Serum-derived factors of breast cancer patients with brain metastases alter permeability of a human blood-brain barrier model

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ABSTRACT

Background: The most threatening metastases in breast cancer are brain metastases, which correlate with a very poor overall survival, but also a limited quality of life of affected persons. A key event for the metastatic progression of breast cancer into the brain is the migration of cancer cells across the blood-brain barrier (BBB).

Methods: We adapted and validated the $CD34^+$ cells-derived human in vitro BBB model (brain-like endothelial cells, BLECs) to analyse the effects of patient serum on BBB properties. We collected serum samples from healthy donors, breast cancer patients with primary cancer, and breast cancer patients with, bone, visceral or cerebral metastases and analysed cytokine levels in these sera utilizing immunoassays and correlated it with clinical data. We used paracellular permeability measurements, immunofluorescence staining, Western blot and mRNA analysis to examine the effects of patient sera on the properties of BBB in vitro.

Results: The BLECs cultured together with brain pericytes in transwells developed a tight monolayer with a correct localization of claudin-5 at the tight junctions (TJ). Several BBB marker proteins such as the TJ proteins claudin-5 and occludin, the glucose transporter GLUT-1 or the efflux pumps PG-P and BCRP were upregulated in these cultures. This was accompanied by a reduced paracellular permeability of Na-fluorescein (400 Da). We then used this model for the treatment with the patient sera. Only the sera of breast cancer patients with cerebral metastases had significantly increased levels of the cytokines fractalkine (CX3CL1) and BCA-1 (CXCL13). The increased levels of fractalkine showed statistically significant correlation with ER/PR receptor status of the tumour. The treatment of BLECs with these sera selectively increased the expression of CXCL13 and TJ protein occludin. In addition, the permeability of Na-fluorescein was increased after serum treatment.

Conclusion: We demonstrate that the $CD34^+$ cell-derived human in vitro BBB model can be used as a useful tool to study the molecular mechanisms underlying cerebrovascular pathologies. We showed that serum from patients with cerebral metastases may affect the integrity of the BBB in vitro, perhaps caused by elevated concentrations of specific cytokines such as CX3CL1 and CXCL13.

Keywords: metastatic breast cancer; blood-brain barrier, in vitro models, CX3CL1, CXCL13

Introduction

The blood-brain barrier (BBB) is a natural barrier that specializes in protecting the brain from harmful substances, including anti-tumour drugs. In vitro models can help to better understand the mechanisms and changes at the BBB in cancer patients and can provide information for future therapies. In vitro BBB models have been used over the past five decades to investigate molecular mechanisms underlying physiological and pathological processes at the BBB and central nervous system (CNS) and to perform drug discovery studies. Central to these models are the brain microvascular endothelial cells, which form a tight barrier through cell-cell contacts, tight, adherens and gap junctions. Specific transporter and receptors are responsible for supplying the brain with energy and clearing it of toxic substances. Rat, mouse, pig and bovine in vitro models are commonly used to study BBB characteristics (1, 2). Human primary brain microvascular endothelial cells rapidly lose their properties when removed from their natural environment (3) and show high permeability when immortalized (1). The development of stem cell-based technologies opened up a new opportunity to generate human in vitro BBB models. Induced pluripotent stem cells (iPSCs)-derived brain-like endothelial cells (BLECs) can be obtained by specific differentiation protocols (4-11). Another published method is the use of umbilical cord blood-derived hematopoietic stem cells for differentiation into endothelial cells, followed by the induction of BBB properties by co-culture with brain pericytes (12-14). These advances in in vitro BBB modelling contribute to advances in the understanding of CNS disorders, such as brain metastases of breast cancer (15-17).

Breast cancer is the world's most common malignant tumour in women and causes the highest tumour-related death among them in western industrialised countries. Fortunately, nowadays primary breast cancer is a well treatable disease with high overall survival. In contrast, tumour metastases such as visceral, bone and cerebral metastases at later stages of this cancer play an important role for overall survival and mortality. However, appearance of metastases also means that a level of systemic tumour disease was reached and therefore describes a palliative situation. Overall, about 10-15 % of the breast cancer patients develop cerebral metastases, one of the most severe metastases types, which is associated with a very poor prognosis with only 20 % one-year survival (18). Particularly, histological cancers of the triple-negative (oestrogen receptor, progesterone receptor and epidermal growth factor receptor 2 negative) and epidermal growth factor receptor 2 (HER2/neu) subtypes have a high potential for cerebral metastases (19, 20). The mechanism of metastatic progression of breast cancer into the brain and the migration of cancer cells via the BBB in detail remains not well understood.

More than 40 chemokines, small proteins of 8-14 kDa, were identified in humans. They are grouped into four classes based on the position of their N-terminal cysteine residues: CC, CXC, XC and CX3C (21). They play a role in numerous biological processes like immune system homeostasis, cell

proliferation and differentiation. Increased levels of chemokines have been identified in inflammatory diseases for instance rheumatoid arthritis, asthma and psoriasis (21). Recent evidence indicates that chemokines also play a role in cancer progression and metastatic dissemination of solid tumours (22).

In the present study, we use a $CD34^+$ cells-derived in vitro BBB model in co-culture with brain pericytes to study the differential effects of breast cancer patient's sera on BBB properties. We analysed chemokine levels in the sera of breast cancer patients with primary cancer, bone, visceral or cerebral metastases relative to a healthy control group. We identified two chemokines that were selectively elevated in the group with cerebral metastases and these sera displayed impairing effects towards the BBB integrity in vitro.

Materials and methods

Patient samples

All human samples were collected from donors only after signing an informed consent form in accordance with German legislation rules. Herby we followed and strictly adhered to the Ethical Guidelines of the University of Würzburg, which are in accordance to the Helsinki Declaration of 1975 and its revision of 1983. We collected 103 blood samples from patients with breast cancer and a healthy control group of 15 women. These samples where pseudonymised and classified into five distinct groups: cancer free individuals (healthy control, HC, n = 15), breast cancer patients with primary cancer (PC, n = 26), breast cancer patients with visceral (VM, n = 30), bone (BM, n = 20) and cerebral metastases (CM, n = 12). The blood specimen were centrifuged at 2000 g, the serum collected and stored at -80°C until use.

Table 1. Summary of clinical data.

	C	PC	VM	BM	CM
Patient characteristics					
total number	15	26	30	20	12
median age	66	57,6	60	66,3	62,1
died					5
premenopausal	1	10	6	2	2
postmenopausal	14	16	24	18	10
Tumour characteristics					
ER/PR positive		15	17	16	1
HER2/neu positive		8	11	3	11
Triple negative		2	2		
Grading:					
well differentiated (G1)		2	4	0	0
moderately differentiated (G2)		18	11	16	6
poorly differentiated (G3)		6	15	4	6
Ki67 staining (median)		25%	40%	15%	30%
other					1

Abbreviations: BM: bone metastases, C: control group of healthy donors, CM: cerebral metastases, ER: oestrogen receptor; Her2/neu: human epidermal growth factor 2, PC: primary cancer, PR: progesterone receptor; VM: visceral metastases.

CD34$^+$ cells-derived human in vitro BBB model

The written informed consent was obtained from the infants' parents prior to collection of the infants' umbilical cord blood. The CD34$^+$ hematopoietic stem cells-derived human endothelial cells were isolated and purified as described previously and adapted in our laboratory using already published procedures. The cells were grown in 6- or 12-well transwells in monoculture or co-culture with brain pericytes for 6 days to induce BBB-like properties and are termed brain-like endothelial cells (BLECs) (12, 14, 23). Shortly, 80 x 10^3 or 325 x 10^3 BLECs were cultured on Matrigel coated 12- or 6-well transwell inserts respectively (pore size 0.4 µm, Corning) for two days in Microvascular Endothelial Cell Growth Medium (ECM) (PLEOBiotech) supplemented with 5% fetal calf serum (FCS). Brain pericytes (12) were cultured on gelatine-coated plates in DMEM (Sigma) supplemented with 20% FCS, 2 mM L-glutamine and 50 µg/ml gentamycin. For co-cultures, 50 x 10^3 brain pericytes were seeded into 12-well plates and grown in ECM along with the BLECs on transwell inserts for five days to induce BBB-characteristics of the latter. Endothelial cells were grown alone or in co-culture with brain pericytes were used to determine paracellular permeability and to isolate RNA and protein. For the incubations experiments, BLECs were treated with ECM supplemented with 2% patient sera for 24 hours before transcriptional, immunofluorescent and permeability studies.

Immunofluorescence

CD34$^+$-derived endothelial cells were grown on their own or in co-culture with pericytes as described above, with or without 2% patient serum in the medium for 24 hours. Separate treatments were done with individual sera. Cells were stained on transwell inserts with anti-claudin-5 antibody, conjugated to Alexa Fluor 488 (Thermo Fisher Scientific) as described previously (24).

Real-time PCR

Real-time PCR was performed as previously described (24-26). Briefly, RNA was isolated using a RNA isolation kit NucleoSpin® RNA (Machery-Nagel) according to manufacturer's instruction. Total RNA (500 ng) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The commercially available TaqMan probes Hs00170162_m1 (OCLN), Hs00533949_s1 (CLDN5), Hs00184500_m1 (ABCB1), Hs00988717_m1 (ABCC4), Hs01053790_m1

(ABCG2), Hs00892681_m1 (SLC2A1), Hs00355476_m1 (CCL20), Hs03676656_m1 (CXCL12), Hs00234140_m1 (CCL2), Hs00757930_m1 (CXCL13), Hs00171086_m1 (CX3CL1) were used with the TaqMan® Fast Advanced Master Mix in the StepOne-Plus Real-Time PCR System (Thermo Fisher Scientific). Calnexin (CANX) (Hs01558409_m1) was used for normalization. Relative expression was calculated by the comparative Ct method.

Western blot analysis

Western blot was performed as recently described (27, 28). Twenty micrograms of protein were subjected to SDS-PAGE followed by transfer to a PVDF membrane (Bio-Rad Laboratories) and blocked with 5% (w/v) non-fat milk in phosphate buffered saline (PBS, pH 7.4). The membranes were incubated with the respective primary antibodies diluted in PBS containing 1% Bovine Serum Albumin (BSA, pH 7, Sigma) at 4°C overnight. Following antibodies were used: anti-BCRP (1:100, Abcam), CLDN5 (1:500, Thermo Fisher Scientific), GLUT-1 (1:2000, Millipore), MCT1 (1:200, Santa Cruz Biotechnology), MRP4 (1:500, ENZO), LRP1 (1:1000, Abcam), P-Glycoprotein (P-GP, 1:20, ENZO), RAGE (1:200, Santa Cruz Biotechnology), Transferrin Receptor (TFR, 1:500, Thermo Fisher Scientific). After incubation with respective secondary antibodies, images were taken using Enhanced Chemiluminescence solution and FluorChem FC2 Multi-Imager II (Alpha Innotech). The intensity of protein bands was measured with ImageJ software.

Endothelial Permeability Measurements

Coated transwell inserts and inserts containing confluent BLECs were placed into a plate with 1.5 ml HEPES-buffered Ringer's solution (pH 7.4). 500 µl of the same solution containing 1 mM Na-fluorescein (Sigma) was added to the upper compartment. During the one-hour assay, the inserts were placed into a new well with fresh buffer solution every 20 min and aliquots from the receiver compartment were collected. Aliquots from the donor solution were taken at the beginning and at the end of the experiment. All samples were measured at 490/516 nm wavelengths using a microplate reader (Tecan). For each treatment condition, at least three inserts with and without cells were tested.

Cytokine Immunoassay

Concentrations of human CCL2, CCL5, CCL20, CXCL12, CXCL13 and CX3CL1 were measured in duplicate in individual patient sera using a multi-analyte immunoassay and Luminex® bead technology with reagent kits (Merck Millipore) according to manufacturer's instructions.

Statistical analysis

GraphPad Prism 7 (GraphPad Software) was used for statistical analyses. Data are expressed as mean ± standard error of the mean (SEM). Differences among groups were analysed either using the ANOVA

with Tukey's multiple comparison test or a non-parametric Kruskal-Wallis test with Dunn's multiple comparison post-hoc test. Mann Whitney U test was used to compare two groups. Statistical significance was assumed for $p < 0.05$ (*). The Spearman-Rho correlation coefficients were calculated for any two measurements of chemokines and of chemokines and tumour properties within the patient population (n = 103). Correlation analysis was performed using the IBM SPSS Statistics 23 Software (IBM Corporation).

Results

Validation of human in vitro BBB model

After 6 days co-culture with brain pericytes expression of TJ proteins occludin and claudin-5 was induced in BLECs (**Figure 1A)**. The solute carrier transporter GLUT-1 was also induced. Among the cellular receptors and efflux pumps, only the LRP-1 and P-GP showed induction. Other analysed proteins were slightly downregulated (**Figure 1 A**). Measurement of paracellular permeability of Na-fluorescein (400 Da) yielded low permeability values ($0.21 \pm 0.04 \times 10^{-3}$ cm/min), which were more than 80% lower than in $CD34^+$-derived endothelial cells monoculture (**Figure 1B**). In addition, we stained the endothelial cells with anti-claudin-5 antibody (**Figure 1C**). Only BLECs in co-culture with pericytes formed a monolayer with the correct localization of claudin-5 at the TJs. Next, mRNA expression of BBB markers was evaluated. All analysed transcripts were significantly altered in BLECs co-cultured with pericytes compared to $CD34^+$-derived endothelial cells monoculture (**Figure 1D**). While ABCB1, SLC2A1 and occludin mRNAs were downregulated, ABCC4, ABCG2 and claudin-5 mRNAs were significantly upregulated. These results indicate that co-culture with brain pericytes is indispensable for the induction of BBB-properties in $CD34^+$-derived endothelial cells.

Quantification of chemokines in patient serum

We compared the concentration of selected chemokines in the sera of healthy controls (C), breast cancer patients with primary cancer (PC), breast cancer patients with visceral (VM), bone (BM) and cerebral metastases (CM) using an immunoassay (**Figure 2**). Mean serum concentrations of BCA-1 (CXCL13) and fractalkine (CX3CL1) were significantly higher in the sera of breast cancer patients with cerebral metastases compared to all other patient populations or controls. CCL20 showed a similar tendency. No differences between the groups were found for the mean serum concentrations of MCP-1 (CCL2), RANTES (CCL5) and SDF-1a/b (CXCL12) (**Figure 2**). In the patient population a statistically significant correlations between MCP-1 and RANTES ($r = 0.238$, $p = 0.04$), BCA-1 and CCL20 ($r = 0.362$, $p < 0.01$), and histological grading and Ki67 staining ($r = 0.456$, $p < 0.01$) were seen (**Table 2**). In addition, a statistically significant correlation was observed between the fractalkine concentrations in patient sera and the ER/PR receptor status ($r = -0.228$, $p = 0.048$) of the tumour (**Figure 3**). Patients with ER

and PR negative tumours had statistically significant higher fractalkine concentrations in serum (**Figure 3 AB**). A statistically significant negative correlation between BCA-1 serum concentrations and the histological grading (r = -0.808, p = 0.003) was seen in patients with cerebral metastases (n = 12).

Table 2. Correlation analysis between chemokine values and chemokine values with tumour properties.

Correlates with:		MCP-1 (pg/ml)	RANTES (pg/ml)	SDF-1a/b (pg/ml)	BCA-1 (pg/ml)	CCL20 (pg/ml)	Grading	Ki67 staining
Fractalkine (pg/ml)	r	-0.03	0.09	0.18	0.19	0.04	0.13	0.18
	p	0.83	0.45	0.12	0.11	0.73	0.33	0.20
MCP-1 (pg/ml)	r		**0.238***	0.13	-0.12	0.00	-0.04	0.02
	p		**0.04**	0.26	0.29	0.99	0.79	0.87
RANTES (pg/ml)	r			0.20	0.04	0.05	-0.09	0.19
	p			0.05	0.68	0.60	0.44	0.11
SDF-1a/b (pg/ml)	r				0.02	-0.12	-0.09	0.17
	p				0.83	0.24	0.41	0.15
BCA-1 (pg/ml)	r					**0.362****	-0.04	0.08
	p					**<0.01**	0.72	0.51
CCL20 (pg/ml)	r						0.00	0.15
	p						0.98	0.24
Grading	r							**0.456****
	p							**<0.01**

Abbreviations: BM: bone metastases, C: control group of healthy donors, CM: cerebral metastases, ER: oestrogen receptor; Her2/neu: human epidermal growth factor 2, PC: primary cancer, PR: progesterone receptor; VM: visceral metastases.

r: correlation coefficient (the positive r means positive correlation, while the negative r means negative correlation; the closer r value to 1, the greater the correlation); p: p value indicating statistical significance; *p<0.05; **p<0.01.

Treatment with patient serum led to reduced barrier properties of BLECs

The identification of two selectively elevated chemokines in the sera of breast cancer patients with brain metastases, prompted us to wonder whether such sera could impair BBB integrity. To test this hypothesis, we treated BLECs for 24 hours with 2% control sera or sera from breast cancer patients with brain metastases (**Figure 4**). Serum-derived factors significantly increased the paracellular permeability of the endothelial monolayer (**Figure 4A**). In addition, staining of serum-treated cells with anti-claudin-5 antibody revealed areas with reduced claudin-5 staining within the monolayer, suggesting a leaky barrier at that site (**Figure 4B, white arrow**). Next, we analysed the mRNA expression of chemokines and BBB-markers after serum treatment (**Figure 4C**). We detected a significant increase in occludin and BCA-1 mRNA levels in cells treated with sera of breast cancer patients with cerebral metastases compared to cells treated with the control serum. Other markers and chemokines such as ABCB1, ABCG2, claudin-5, CCL2, CCL20 and CX3CL1 were not significantly

altered (**Figure 4C**). These results suggest that even low levels of patient serum (2%) can increase paracellular permeability and alter the expression and cellular localization of TJ proteins responsible for sealing of the BBB.

Discussion

The integrity of the BBB is a key feature responsible for protecting the brain from harmful substances, regulating entry and efflux of macromolecules and immune cells to and from the brain, and maintaining homeostasis of the CNS (29, 30). Inflammation can affect the barrier properties of the BBB. However, clinical and in vitro data on inflammation and underlying pathomechanisms at the BBB in breast cancer patients with cerebral metastases are limited. Therefore we designed the present study to analyse serum-derived chemokines in breast cancer patients with different cancer characteristics and to test the effects of patient sera on the BBB properties in vitro.

We utilized the $CD34^+$ cells-derived in vitro BBB model first published by Cecchelli et al. in 2014 (12). Similar to the first report on this model, we observed the induction of TJ proteins, multiple transporters and cellular receptors either at the protein or mRNA level when the cells were co-cultured together with pericytes. Pericytes have been chosen, because they induce BBB characteristics in $CD34^+$ cells-derived hematopoietic stem cells (12) and they are known to play a role in the maturation and stabilization of the BBB (31, 32). Induction of TJ-protein expression was accompanied by low paracellular permeability for Na-fluorescein. The permeability values were similar to previously published values for this (12) and also for other in vitro BBB models (33). It can be assumed that a 6 day-co-culture with brain pericytes is sufficient to induce BBB properties in $CD34^+$ cells-derived hematopoietic stem cells. Differentiated BLECs can be used to study molecular mechanisms underlying brain disorders, such as brain metastases.

In general, inflammatory mediators are mainly expressed by macrophages such as microglia, but also by astrocytes, oligodendrocytes and vascular endothelial cells (34). Numerous mediators play a role in this out-balanced process, including anti-inflammatory cytokines, pro-inflammatory cytokines, chemokine-ligands and receptors. A correlation between cancer metastases and cytokine expression is discussed for various tumour entities (35, 36), including breast cancer (37, 38). Therefore, we examined chemokines in the sera of breast cancer patients with and without cerebral metastases in comparison to healthy donors. Among the chemokines tested, CX3CL1 and CXCL13 were selectively and significantly increased in patients with cerebral metastases of breast cancer. CX3CL1 (also known as fractalkine or neurotactin) is a membrane-bound chemokine that can facilitate intercellular interactions, interacts with the TNFα-converting enzyme ADAM17 and is released in its shed form by apoptotic cells to recruit professional phagocytes to the site of cell death (39, 40). Fractalkine serum

concentrations were higher in patients with the ER/PR negative tumours, which corresponds to the literature. Andre et al. showed in a study with 142 patients that a high CX3CL1 expression in the primary cancer correlates with brain metastases in a 13-year median-follow up (41). Similarly, Tsang et al. postulated that CX3CL1 expression is associated with poor outcome in breast cancer patients (42). High levels of CX3CL1 in cells can attract those cancer cells expressing its receptor CX3CR1 and trigger them to invade the tissue and form metastases as seen e.g. for breast cancer spinal metastases (43). In our study, we showed that CX3CL1 is selectively elevated in the serum of breast cancer patients with cerebral metastases. CX3CL1 may therefore be involved in the formation of metastases in the brain, but further investigations are needed to fully elucidate the underlying mechanisms. Another elevated chemokine in the serum of breast cancer patients with brain metastases was CXCL13 (also known as B cell-attracting chemokine 1, BCA-1). Our results are consistent with other reports showing elevated BCA-1 serum concentrations in patients with metastatic disease (44). A negative correlation between BCA-1 and the histological grading in patients with brain metastases suggests that high BCA-1 serum concentrations can lead to brain metastases from moderately differentiated tumours with low histological grading. However, our analysis is limited by a small number of samples. In breast cancer cell lines, CXCL13 induced changes of epithelial-to-mesenchymal transition marker expression. It upregulated vimentin, Snail, Slug, N-cadherin, MMP9 and RANKL and downregulated E-cadherin (45). The endothelial-to-mesenchymal transition of brain endothelial cells has been described (46) and could also play a role in our in vitro model.

One of the limitations of our study is the relatively low number of serum samples from breast cancer patients. It will be interesting to increase the number of specimen for chemokine analyses and the evaluation of serum effects on BBB properties. In vitro BBB models are valuable tools for studying cellular responses to factors such as patient sera. However, we must consider that they are model systems not reconstituting the microenvironment existing in the human body.

Conclusion

The $CD34^+$ cell-derived human in vitro BBB model shows high barrier properties, accompanied by the expression of BBB and endothelial cell markers. It was used in the present study to analyse the mechanisms underlying the formation of cerebral metastases in breast cancer. Sera with elevated CX3CL1 and CXCL13 levels displayed barrier-compromising effects in vitro and therefore could contribute to the formation of brain metastases by breast cancer cells in vivo.

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Authors' contributions

CJC, CS, JF, CH, SLH, MB performed and analysed experiments and wrote the manuscript. FG, NS, NR, AW, PM provided crucial reagents. All authors were involved in critical revision and final approval of the manuscript.

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Conflict of interest

None

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Figure legends

Figure 1. BLECs in co-culture with pericytes develop a tight barrier. $CD34^+$-derived endothelial cells were cultured as monoculture or co-culture with brain pericytes for 6 days in a transwell system. (**A**) Expression of selected BBB marker proteins was induced in co-culture (Co) in comparison to monoculture (Mono) as shown by Western blot. Numbers under the representative bands indicate protein levels normalised to β-actin and to monoculture control. (**B**) Paracellular permeability for Na-fluorescein of $CD34^+$-derived endothelial cells monolayers either cultured alone or together with pericytes. Data are shown as mean ± SEM (n = 3), ***p < 0.001. (**C**) Expression of claudin-5 in $CD34^+$-derived endothelial cells cultured alone or in co-culture with brain pericytes shown by immunofluorescence. Magnification 400x, green: claudin-5, blue: DAPI nuclear staining. (**D**) Messenger RNA expression of transporters and tight junction proteins in BLECs was quantified by qPCR. Target gene expression was normalized to endogenous controls and shown as fold over control, which was arbitrarily set as 1 (control level marked in graph). Data are shown as mean ± SEM of three experiments. *p < 0.05 statistically significant versus control. BCRP: Breast Cancer Resistance Protein (ABCG2), CLDN5: claudin-5, GLUT-1: Glucose Transporter Type 1 (SLCA1), LRP1: LDL Receptor Related Protein 1, MCT1: Monocarboxylate Transporter 1 (SLC16A1), MRP4: Multidrug Resistance-Associated Protein 4 (ABCC4), OCLN: occludin, P-GP:P-Glycoprotein 1 (ABCB1), RAGE: Receptor For Advanced Glycation End-Products (AGER), TFR: Transferrin Receptor (TFRC), ZO1:Zonula Occludens 1 (TJP1).

Figure 2. Serum levels of cytokines and chemokines measured in serum from healthy donors and breast cancer patients. Cytokines and chemokines were measured in the sera by multi-analyte immunoassay in following groups: persons without cancer (C), breast cancer patients with primary cancer (PC), breast cancer patients with visceral (VM), bone (BM) and cerebral metastases (CM). Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 3. Correlation between fractalkine serum concentrations and tumour receptor status. The values of fractalkine concentrations (pg/ml) are converted into logarithmic values. ER: oestrogen receptor, PR: progesterone receptor, *p < 0.05.

Figure 4. BLECSs treated with serum from patients with cerebral metastases show decreased barrier properties. BLECS were co-cultured with brain pericytes for 6 days in a transwell system. (**A**) BLECs were treated with the control sera (C) and sera from breast cancer patients with cerebral metastases (CM) for 24 hours followed by measuring the paracellular permeability for Na-fluorescein. Data are shown as mean ± SEM of three independent experiments, *p < 0.05. (**B**) Immunostaining of claudin-5 in BLECs co-cultured with pericytes and treated either with serum from healthy donors (C) or breast cancer patients with cerebral metastases (CM). The arrow indicates loss of claudin-5 staining at the tight junction of BLECs treated with serum from breast cancer patients with CM. Shown is one representative image of n = 6. Magnification 400x, green: claudin-5, blue: DAPI nuclear staining. (**C**)

Messenger RNA Expression of chemokines and BBB-markers in BLECs treated with control serum and serum from breast cancer patients with cerebral metastases. The mRNA was quantified by qPCR. The target gene expression was normalized to endogenous control and shown as fold over control, which was arbitrarily set as 1 (control level marked in graph). Data are shown as mean ± SEM of n = 4 (control sera) and n = 5 (CM). **p < 0.01 statistically significant versus control.

Figure 1

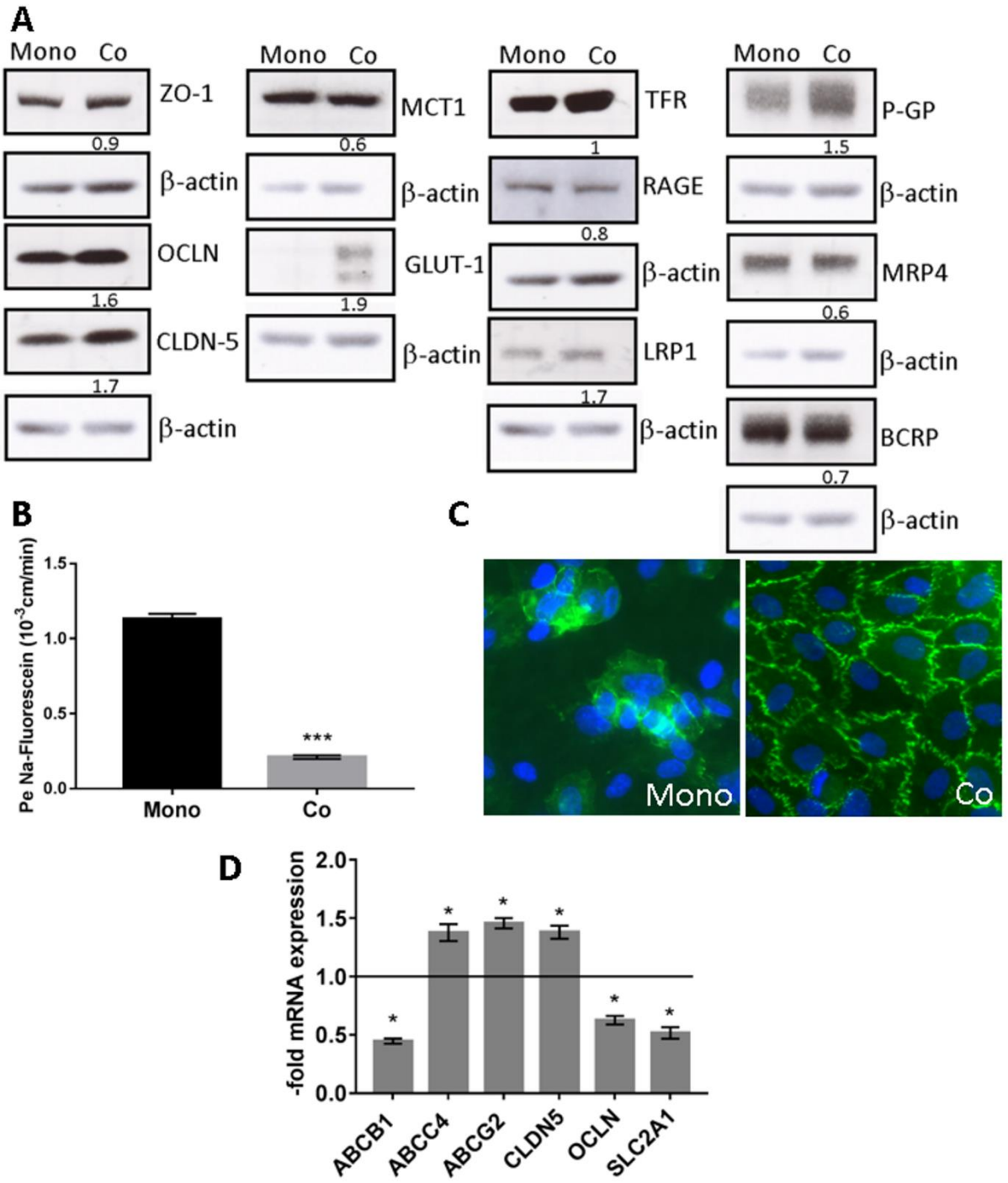


Figure 2

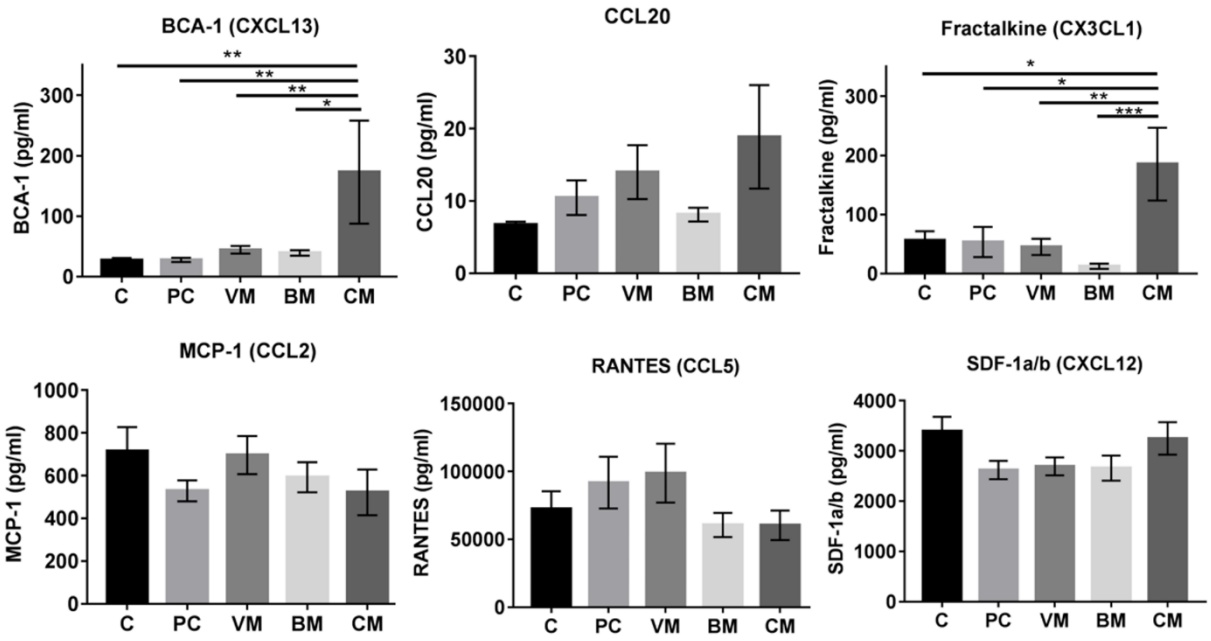


Figure 3

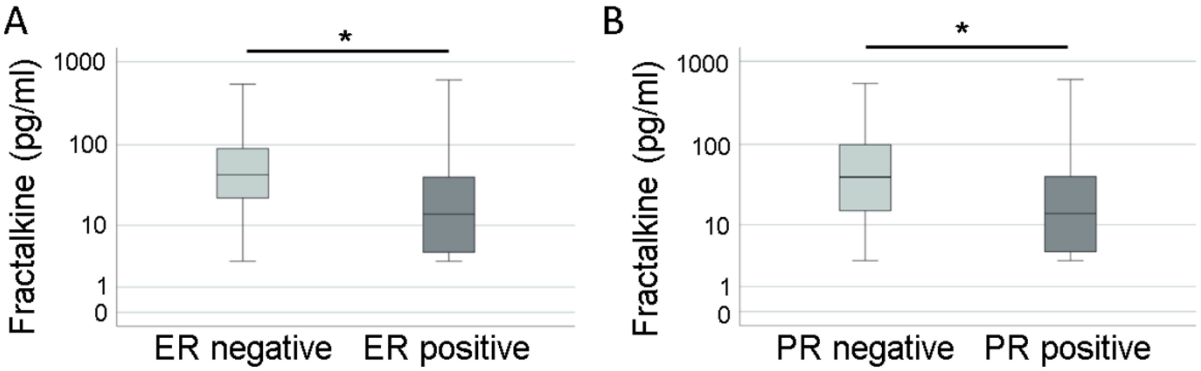
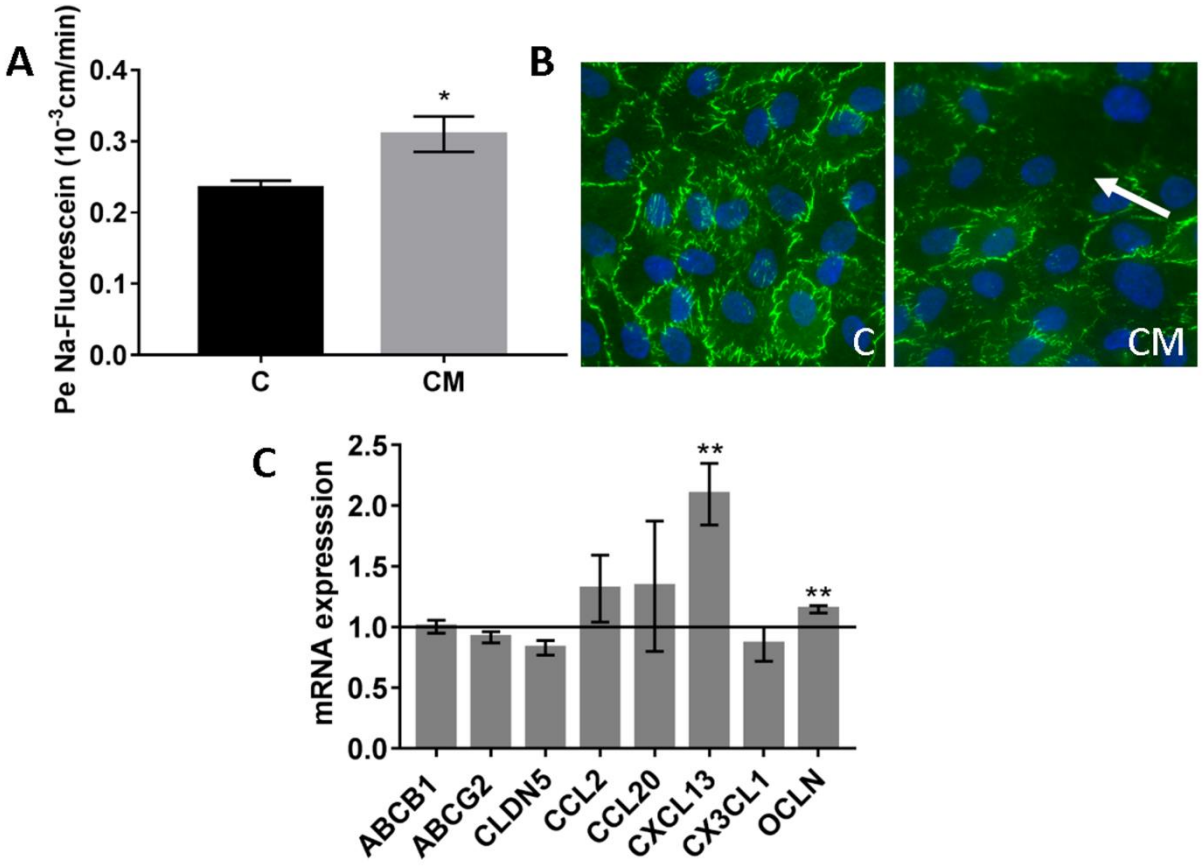


Figure 4


A
Pe Na-Fluorescein (10^{-3}cm/min)
0.4
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0.2
0.1
0.0
*
C
CM
B
C
CM
C
mRNA expresssion
2.5
2.0
1.5
1.0
0.5
0.0
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ABCB1
ABCG2
CLDN5
CCL2
CCL20
CXCL13
CX3CL1
OCLN