all specific measurements. Images were taken from 50 cells of each treatment and were stored electronically. To quantify the fluorescence intensity of vimentin and keratins, the images were subjected to java-based image processing with the use of Image J program (NIH). Cells immunostained in suspension were also analyzed by FACS (Attune® Acoustic Focusing Cytometer, Applied Biosystems) at a cell population of at least 20000 cells.

Wound healing assay

Cells were seeded in12-well plates and cultured until a monolayer was formed. At that point, cells were incubated for 1 hour with mitomycin C (10 μg/ml), so as to prevent further cell proliferation. Afterwards a thin wound was drawn in the middle of each well using a sterile micropipette tip and cells were washed with PBS in order to remove any remaining cell debris. Fresh medium containing BAFF or APRIL (100 ng/ml) with or without Testosterone-BSA (10^{-6} M), was added. The subsequent colonization of the denuded area was photographed with an inverted microscope (DM IRE2, Leica), at different time-intervals (at time 0, just before adding the tested agents 24 hours and 48 hours afterwards, see Results for details) always at predefined points of the well. The photographs were analyzed and the covered distance was measured, and compared to the control cells.

Actin cytoskeleton staining and visualization

Cells were grown on 8 -well chamber slides. After incubation with the different agents for 10 min, cells were washed with PBS twice, fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature, permeabilized with 0.5% Triton X-100 for 10 min and incubated in blocking buffer (2% BSA in PBS) for 15 min. Actin cytoskeleton was visualized with rhodamine-phalloidin staining (1:400 in PBS containing 0.2% BSA) for 45 min. Specimens were analyzed in a Leica SP confocal microscope.

Knock down experiments (BCMA, JNK)

T47D cells were plated in six-well plates (2.3 10^5 cells/well) and left to adhere for 24 h. The medium was changed, and transfection with si RNA for BCMA (NM_001192, Sigma-Aldrich) or shRNA against JNK1 or JNK2 prepared by our group [19] for JNK was performed with a standard Lipofectamine 2000 protocol (Invitrogen; 0.8 mg

DNA, 1 ml Lipofectamine 2000 in Optimem medium, for each well of a 24-well plate, scaling up for 6-well plates). For the later transfection efficiency was 85%, as estimated by counting GFP-positive cells.

JNK inhibition

T47D cells were cultured in six-well plates and treated with APRIL or BAFF (100ng/ml) for 4 days in the presence of the specific JNK inhibitor, SP600125 (10μM).

NFk-B activation assay

Cells were cultured in 24-well plate, and were transfected with 0.2μg/well of pNFκB-Luc plasmid (Clontech, Mountain View, CA), carrying NFκB response elements, in front of the 5' end of the firefly luciferase gene, together with 0.2μg/well of a Renilla luciferase vector (pRL-CMV, Promega, Fitchburg, WI), using Lipofectamine 2000 (Invitrogen, 1 ml per well) in Optimem medium. Cells were incubated for 24h and then treated with BAFF or APRIL for 24 hours. Luciferase activity was assayed with a Dual-Luciferase Reporter 1000 Assay System (Promega, Fitchburg, WI), in a Berthold FB12 Luminometer (Bad Wildbad Germany).

Data from public databases

Data for BAFF, APRIL, BCMA, AR, SOX2, KLF4, ALDH1A, NANOG2 were retrieved from www.cbioportal.org (version 1.4.2 snapshot) from the METABRIC breast cancer study (2509 samples [23, 24]. Co-occurrence and/or mutual exclusivity, as well as correlation with breast cancer subtype and ER status were interrogated. In addition, study GDS3116 [25, 26], referring to 53 letrozol-treated patients was interrogated for the expression of APRIL, before and 3-months after therapy.

Statistical analysis

Statistics were performed by the use of SPSS v21 (SPSS/IBM Inc, Chicago, IL) and Prism v6.05 (GraphPad Inc, La Jolla, CA). Parametric tests were used, as appropriate and a $p<0.05$ level was retained for significance.

Results

BAFF and APRIL expressed in breast cancer cell lines, increase cell migration and epithelial-mesenchymal transition (EMT)

Previous data from our [15] and other groups [17] have verified the presence of APRIL and BAFF and their cognate receptors on breast epithelial cells, both at the mRNA and protein level. Here, we have assayed the presence of these TNFSF members in an ERα-positive (T47D) and a triple negative breast cancer cell line (MDA-MB-231 cells, named thereafter MDA cells). mRNA for BAFF, APRIL and BCMA and BAFFR was present in both cell lines, while TACI transcript was constantly absent (Figure 1A). APRIL expression was higher in T47D cells, suggesting a possible implication of sex steroids (correlation with ER status) in the expression of APRIL.

APRIL has been reported to be associated with increased tumor growth and metastasis [17]. A critical factor promoting metastasis is enhanced cell migration, accompanied by EMT of cells in transition towards metastatic sites. In our cell lines both APRIL and BAFF enhance cell migration (the effect is more prominent after a 48h-incubation, Figure 1B). Additionally, the EMT cell status of breast cancer cells was enhanced when cells were treated with either APRIL or BAFF (100ng/ml) (Figure 1 C and D). This was accompanied by actin cytoskeleton rearrangements, characteristic of migrating cells (Supplementary Figure 1 in Additional File 1). It is to note that APRIL

and BAFF induce a similar modification of EMT markers and migratory activity, suggesting an interaction occurring through the same receptor or receptors.

Our data therefore suggest that after prolonged incubation at equimolar concentrations APRIL and BAFF induce similar effects on cellular migration and EMT related phenomena.

APRIL and BAFF induce breast cancer cell stemness

In addition to EMT, another factor necessary for metastatic potential and disease progression is a combination of genetic alterations and epigenetic events that recapitulate normal developmental processes including stem cell self-renewal [18] and the acquisition of ‘‘stem cell’’ properties through a ‘‘dedifferentiation’’ process. Recently, we have reported that short term exposure of breast cancer cells to tamoxifen may act as such an epigenetic stimulus by increasing stem cells [27]. We therefore investigated whether APRIL and BAFF may also be associated with the expansion of the stem cell population of breast cancer cell lines.

T47D and MDA cells, treated with APRIL or BAFF (100ng/ml) exhibited an increased ability to form mammospheres. More specifically, the number of primary mammospheres increased by 40% after 9 days of incubation (Figure 2A). The effect of these two agents is more prominent in ERα-positive T47D than in triple negative MDA cells, suggesting a possible role of steroids in this phenomenon. In the following experiments, we have focused in the T47D cell line, in which the effect of APRIL and BAFF was more prominent. After dispersion of primary mammospheres and culture of cells under the same conditions (formation of secondary mammospheres) the effect of both cytokines was further enhanced, with APRIL being slightly more potent than BAFF (Figure 2B).

The effect of BAFF and APRIL on the induction of stemness was further verified by taking advantage of the recently reported intrinsic autofluorescence of epithelial cancer stem cells, due to riboflavin accumulation in membrane-bounded cytoplasmic structures bearing ATP-dependent ABCG2 transporters [20]. Incubation of breast cancer cells with APRIL or BAFF (100 ng/ml) for at least four days induced a significant increase in autofluorescence, from ~2% in control (untreated) cells, to ~5% in APRIL or BAFF-treated cells Figure 2C). This finding suggests that the epigenetic changes required for the induction of this phenotype need a prolonged exposure to these agents. This increase in autofluorescence correlates and is of similar amplitude with the increase of ALDH1A1 activity, assayed by flow cytometry and the ALDEFLUOR® kit (see Supplementary Figure 2 in Additional File 1 for an example and Figure 2D). When dispersed T47D cell mammospheres were assayed for a stem cell signature and they were found to have significantly more stem cells compared to cells from normal culture (7.2% vs 2.1%). BAFF or APRIL treatment further increased this autofluorescent stem cell signature (17.9 vs 4.5 for APRIL and 11.2 vs 3.6 for BAFF respectively) suggesting that these cytokines have the capacity to induce pluripotency in T47D cells (Figure 2E).

Stem cells (including cancer stem cells) express a specific set of genes, related to pluripotency. These genes include SOX2, c-MYC, KLF4 and NANOG, which are sequentially activated transcription factors, leading to the emergence of a stem cell-like phenotype. Additionally, ALDH1A1 is another established marker of stemmness, having a possible role in tumor-initiating cell biology and as a prognostic factor in breast cancer [28]. In order to further verify the emergence of stemness after 4 days incubation with APRIL or BAFF (100 ng/ml), the transcription of these factors was investigated in T47D cells. The expression of the above genes was examined both

at the mRNA (by Real Time PCR, Figure 3A) and protein level (by immunocytochemistry, Figure 3B). As shown, APRIL and BAFF mainly increase the expression of ALDH1A1, KLF4 and NANOG.

APRIL and BAFF modulate stemness markers expression through BCMA and JNK signaling

Results presented so far suggest that APRIL and BAFF promote in a similar way the emergence of pluripotency in breast cancer cells. Taking into consideration that T47D cells express BCMA, on which both cytokines can bind, and BAFFR, an exclusive BAFF receptor (see Figure 1) [10], we have assumed that this effect might be mediated by BCMA. In order to confirm this hypothesis, we transfected them with a specific anti-BCMA siRNA. Transfected cells were then incubated with BAFF or APRIL and pluripotent/stem cells were assayed by their autofluorescence. As depicted in Figure 4A, cancer stem (autofluorescent) cells were dramatically decreased, suggesting the mediation of this effect through BCMA. Previous work, has shown that BAFF and APRIL can mediate their post-receptor effects through activation either through TRAF signaling and NFκB activation [29], or through a BCMA-specific signaling, mediated by JNK [19]. Here, we have explored these two pathways, in order to decipher the one implicated in BAFF/APRIL signaling in breast cancer cells, and related to the induction of pluripotency. As shown in Figure 4B, T47D cells, transfected with a specific plasmid, carrying NFκB response elements in front of the firefly luciferase gene, do not show any activation after a 24h BAFF or APRIL incubation. On the other hand, the JNK specific inhibitor, SP600125 or shRNAs against JNK1 and JNK2, significantly blocked the effects of APRIL and BAFF on ALDH1A1 and KLF4 (Figure 4C and D). We have therefore concluded that, in breast cancer cells, APRIL and BAFF binding to BCMA signal towards

pluripotency is mediated by JNK, and especially JNK1, as we have previously reported for APRIL effects in human HepG2 hepatocellular carcinoma [19]. Furthermore, the complete inefficiency of BAFF to modify NFκB further suggests that, in our settings, BAFFR signaling, might be ineffective in this cell line.

Regulation of APRIL production in breast cancer cells by sex steroids

Our data so far indicate that APRIL and BAFF, acting through BCMA, induce pluripotency in breast cancer cells, this effect being more prominent in the steroid receptor positive cell line T47D. To explore whether there is a relation between steroids and APRIL and BAFF production on one hand and induction of pluripotency on the other we have at a first approach interrogated our previously reported transcriptome data on the effect of androgen (GSE18146) and estrogen (GSE32666 and GSE32668) on T47D and MDA cells. As depicted in Figure 5A a significant increase of APRIL transcription was observed in both T47D and MDA cells especially after a 3h-incubation with testosterone. Interestingly, this increase was observed with the membrane-only acting androgen (Testosterone-BSA) suggesting an extranuclear androgen action. In contrast, estrogen had a minimal effect on APRIL transcription in both cell lines. Conversely, no significant modification of the BCMA transcript was found (not shown).

Exploring the promoter region of APRIL, with the rVista V 2.0 web resource (https://rvista.dcode.org/), we have identified four putative androgen response elements in the promoter of the APRIL gene (Supplementary Figure 3 in Additional File 1). We therefore explored the possible modification of APRIL transcription after cell incubation with membrane-only acting testosterone (testosterone-BSA). Incubation of T47D cells with testosterone-BSA resulted in a significant increase of APRIL gene product at 3 and 6 hours (Figure 5B). Interestingly, this effect was

inhibited by the antiandrogen cyproterone acetate (which was ineffective *per se*), a result suggesting the membrane-initiating androgen action observed in this case may actually be mediated via the classical androgen receptor, possibly through its ability to anchor to the cellular membrane via palmitoylation and not by other membrane androgen-binding receptors, as the OXER1, we have recently reported [30]. This mechanism that implicates the classic steroid receptors in membrane actions of steroid hormones has been extensively studied in the recent years [31].

The significant increase of APRIL by testosterone-BSA could result in the possible transformation of cells, with an induction of pluripotency. Indeed, testosterone-BSA per se increases mammosphere formation in T47D cells (Figure 5C). When cells were incubated with testosterone-BSA and APRIL, an additive effect was observed, suggesting that the system is not saturated with the provided concentrations of the ligands.

APRIL in aromatase-inhibitor treated patients' samples

Our data suggest that androgen enhance APRIL production in breast cancer cells regardless of estrogen receptor status. In a relevant clinical setting, increased local androgen concentration could occur in patients treated with aromatase inhibitors. In this setting, increased local androgen could enhance APRIL production and therefore (under circumstances to be determined) could increase cancer pluripotency. To provide a proof of principle of this hypothesis, we extracted APRIL data from the GEO-deposited GDS3116 study [25, 26]. In this series, transcriptome data of a series of 53 letrozol (an aromatase inhibitor) treated patients is included, before and 3-months after therapy. 37/53 patients responded to the therapy (as estimated by changes in estrogen responsive genes and by ultra sound detected changes in tumor volume) while 16/53 were non-responders. In responders, in who an increased local

androgen concentration is expected due to the action of the drug, APRIL transcripts are significantly higher, while in non-responders no change is observed (Figure 6).

Furthermore, cBioPortal® for Cancer Discovery [32-34] data analysis from a large breast cancer study (2509 samples, MetaBRIC study) [23, 24] show that BAFF, APRIL and BCMA have a significant tendency to co-express with androgen receptor AR ($p<0.001$ for BAFF and APRIL, $p=0.012$ for BCMA) further conforming the association with androgen (Figure 7 and Supplementary Figure 4 in Additional File 1). The TNFSF members' mRNA had higher expression in ER-negative tumors and especially the highly aggressive claudin-low subtype. Significant correlations were retrieved for the stemness markers we have tested in the breast cancer cell lines.

Discussion

During the last decade, the cancer stem cell hypothesis has been widely studied and even though the results are still controversial on the biomarkers for their detection, it is generally accepted that tumors contain a sub-population of cells with stem cell properties like tumor initiation, self-renewal and differentiation [18]. Moreover, cancer stem cells (CSCs) seem to be even more clinically important, since they have been reported to be involved in resistance to therapy. Recently, the higher expression of the TNF superfamily member APRIL was shown to be associated with tumor aggressiveness in triple negative breast cancer [17]. In the present work, we investigated the possible role of APRIL and BAFF in inducing breast cancer stem cells (BCSC) and thus contributing to an aggressive tumor cell behavior with poor clinical outcomes.

BCSCs were first identified as a cellular population expressing CD44+/CD24−/low [35] with the ability to have unlimited self-renewal capacity and to generate

differentiated descendants [36]. Later on, other markers such as aldehyde dehydrogenase 1 (ALDH1), SOX2, CD133 and the ability to grow as anchorage independent spheres have been utilized for their characterization [37, 38]. Recently, CSC autofluorescence due to rivoflavin accumulation in cytoplasmic structures was characterized as a new marker to identify CSC cells [20]. However, it seems that the different markers identify slightly different sub-populations and it is therefore necessary to utilize more than one specific marker and/or to test for unique CSC properties such as tumor initiation (sphere formation or xenografted tumor development, etc). In the present work, in order to investigate the effect of APRIL and BAFF, two TNF superfamily members that have been previously described by our group [15] and others [17] to have a significant role in breast cancer promotion, we utilized multiple approaches to detect CSCs.

Our results show that both BAFF and APRIL have the ability to increase the percentage of breast cancer stem cells. Indeed, when T47D breast cancer cells were treated for 4 days with either BAFF or APRIL a two to three-fold increase in the BCSC population was observed, as assayed by different methods. Breast cancer cells, treated with APRIL or BAFF present: (1) Increased migration and modifications of the actin cytoskeleton; (2) increased mesenchymal markers and signs of the acquisition of a mesenchymal phenotype that is linked to the generation of stem-like cells [39, 40] and is associated with metastasis and a higher migratory capacity [41, 42]; (3) increased stem cell population, as assayed by CSC autofluorescence and ALDH1A1 activity, and (4) increased mammosphere formation, with a significant increase of CSC population. These results are in accordance with previously reported findings that APRIL promoted breast cancer lung metastasis [17].

An important finding supporting the observed BCSC enrichment after treatment with APRIL or BAFF is the significant increase observed in a number pluripotency markers KLF4, NANOG, c-MYC and ALDH1A1. Recent progress in the reprogramming of somatic cells into pluripotent cells has also identified a number of oncogenes (c-MYC, KLF4, SOX2, OCT4, and NANOG) [43-45], that when properly modified may act as transcription factors that promote a stem cell phenotype, maintain pluripotency and prevent differentiation [43, 44, 46]. Interestingly, Sox and Oct genes are positive regulators of their own and alternative transcription, acting also as rheostats of stem cell proliferation (at low concentrations) or differentiation [47], while they subsequently activate NANOG transcription [48]. Finally, these three genes regulate a set of transcripts, necessary for the induction or the maintenance of pluripotency and stem cell phenotype. The same is also observed for another transcription factor related to the induction or the maintenance of pluripotency, MYC [see 49, for a recent review]. Interestingly, a recent study [50] suggests that c- MYC acts early during reprogramming, at least in part to repress the expression of differentiation genes, and may therefore have an early role in this process. This function is possibly related to global alterations in chromatin, such as histone acetylation [51] and happens prior to the activation of the other stem cell-specific factors [Oct4/Sox2/Klf4, reviewed in 46].

The fact that in the studied cell lines both BAFF and APRIL induced similar effects suggests that their action should be mediated through BCMA, a cognate receptor for both ligands [10]. This was further supported by the inhibitory action of the specific anti-BCMA siRNA on their effects and the fact that the BCMA specific signaling involving JNK activation was found to be involved. Indeed, their effect on ALDH1A1 and KLF4 was significantly reduced in the presence of the JNK inhibitor, SP600125

or shRNAs against JNK1 and JNK2 (Figure 4C). Additionally, no NFκB activity was detected. Therefore, it seems that in breast cancer cells, APRIL-BCMA signal towards pluripotency is mediated by JNK, a signaling pathway also reported previously by our group for the effect of APRIL in hepatocellular carcinoma cell lines [19].

Moreover, the fact that the effect of these two agents was more prominent in ERα-positive T47D cells than in triple negative MDA cells, suggested a possible role of steroids in this phenomenon. We therefore explored whether there is a relation between steroids and APRIL production as well as induction of pluripotency. By analyzing our previously reported transcriptome data on T47D and MDA cells [52-54], we found that indeed APRIL transcription was significantly increased in both T47D and MDA cells after a 3h-incubation with testosterone while surprisingly estradiol had only a minimal effect. Interestingly, a significantly higher change was induced by membrane-only acting testosterone (Testosterone-BSA) suggesting a membrane-initiated action.

The above findings that androgen can increase APRIL production and subsequently induce stemness and tumor aggressiveness could possibly have a clinically importance in the case of breast cancer patients that are treated with aromatase inhibitors which can result at increased local androgen concentration. In order to provide a proof of principle of this hypothesis, we extracted APRIL data from a series of 37 letrozol-responders and 16 non-responders before and after therapy (GEO-deposited GDS3116 study) [25]. Indeed, APRIL expression was significantly higher in the responders group (expected to have increased local androgen levels), while it was not changed in the non-responders, verifying our hypothesis on testosterone-control of APRIL in breast cancer.

We have also interrogated one large MetaBRIC breast cancer study [23, 24]. APRIL mRNA downregulation was evidenced in 7% of cases, confirming our previous report on protein expression [15]. Moreoever, it is evident that BAFF, APRIL and BCMA tend to co-occur with AR and stemness markers. They are preferentially expressed in claudin-low tumors, that display a highly aggressive behavior.

Our study presents a number of limitations. First, only two breast cancer lines are tested. Larger studies in more breast cancer cell lines and in vivo models are required to validate our data. However, evidence from a number of clinical studies, presented above, referring to treated patients provide promising validation in patients' data. Our omics approaches based on a retrospective analysis of publicly available data. Future prospective studies could provide more refined data on the mechanism presented here. The clinical implications could be potentiated, as aromatase inhibition is widely used to treat ER-positive tumors and AR profiling is part of cutting-edge approaches in breast cancer.

Conclusions

Our findings clearly indicate that both BAFF and APRIL have the ability to increase the percentage of breast cancer stem cells through BCMA-JNK mediation, pointing out for the first time that APRIL and BAFF not only modify breast cancer cell proliferation but they can also contribute to the re-formation of the tumor. Additionally, they provide evidence for a new possible mechanism of therapy resistance that involves increased stemness by high APRIL levels as a result of the accumulation of androgens that could occur in aromatase inhibitors treated patients. They further provide additional evidence that, in order to establish personalized, immune-related therapies in breast cancer patients, one should, in addition to the

targeting of the stroma and cancer-infiltrating immune cells [5], also investigate and target tumor cells, as was recently reported for another member of the TNFRSF, TNFR2 [7, 8].

Declarations

Ethics approval and consent to participate: Not Applicable

Consent for publication: Not Applicable

Availability of data and material: All data generated and analyzed during this study are included in this published article and its additional information file 1. The datasets generated and analyzed during the current study are available in the GEO database repository https://www.ncbi.nlm.nih.gov/geo/ (GDS3116 study) and the cBioPortal® for Cancer data analysis http://www.cbioportal.org/data_sets.jsp (MetaBRIC study).

Competing interests: The authors declare that they have no competing interests

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Authors' contributions: MK, EC and AT conceived and designed the experiments and wrote the paper. VP, GN, PA, KA, FK, NK, KK, EK, HP and PT performed the experiments and analyzed the data. GN, AT and EC participated in its design and coordination and helped to draft the manuscript.

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FIGURES

Figure 1. **Expression levels of BAFF, APRIL, BAFF-R, BCMA and TACI in breast cancer cell lines and the effect of APRIL and BAFF on migration and EMT status A.** The expression of BAFF, APRIL, BAFF-R, BCMA and TACI investigated by PCR in T47D and MDA-MB-231 cells breast cancer cell lines. **B.** Effect of APRIL and BAFF (100ng/ml) on migration of T47D and MDA cells after 24 and 48 hours of treatment (expressed as percentage of control=100). **C.** Representative photos of control (untreated) T47D cells and APRIL or BAFF (100ng/ml) treated cells for 4 days immunostained for keratins (upper panel, green) and vimentin (lower panel, red). Fluorescence intensity was calculated using Image J. Values represent the ratio of vimentin/keratins expression in these cells. **D.** Graphical presentation of EMT status changes obtained by calculating the ratio of vimentin/keratins expression of 50 cells for each treatment condition. All samples were analyzed with t-test. *** denotes $p< 0.0001$.

Figure 2. Effect of APRIL and BAFF on mammosphere formation and breast cancer stem cell population. A. Percentage of mammospheres (primary) formed after treatment of breast cancer cells (T47D and MDA) with either BAFF or APRIL (100ng/ml) for 9 days. Results of three independent experiments are expressed as a percentage of control (untreated) cells performed (*denotes $p<0.05$). **B.** Percentage of mammospheres formed after treatment of T47D cells with either BAFF or APRIL (100ng/ml) for 9 days (primary mammospheres) compared to secondary mammospheres and a subsequent 7-day period. Results of three independent experiments are expressed as a percentage of control (untreated) cells. (* $P<0.05$) **C.** Percentage of breast cancer stem cells (T47D and MDA) with or without treatment with BAFF or APRIL (100ng/ml) for 4 days as estimated by assaying them with high green autofluoresence in a BL2-A/BL1-A dot plot. Three independent experiments

were performed and the results of a representative one is presented. **D.** Comparison of the percentages of breast cancer stem cells as estimated by autofluorescence in a BL2-A/BL1-A dot plot and ALDEFLUOR kit with or without treatment with BAFF or APRIL (100ng/ml) for 4 days. Three independent experiments were performed (*denotes $p<0.05$). **E.** Comparison of the percentages of breast cancer stem cells (identified by autofluorescence in a BL2-A/BL1-A dot plot) in T47D cells and mammospheres treated or not with BAFF or APRIL (100ng/ml) for 4 and 12 days respectively. Three independent experiments were performed (*denotes $p<0.05$).

Figure 3. Expression levels of pluripotency markers in T47D cells after APRIL or BAFF treatment. A. Expression levels of ALDH1A1, KLF4, SOX-2, and c-MYC, genes (quantified by Real-Time PCR and expressed as percentage of control) after treatment of T47D cells with APRIL or BAFF (100ng/ml) for 4 days. Three independent experiments were performed (*denotes $p<0.05$). **B. and C.** Expression of SOX-2, c-MYC, ALDH1A1 and NANOG proteins (assayed by immunocytochemistry). Representative images of three independent experiments are presented **(B)** Quantification of the proteins' expression levels was performed in at least 10 cells in three different photos for both control and treated cells using Image J and is expressed as percentage of control **(C).**

Figure 4. A. Effect of APRIL and BAFF on breast cancer stem cells in the presence of siBCMA. Percentage increase in the number of breast cancer stem cells transfected or untransfected with siBCMA and treated with BAFF or APRIL (100ng/ml) for 4 days. (Breast cancer stem cell number was estimated by counting the cells with high green autofluoresence in a BL2-A/BL1-A dot plot). **B. NFk-B activity** of untreated (control) and treated with APRIL or BAFF (100ng/ml, for 24hrs)

T47D cells. Experiments were performed in triplicate. **C. and D. Effect of APRIL and BAFF on ALDH1A1 and KLF4 expression in the presence of a JNK inhibitor or sh RNA for JNK1 or JNK2**. Expression levels of ALDH1A1 and KLF4 after treatment of T47D cells with APRIL or BAFF (100ng/ml) for 4 days in the presence of a JNK inhibitor SP600125 (10μM) **(C)** or sh RNA for JNK1 or JNK2**(D).** Data are presented as a mean ±SEM of three independent experiments (*denotes $p<0.05$).

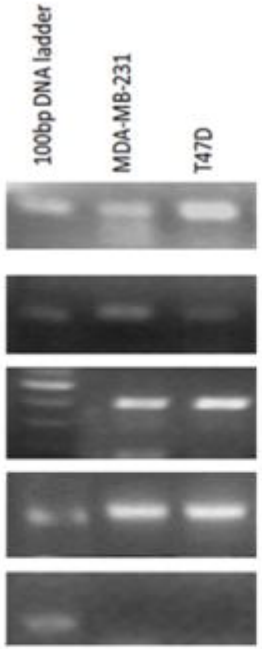
Figure 5. Effect of steroid hormones on APRIL expression levels and of testosterone on the effect of APRIL and BAFF on mammosphere formation. A. APRIL expression (from Gene Chip analysis) after treatment of breast cancer cells for 3hrs with steroid hormones, testosterone (testo) or estradiol (E2), unconjugated or in their membrane impermeable form conjugated to BSA (steroid-BSA). **B.** The effect of testosterone-BSA on APRIL expression (investigated by PCR) in T47D breast cancer cells in the presence or absence of cyproterone acetate (C). **C.** Percentage of mammospheres (primary) formed after treatment of breast cancer cells (T47D and MDA) with either BAFF or APRIL (100ng/ml) in the presence or absence of testosterone-BSA, for 9 days. Results of three independent experiments are expressed as a percentage of control (untreated) cells performed (*denotes $p<0.05$).

Figure 6. APRIL expression in breast cancer specimens from patients A. patients that have responded to letrozol therapy (R) and **B.** patients that have not responded (NR). The levels of APRIL expression are given before and after therapy. (**denotes $p<0.001$).

Figure 7. Results of the analysis of the MetaBRIC study in cBioPortal®. **A**. The distribution of BCMA (TNFRSF17, left panel), APRIL (TNFSF13, middle panel) and

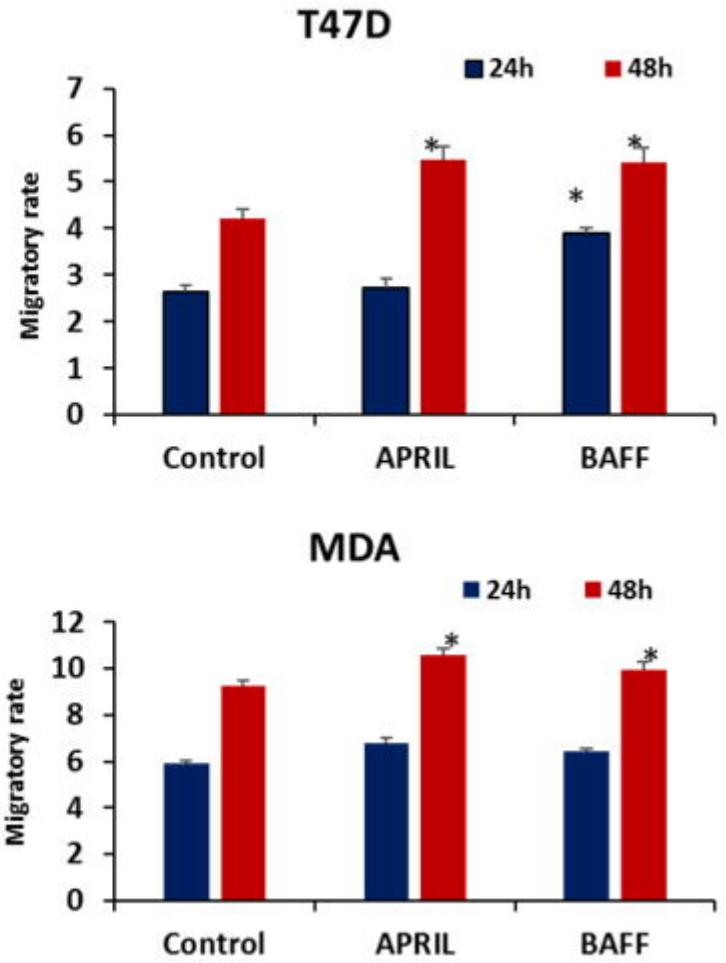
BAFF (TNFSF13B, right panel) are shown, in relation to cancer subtypes, identified by the Pam50 geneset. **B.** A heatmap of the annotated gene expression heatmap is presented.

A.

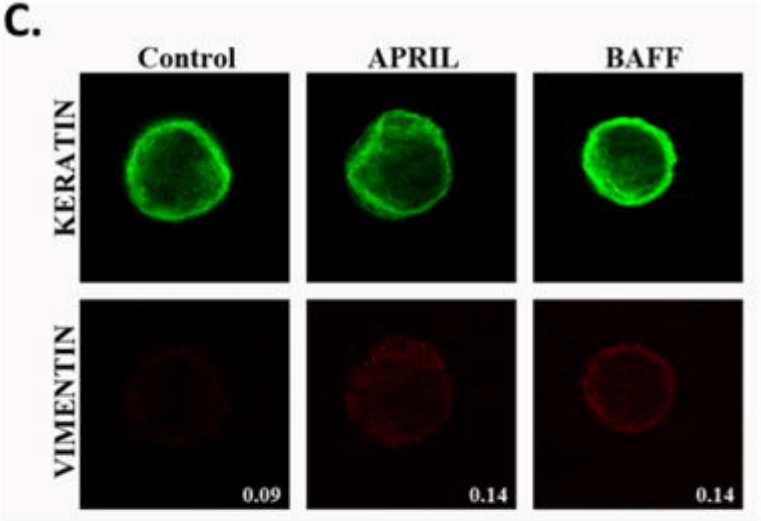


	MDA	T47D
APRIL	+	+
BAFF	+	+
BAFFR	+	+
BCMA	+	+
TACI	-	-

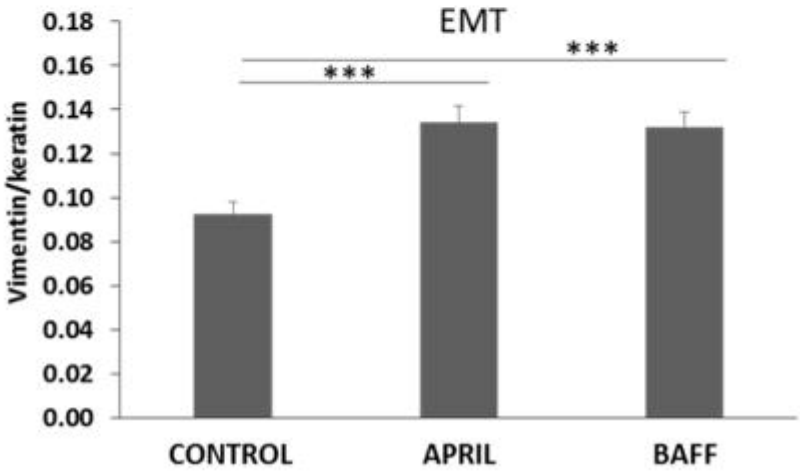
B.

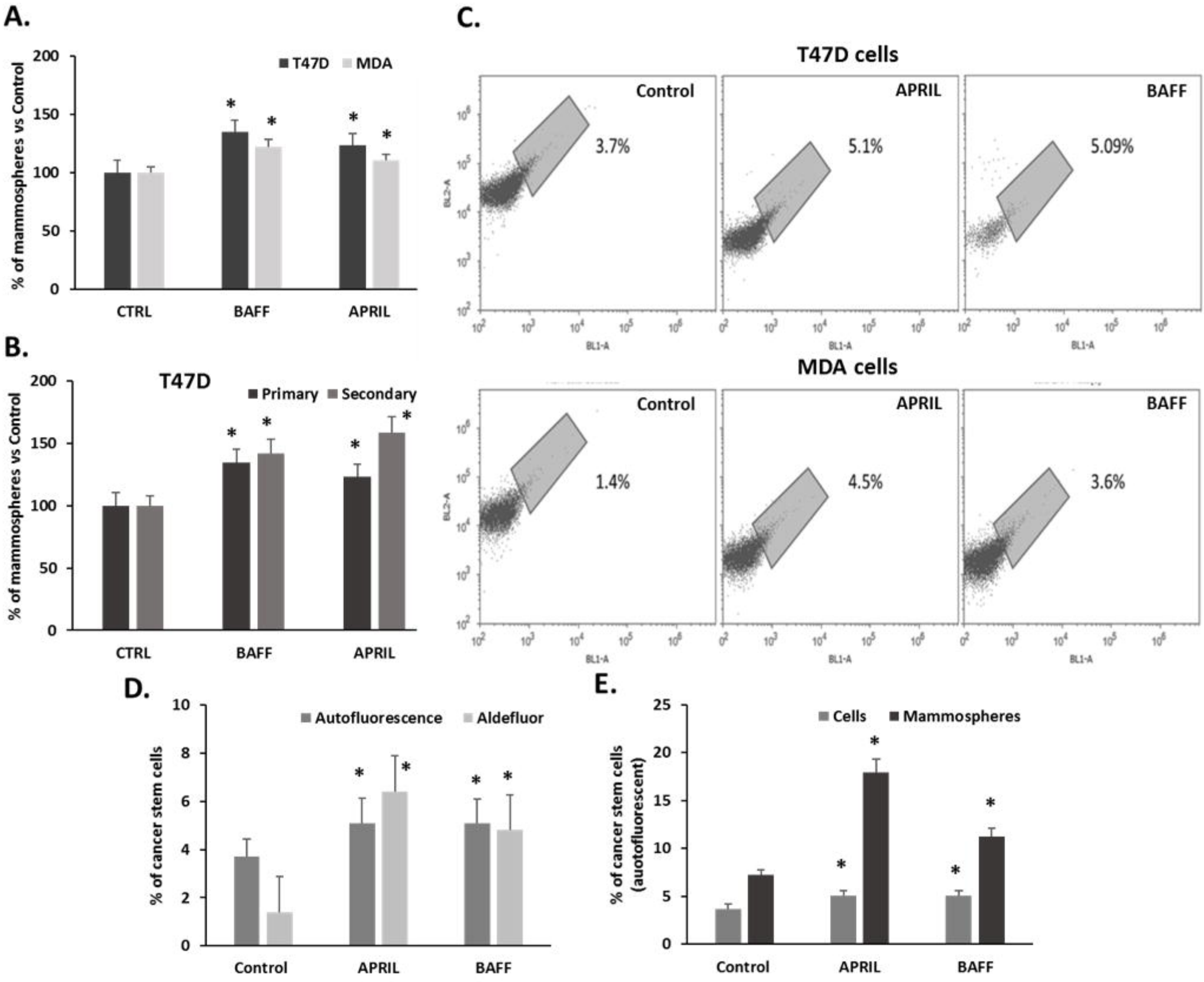


C.

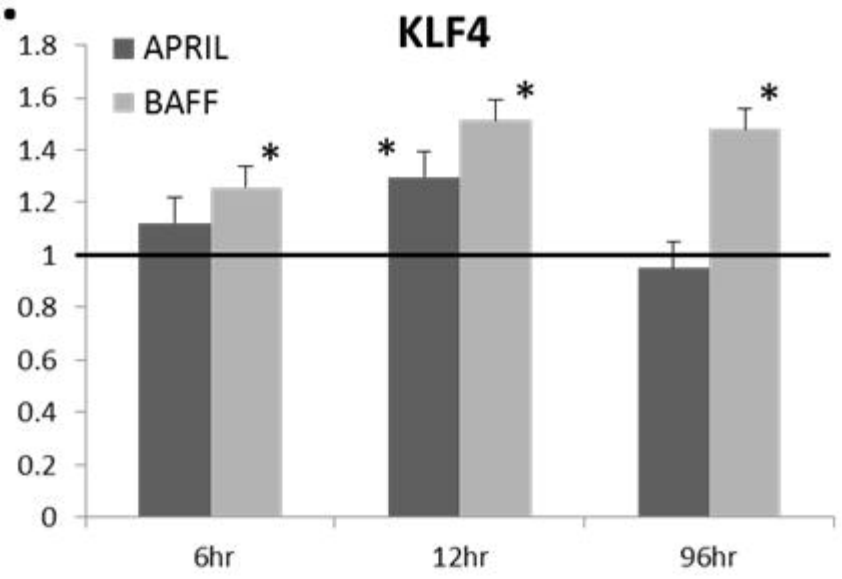


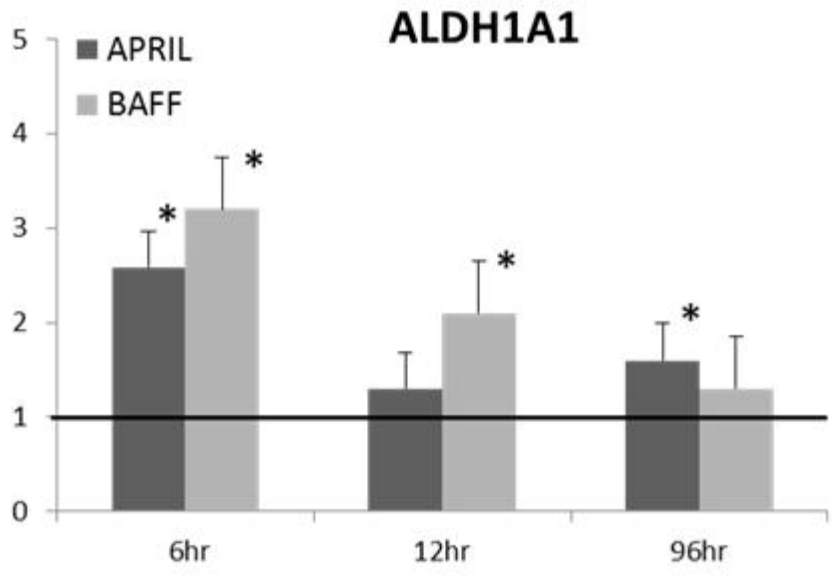
D.

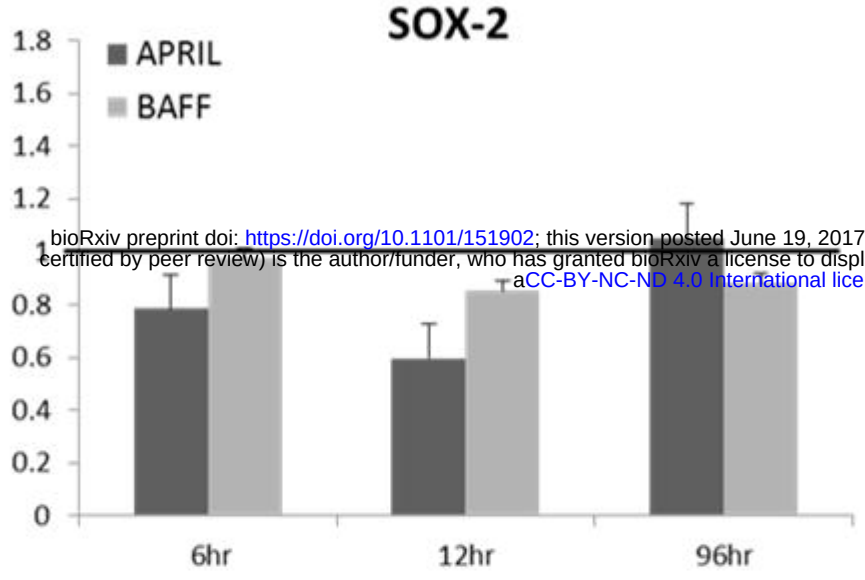


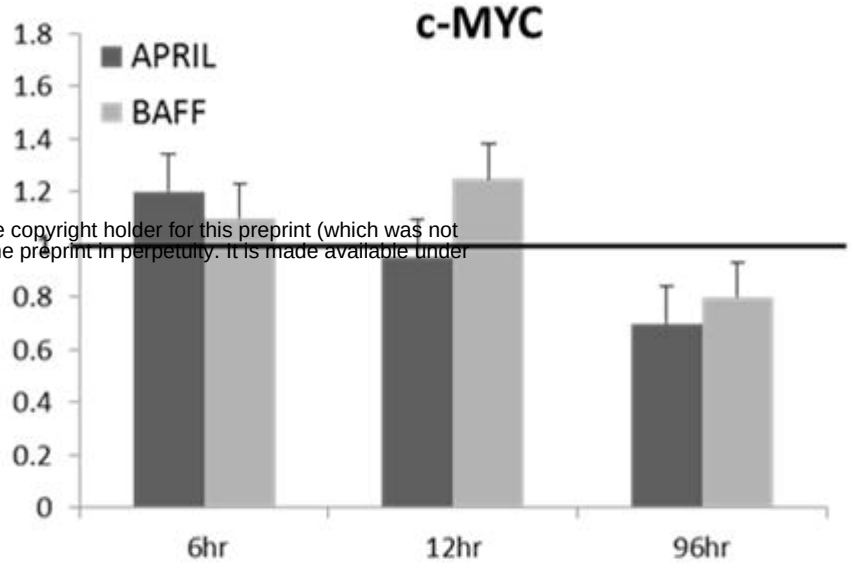

A.
T47D
MDA
% of mammospheres vs Control
200
150
100
50
0
CTRL
BAFF
APRIL
B.
T47D
Primary
Secondary
% of mammospheres vs Control
CTRL
BAFF
APRIL
C.
T47D cells
Control
APRIL
BAFF
3.7%
5.1%
5.09%
MDA cells
1.4%
4.5%
3.6%
BL2-A
BL1-A
D.
Autofluorescence
Aldefluor
% of cancer stem cells
Control
APRIL
BAFF
E.
Cells
Mammospheres
% of cancer stem cells (autofluorescent)
Control
APRIL
BAFF

A.









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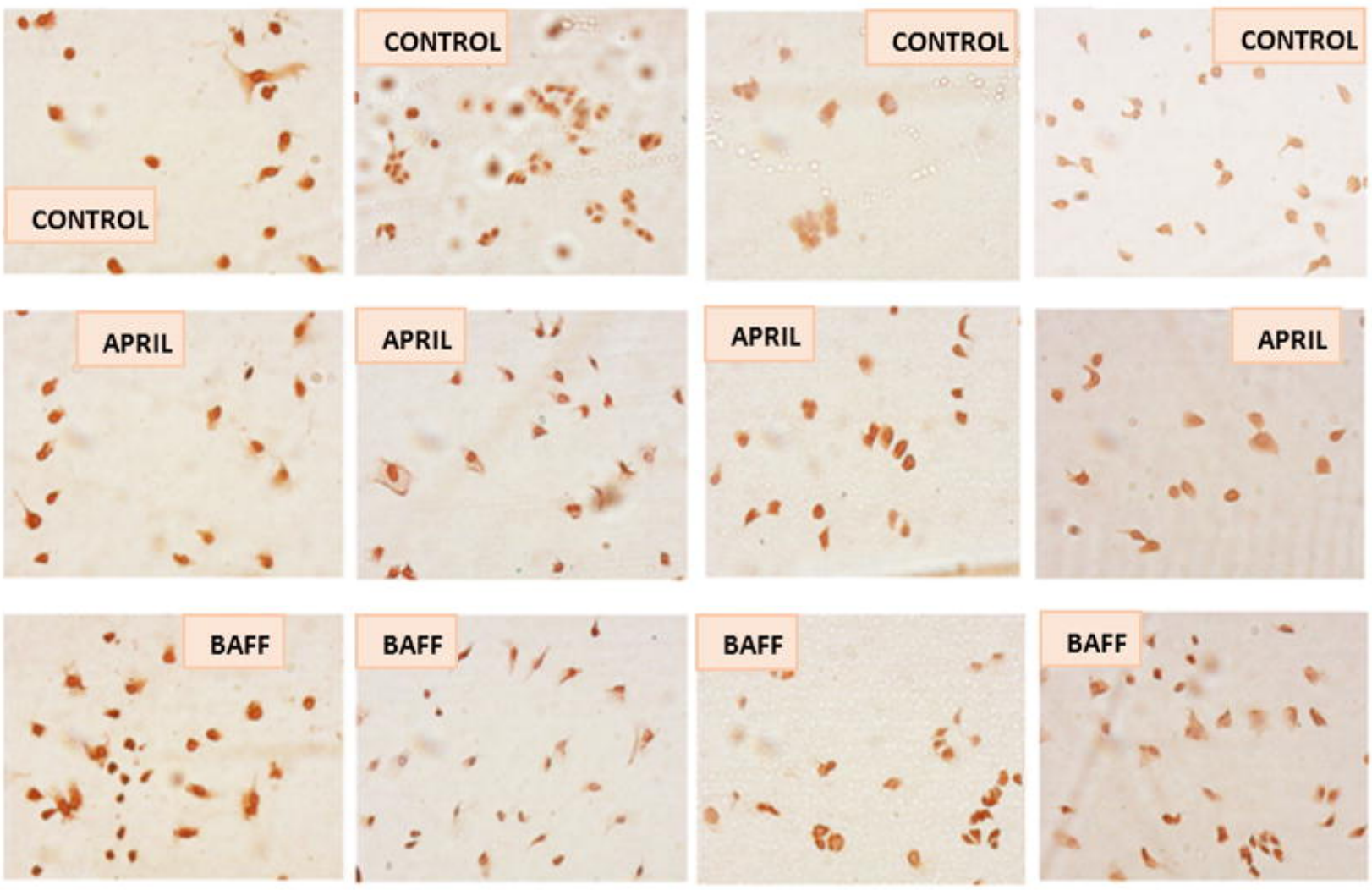
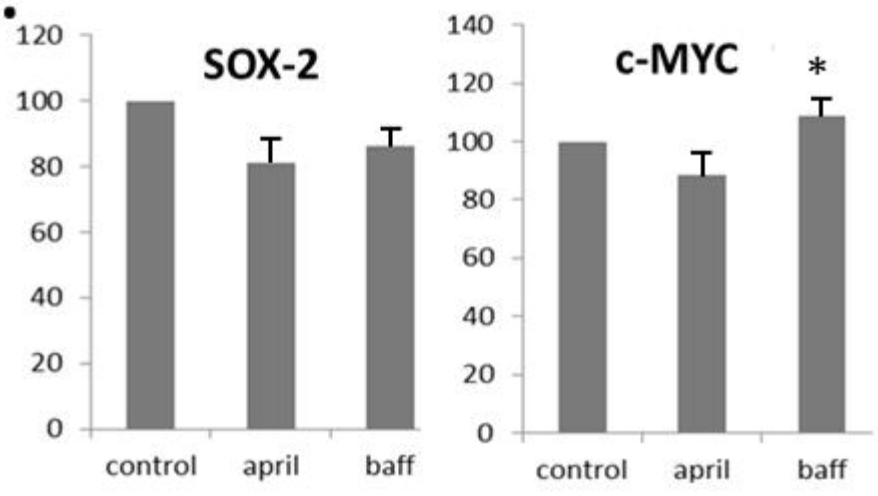
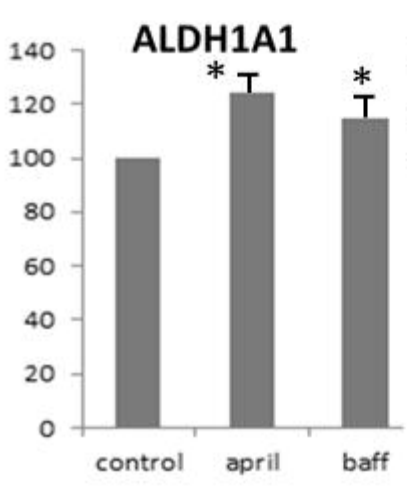
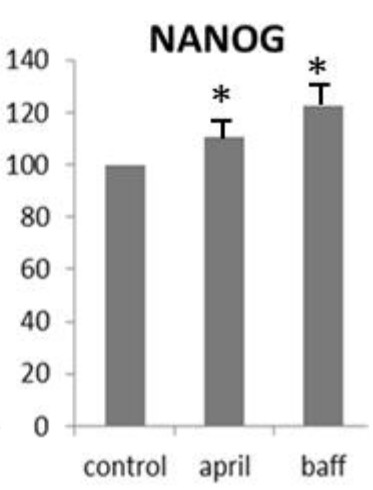
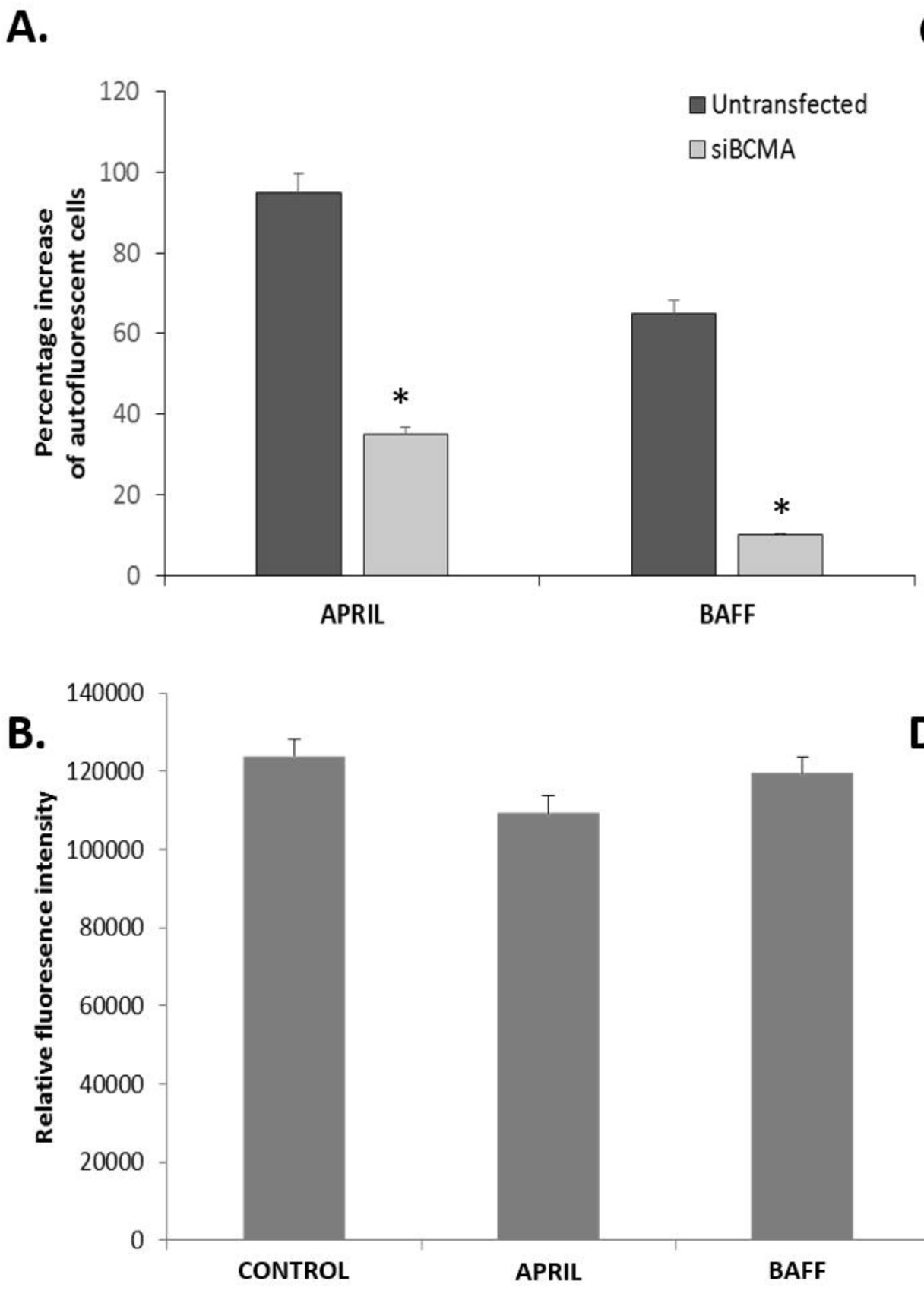


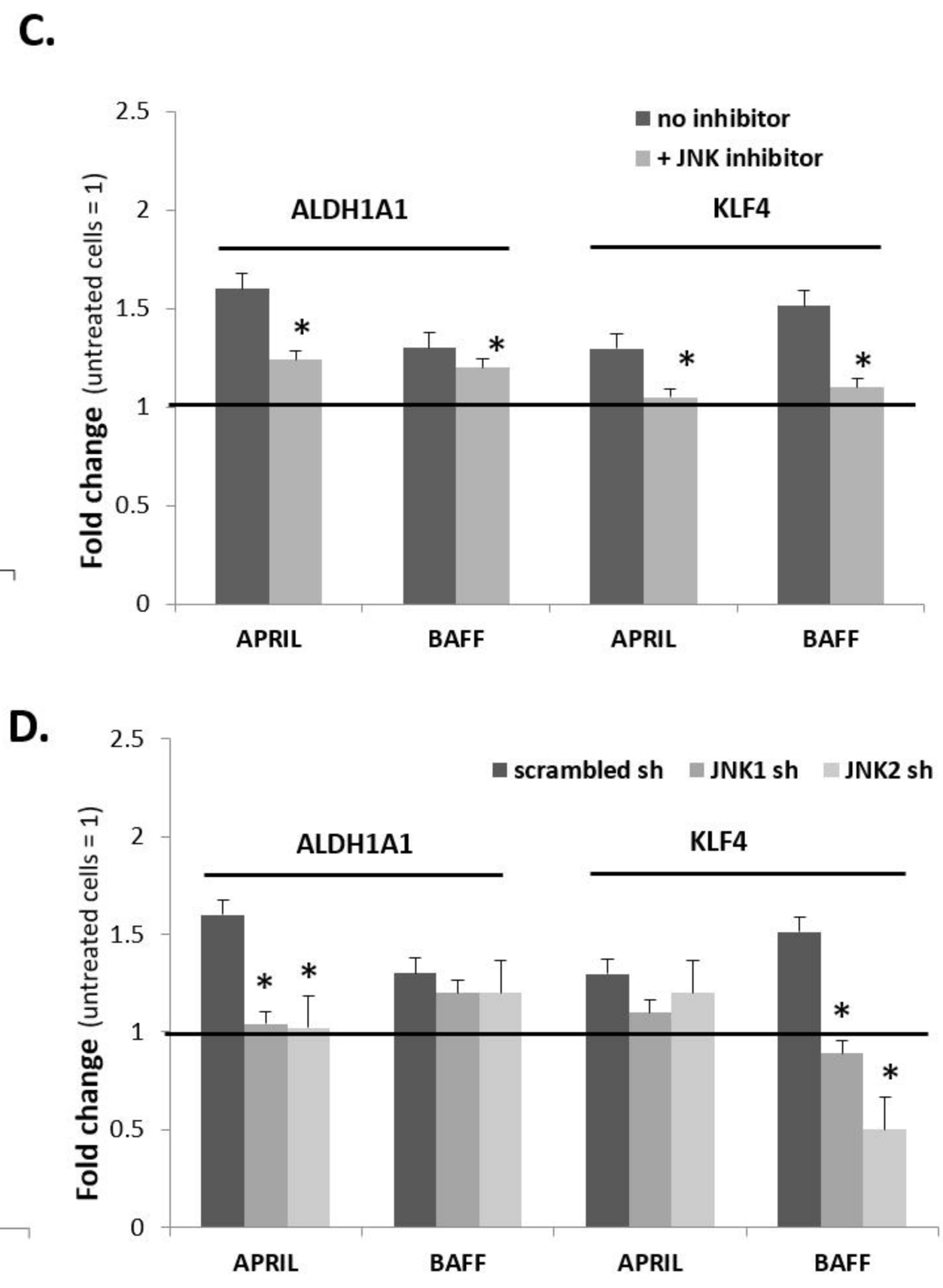
C.

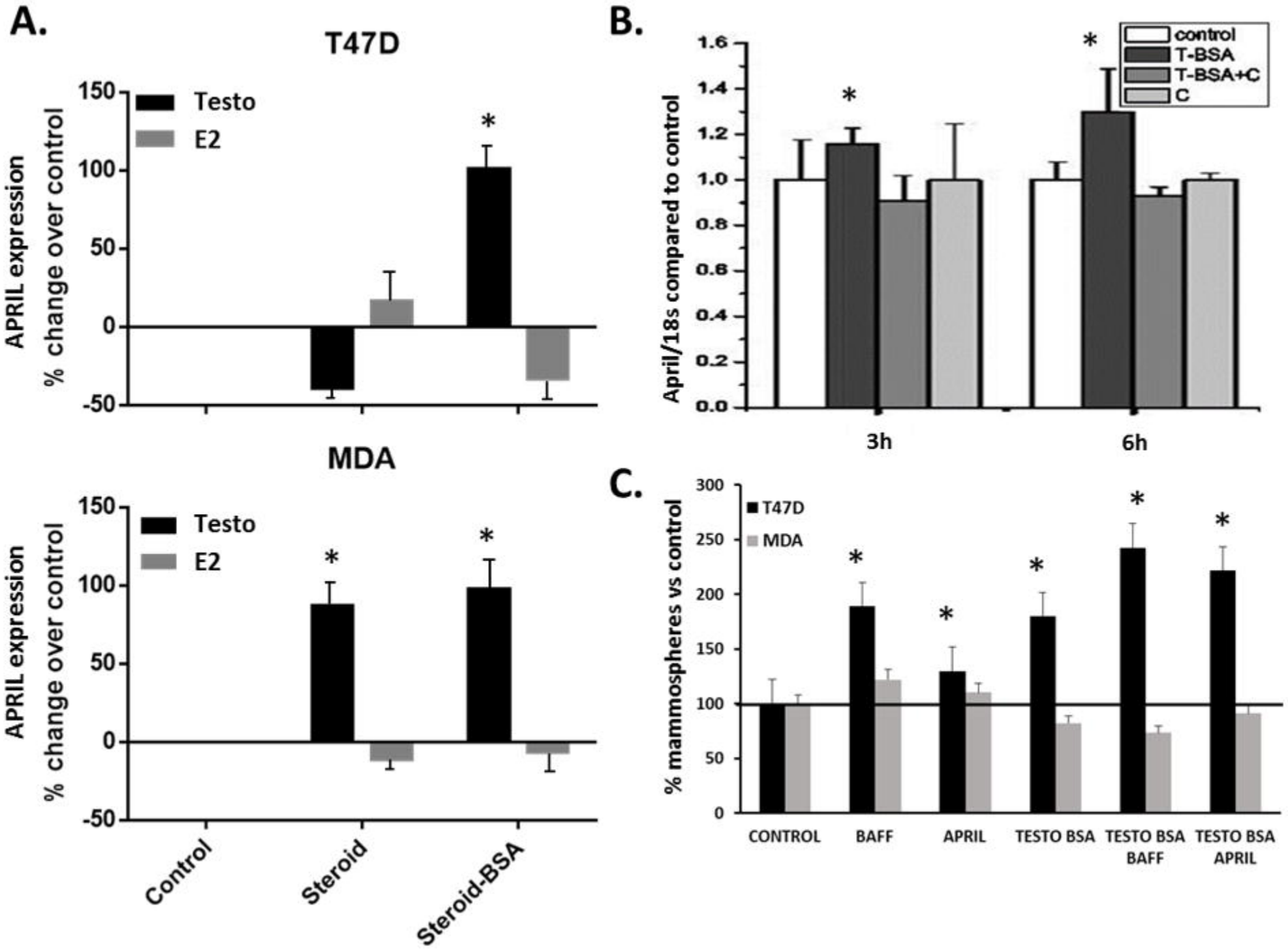


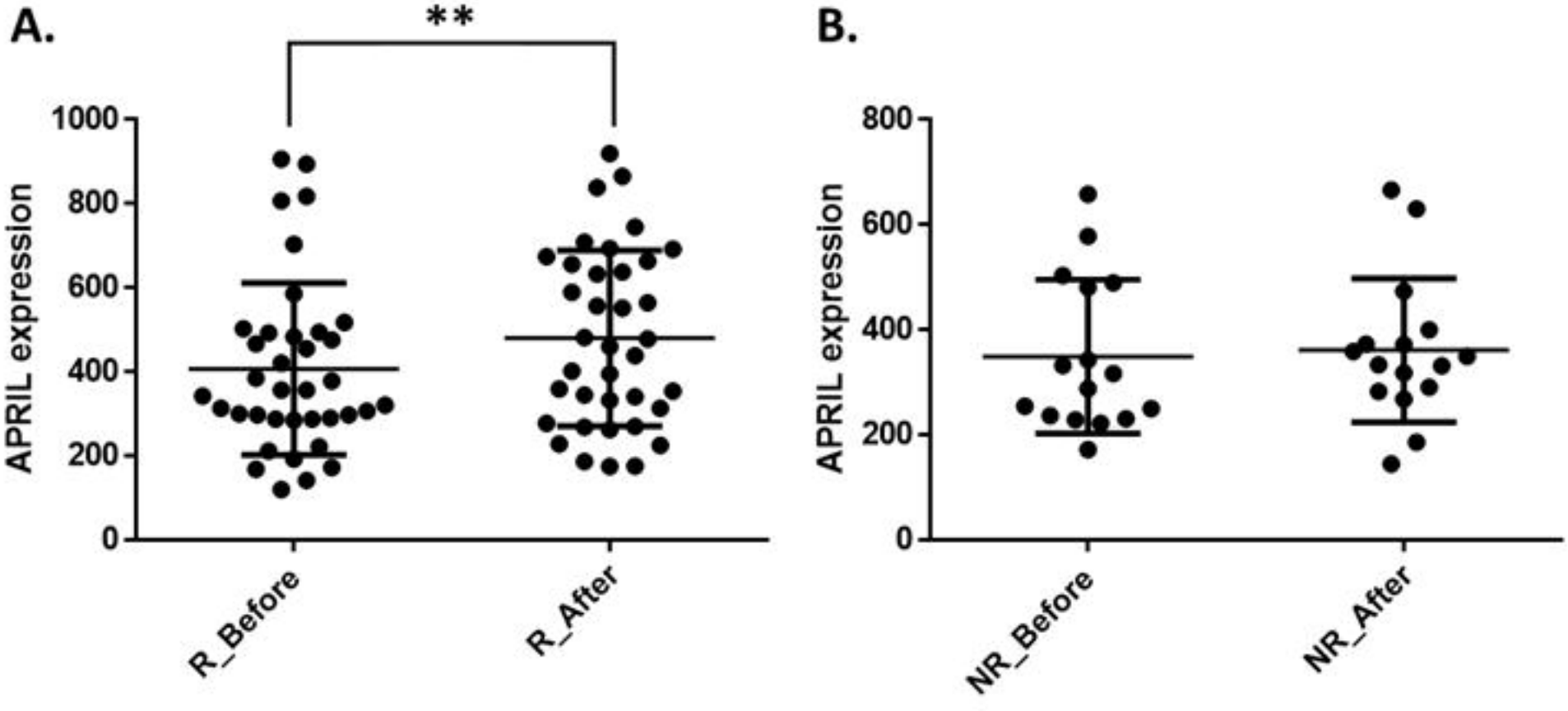


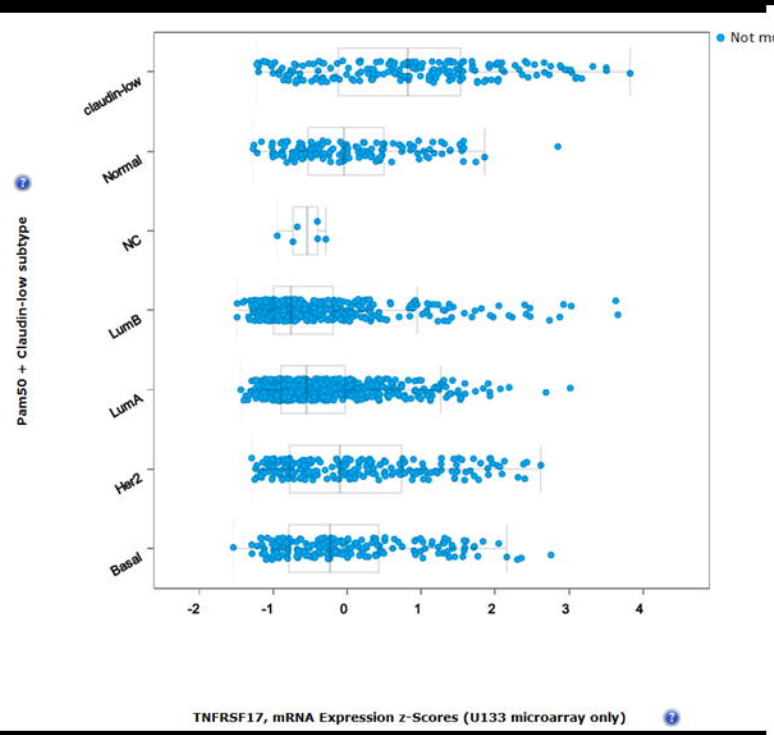


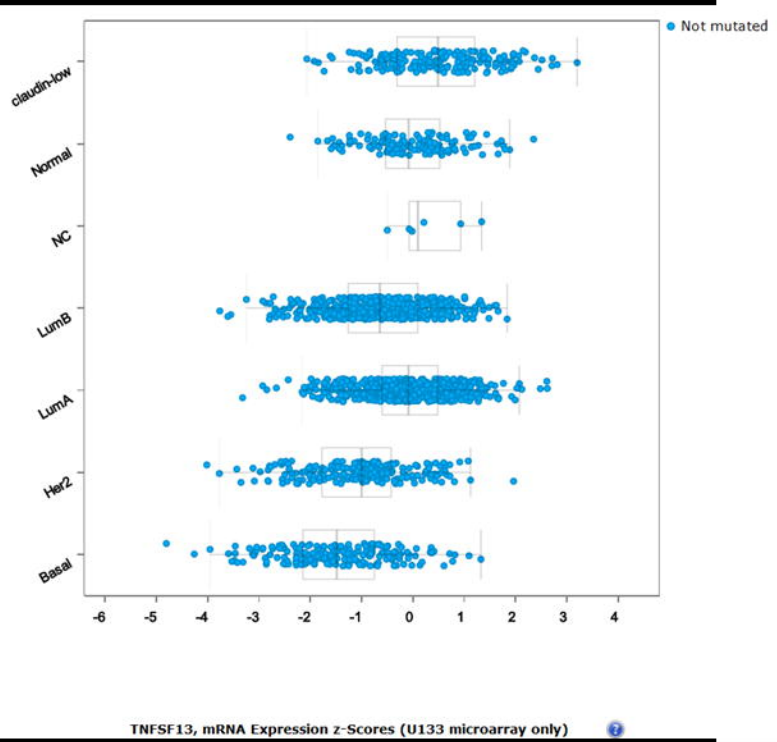


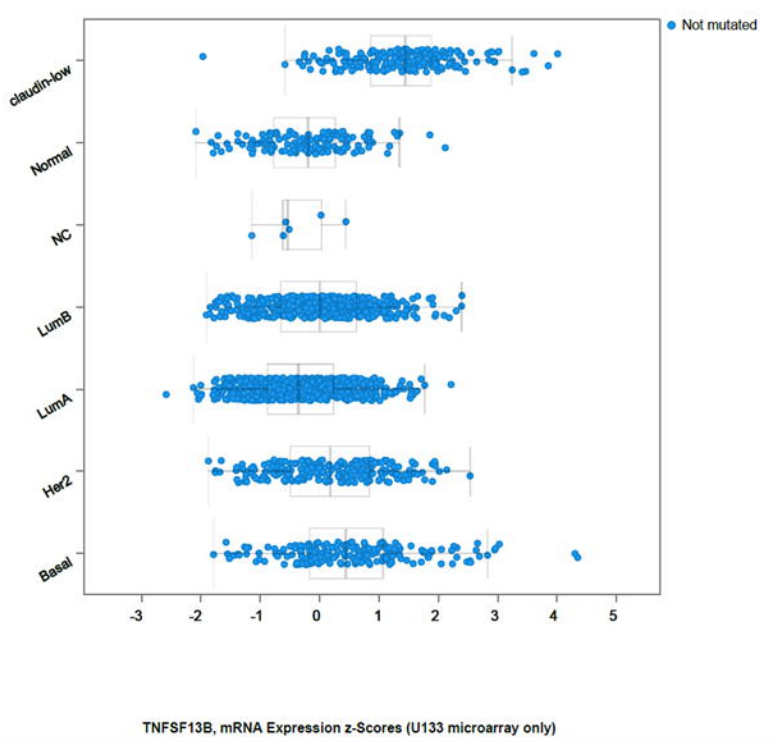


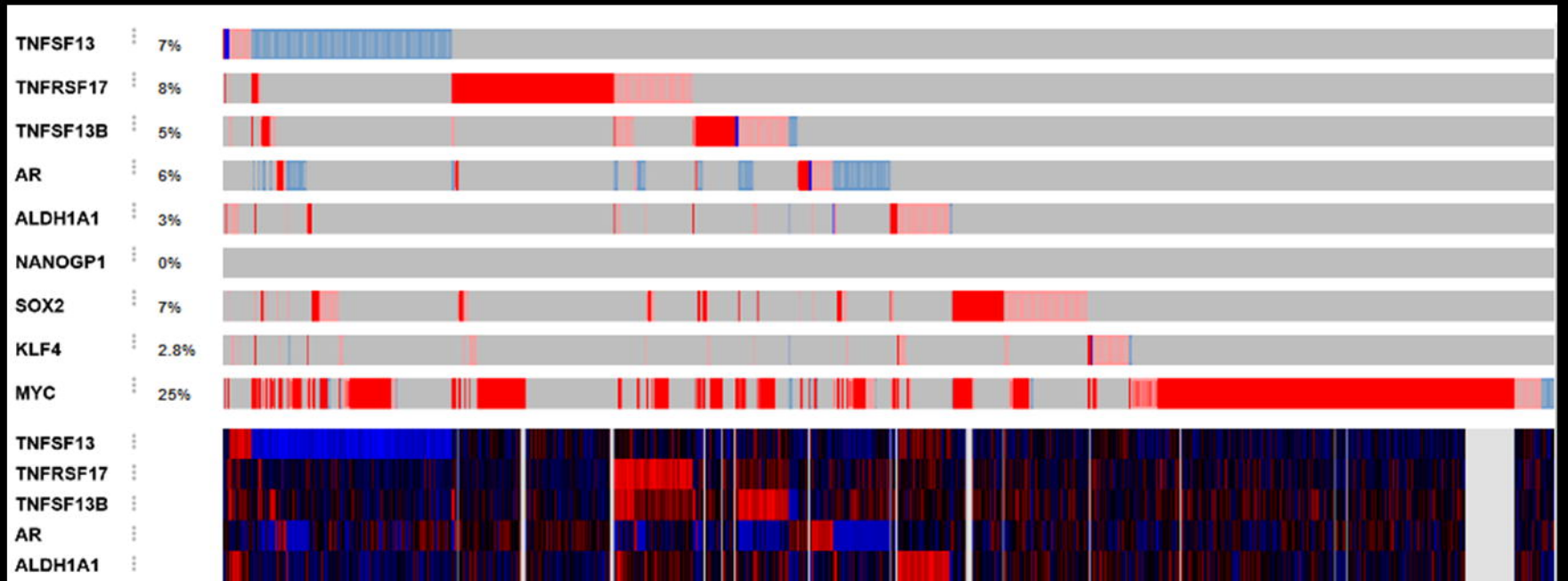

A.
T47D
Testo
E2
APRIL expression
% change over control
150
100
50
0
-50
*
MDA
Testo
E2
APRIL expression
% change over control
150
100
50
0
-50
*
*
Control
Steroid
Steroid-BSA
B.
control
T-BSA
T-BSA+C
C
April/18s compared to control
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
3h
6h
C.
T47D
MDA
% mammospheres vs control
300
250
200
150
100
50
0
*
*
*
*
*
CONTROL
BAFF
APRIL
TESTO BSA
TESTO BSA BAFF
TESTO BSA APRIL


A.
**
APRIL expression
1000
800
600
400
200
0
R_Before
R_After
B.
APRIL expression
800
600
400
200
0
NR_Before
NR_After


Not mutated
Pam50 + Claudin-low subtype
claudin-low
Normal
NC
LumB
LumA
Her2
Basal
-2 -1 0 1 2 3 4
TNFRSF17, mRNA Expression z-Scores (U133 microarray only)


Not mutated
claudin-low
Normal
NC
LumB
LumA
Her2
Basal
-6 -5 -4 -3 -2 -1 0 1 2 3 4
TNFSF13, mRNA Expression z-Scores (U133 microarray only)


Not mutated
claudin-low
Normal
NC
LumB
LumA
Her2
Basal
-3 -2 -1 0 1 2 3 4 5
TNFSF13B, mRNA Expression z-Scores (U133 microarray only)


TNFSF13 7%
TNFRSF17 8%
TNFSF13B 5%
AR 6%
ALDH1A1 3%
NANOGP1 0%
SOX2 7%
KLF4 2.8%
MYC 25%
TNFSF13
TNFRSF17
TNFSF13B
AR
ALDH1A1