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Research Article

VEGF-R1 prognostic role in breast cancer. A possible target for anti- angiogenic therapy? A randomized controlled trial

[1]Elena Galoș, [2]Rareș Buiga, [3]Laura Maja, [4]Tiberiu Tat, [4]Răzvan Popa, [5]Anca I. Ciurea, [6]Ștefan C. Vesa, [7]Daniela Ionescu, [8]Carmen M. Mihu

[1] Department of Anesthesia and Intensive Care, Clinical Hospital CF Cluj-Napoca, România, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, România; [2] Department of Pathology, "Prof Dr Ion Chiricuță" Institute of Oncology, Cluj-Napoca, România, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, România; [3] Department of Pathology, "Prof Dr Ion Chiricuță" Institute of Oncology, Cluj-Napoca, România; [4] Department of Anesthesia and Intensive Care, "Prof Dr Ion Chiricuță" Institute of Oncology, Cluj-Napoca, România, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, România; [5] Department of Radiology, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, România; [6] Department of Pharmacology, Toxicology and Clinical Pharmacology, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, România; [7] Ist Department of Anesthesiology and Intensive Care, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, România; [8] Department of Histology and Morphological Sciences, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, România.

Abstract. Objective: With breast cancer being one of the world's most prevalent cancers, discovering new treatment targets is of paramount importance. The study's purpose is to assess if Vascular Endothelial Growth Factor Receptor 1(VEGF-R1) is a possible treatment target and its prognostic role. Material and method: 120 patients undergoing surgery for breast cancer resection were enrolled and randomly assigned to one of the four types of anaesthesia: general anaesthesia (GA) with either sevoflurane or propofol, associated or not with a continuous 1% lidocaine infusion. For serum level assessment of proangiogenic factors Vascular Endothelial Growth Factor A (VEGF-A), matrix metalloproteinases 3 and 9 (MMP-3 and MMP-9), blood samples were drawn before surgery. The excised tumour underwent histological analysis for diagnosis and tumoral grading. VEGF-R1 expression in the tumoral tissue was assessed by immunohistochemistry and graded as high positive, positive, low positive and negative. Results: No corelations were found between preoperatively serum levels of proangiogenic factors and histological expression of VEGF-R1. High positive and low positive VEGF-R1 expression was homogenous throughout all Nottingham grades. Significant differences were observed when comparing Nottingham grades II and III for low positive VEGF-R1 expression (higher percentage of low positive being associated with a worse outcome) and Nottingham grade III and II for negative VEGF-R1 expression. VEGF-R1 positivity did not correlate with higher recurrence rates. Conclusion: Tissue positivity for VEGF-R1 is not associated with a higher proangiogenic potential, higher recurrence rates, and it is unlikely that it can represent a marker of tumoral aggressiveness. It is possible that the lack of VEGF-R1 expression is linked to a better outcome.

Key Words: angiogenesis, VEGF-R1, anaesthesia, breast cancer prognosis

Corresponding Author: E. Galoș, email: draghiciuelena@gmail.com

Introduction

Breast cancer is the second most frequent neoplasia worldwide and the 5th cause of death by neoplasia (World Health Organisation [Internet] 2021). In 2020, out of the 19 292 789 million cases of cancer worldwide, 11.7% were due to breast neoplasia, with the number of deaths being 6.9% out of the 9 958 133 of total cancer deaths (International Agency for Research on Cancer 2021). Surgical excision of the tumoral process remains a mainstay of treatment for breast cancer patients. After the surgical removal of the tumoral process, cancer treatment includes a combination of chemotherapy, radiotherapy and hormonal therapy meant to eliminate possible residual tumoral tissue or distant metastasis not identified before the tumoral resection.

Death due to cancer occurs mainly because of disease relapse, which is promoted by proangiogenic processes that favour tumoral development. Chemotherapy, hormonal therapies and immunotherapies, work mainly by interfering with cellular DNA (deoxyribonucleic acid), disrupting molecular mechanisms essential for cellular division and growth and by lowering hormonal levels that promote tumoral growth (American Cancer Society 2021).

Role of VEGF-A and its receptors

In the process of tumoral dissemination and progression, angiogenesis is an essential step. VEGF-A represents a main pathway for proangiogenic processes playing a role in increasing vascular

permeability and stimulating vascular endothelium proliferation (Jaura AI et al. 2014). Treatments that target VEGF-A (like bevacizumab) have an important role in cancer therapy, but the emerging data reveals the development of tumoral resistance to anti-angiogenic therapies, creating the need for identifying different targets for cancer treatment (Masoud et Pagès 2017). VEGF-A acts through its receptors VEGF-R1 and VEGF-R2 (vascular endothelial growth factor receptor 2) to regulate angiogenesis: VEGF-R1 receptors are responsible for negative regulation of angiogenesis during early development, the migration of monocytes/macrophages, stimulation of inflammation, cancer metastasis and atherosclerosis (Shibuya 2006), and VEGF-R2 receptors mediate the mitogenic, angiogenic and permeability-enhancing activity of VEGF-A (Takahashi et Shibuya 2005). The results of therapies that target VEGF-A receptors by using Tyrosine Kinase Inhibitors (TKI) in order to inhibit VEGF-A signal transduction have produced mixed results so far (Madu et al. 2020), thus creating a need for further studies in order to assess the role of VEGF-A receptors in tumoral progression.

Metalloproteinases 3 and 9

Metalloproteinases are a part of zinc-endopeptidases family that have a proteolytic activity against substrates located on the extracellular matrix (ECM) (Quintero-Fabián et al 2019) facilitating in this cell migration in nearby tissues. In humans there are 23 genes that code MMPs (Nagase et Woessner 1999) each with multiple functions.

MMP-3 plays a role in the epithelial-mesenchymal transition (EMT) process (Gilles et al 2000-2013), degradation of ECM proteins and activating MMP-9 (Ramos-DeSimone et al 1999). MMP-9 is one of the most investigated MMPs, known for its role in degrading ECM, activation of multiple cytokines which influence tissue remodelling (Yabluchanskiy et al 2013) and releasing VEGF from the ECM (Klein et Bischoff 2010). Considering their part in the processes that favour cell migration and release of proangiogenic factors, MMPs may have an important role in promoting tumoral development and metastasis.

In this study we aimed to evaluate if VEGF-R1 has a prognostic role in breast cancer and the possibility of it becoming a potential target for anti-angiogenic therapy.

The primary objective was to analyse if higher VEGF-R1 positivity on excised tumoral tissue is associated with higher serum levels of VEGF-A, MMP-3 and MMP-9.

The secondary objective was to assess if higher expression of VEGF-R1 in tumoral tissue is associated with a higher tumoral aggressiveness and a worse prognostic (assessed through the use of Nottingham scale).

The third objective was to evaluate if there is a connection between VEGF-R1 expression in tumoral tissue and the frequency of relapse 1 year postoperatively.

This study is comprised in a larger study designed to investigate the influence of different types of anaesthesia in breast cancer surgery on factors that may influence prognosis and metastasis (NETosis and angiogenesis expression after intravenous or inhalation anaesthesia with or without i.v. lidocaine for breast cancer surgery: a prospective, randomised trial, published by Bristish Journal of Anaesthesia (Galoș et al 2020); Continuous intravenous infusion of lidocaine for postoperative pain in breast cancer surgery patients. Effect on acute and chronic pain published by Human and Veterinary Medicine, International Journal of the Bioflux Society (Galoș et al 2020).

Material and Methods

After obtaining the Iuliu Hațieganu University of Medicine and Pharmacy ethics comitee approval (No 54/14.03.2016), the study was registered with ClinicalTrials.gov (NCT02839668). The study took place in the setting of Prof. Dr. I Chiricuță Institute of Oncology of Cluj-Napoca, Romania and enrolled 120 patients aged 18 to 80 years of age and an American Society of Anesthesiology (ASA) physical status classification of I to III, that were not proven to have disseminated disease before surgery. Criteria that did not permit inclusion in the study were: refusal to participate, incapacity of understanding study protocol or giving informed consent (neuropsychiatric diseases), allergies to the substances used in the study, existence of chronic inflammatory diseases, presence of ischemic cardiovascular disease and peripheral vascular disease, diabetes and endometriosis (pathologies that could interfere with serum VEGF-A levels). After giving informed consent based on the Helsinki declaration, patients were computer-randomised in a 1:1:1:1 ratio to one of the four types of anaesthesia groups: GA using Sevoflurane (group S), GA with Sevoflurane and a continuous infusion of intravenous (iv) 1% lidocaine (group SL), GA with propofol using total intravenous anaesthesia target controlled infusion (TIVA-TCI) technique (group P), and propofol TIVA-TCI general anaesthesia associated with a continuous iv infusion of 1% lidocaine (group PL) (Table 1– patients demographic data, Figure1- patients randomisation).

Blood samples (20 ml) were collected by peripheral venous puncture just before initiation of anaesthesia and 20-24 h after the end of the surgical procedure. Samples were centrifuged at 4000x rotations per minute (rpm) within 1h of collection and stored in 2ml aliquotes at - 80°C for ulterior determination of VEGF-A, MMP-3 and MMP-9. The tissue that was removed during the surgical procedure was labeled and sent for histologycal analysis to the pathology laboratory. Excised tissue was fixed and embedded in paraffin blocks for subsequent analysis untill the enrollement of all patients. After enrollement completion, serum levels of VEGF-A , MMP-3 and MMP-9 were determined using comercially available ELISA kits in accordance with manufacturere's instructions: VEGF-A (Human VEGF-A, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA; assay range: 23,4-1500pg ml^{-1}, sensitivity: ≤5 pg ml^{-1}), MMP-3 (Human MMP-3, Bender MedSystems GmbH, Vienna, Austria; assay range: 2-28ng ml^{-1}, sensitivity: 0.0005 ng ml^{-1}) and MMP-9 (Human MMP-9, Bender MedSystems GmbH, Vienna, Austria; assay range: 0.23-15.0 ng ml^{-1}, sensitivity: 0.05ng ml^{-1}). For VEGF-R1 determination 3µ sections were deparaffinized and subjected to immunohistochemistry using a rabbit anti-human, mouse VEGF-R1 poyclonal antibody (My BioSource MBS 9410242) according to the immunohistochemistry protocol.

Immunohistochemical staining was assesed in tumoral tissue after image acquisition with a microscope equipped camera and using CaseViewer 2.4.0.119028 digital microscopy programme wich permitted using a 15.0x zoom of the sections (CaseViewer 2020). Analysis of VEGF-R1 staining was done by randomly selecting 5 areas of each image and quantifing the cytoplasmic positivity for VEGF-R1 (by using ImageJ processing programme;

Table 1. Patients demographic data. Data is expressed as mean and percentage of total

Trial groups	Sevoflurane (S)(n=17)	Sevoflurane + Lidocaine (SL)(n=22)	Propofol (P)(n=18)	Propofol + Lidocaine (PL)(n=18)
Age (mean)	56.82	58.54	52.16	59.94
Body-mass index (kg m^2)	26.92	29.57	26.63	26.94
ASA physical status (n,%)				
ASA I	6 (35%)	6 (27%)	10 (55.5%)	8
ASA II	10 (59%)	12 (54%)	8 (44.4%)	10
ASA III	1 (6%)	0	0	0
Not mentioned on the chart	0	4	0	0
Preoperative treatment (n,%)				
Previous chemotherapy	8(47%)	9 (41%)	9 (50%)	9 (50%)
Previous radiation	0	0	0	0
Previous hormonal therapy	1(6%)	3 (14%)	0	0
Not mentioned on the chart	0	2 (9%)	0	1 (5.5%)
Tumour site (n, %)				
Right	6 (35%)	10 (45%)	9 (50%)	4 (22.2%)
Left	11 (65%)	11 (50%)	9 (50%)	13 (72.2%)
Bilateral	0	1 (5%)	0	1 (5.5%)
TNM classification				
Pathology stage, tumour (n,%)				
Tx	0	0	0	0
Tis	2 (11.7%)	0	0	1 (5.5%)
T0	0	0	0	0
T1	6 (35.2%)	11 (50%)	10 (55.5%)	8 (44.4%)
T2	6 (35.2%)	10 (45.5%)	3 (16.6%)	9 (50%)
T3	2 (11.7%)	0	3 (16.6%)	0
T4	1 (5.8%)	0	1 (5.5%)	0
Not fund/ Not mentioned/ Missing	0	1 (4.5%)	1 (5.5%)	0
Pathology stage, nodes (n, %)				
Nx	0	1 (4.5%)	0	0
N0	8 (47%)	10 (45.5%)	8 (44.4%)	6 (33.3%)
N1	4 (23.5%)	2 (9%)	6 (33.3%)	8 (44.4%)
N2	4 (23.5%)	6 (27.3%)	2 (11.1%)	4 (22.2%)
N3	1 (5.8%)	2 (9%)	1 (5.5%)	0
Not fund/ Not mentioned/ Missing		1 (4.5%)	1 (5.5%)	0
Pathology stage, metastasis (n,%)				
Mx	14 (82.3%)	17 (77.3%)	15 (83.3%)	16 (88.8%)
M0	3 (17.6%)	3 (13.6%)	1 (5.5%)	2 (11.1%)
M1	0	1 (4.5%)	1 (5.5%)	0
Not fund/ Not mentioned/ Missing		1 (4.5%)	1 (5.5%)	0
Notthingham Score				
Nottingham I	4 (23.5%)	5 (22.7%)	3 (16.6%)	7 (38.8%)
Nottingham II	5 (29.4%)	12 (54.5%)	8 (44.4%)	8 (44.4%)
Nottingham III	3 (17.6%)	2 (9%)	5 (27.7%)	2 (11.1%)
Not mentioned	5 (29.4%)	3 (13.6%)	2 (11.1%)	1 (5.5%)

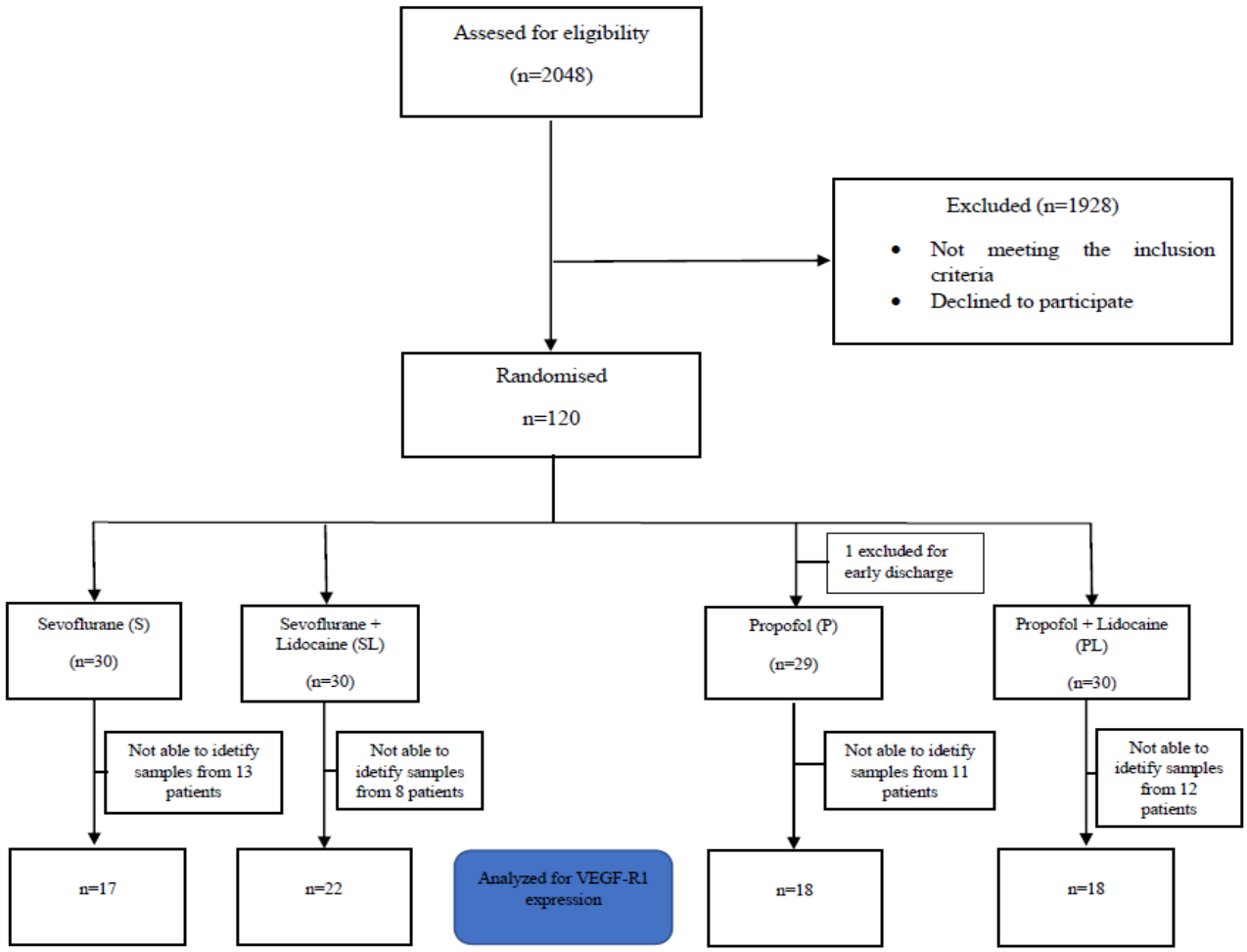
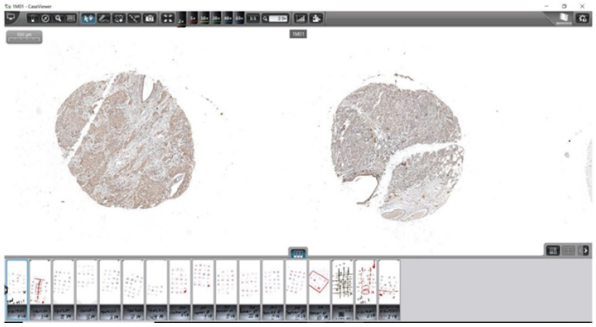


Figure 1. Patient randomisation



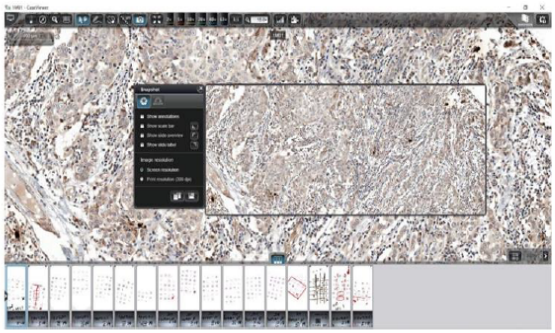


Figure 2. The process of image acquisition and determination of percentage of positivity for VEGF-R1 cytoplasmic expression Figure 2 a. Identification of the tumoral tissue belonging to a patient with the CaseViewer programme

https://imagej.net/ImageJ) (Image J. 2020) (Figure 2, Figure 3) as: high-positive, positive, low-positive and negative (Figure 4). A mean of the 5 acquired results was obtained and considered as the final result for each analysed image. Results were expressed as percents.

Apart from TNM (tumour, nodes, metastasis) staging, Nottingham score was used to evaluate tumoral aggressiveness. Nottingham score relyes on 3 tumoral features that can be identified under microscopy: gland formation, nuclear grade and mitotic count. Each characteristic recieves a grade from 1 to 3, then the grades

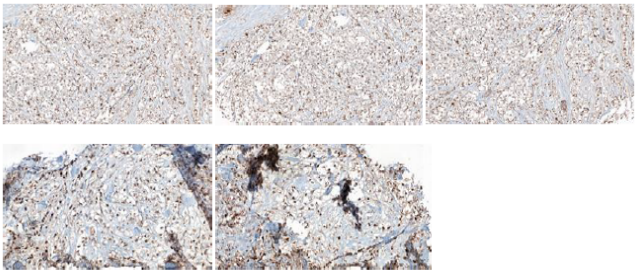


Figure 2b. Selection of 5 regions of the tumoral tissure in order for it to be analyzed

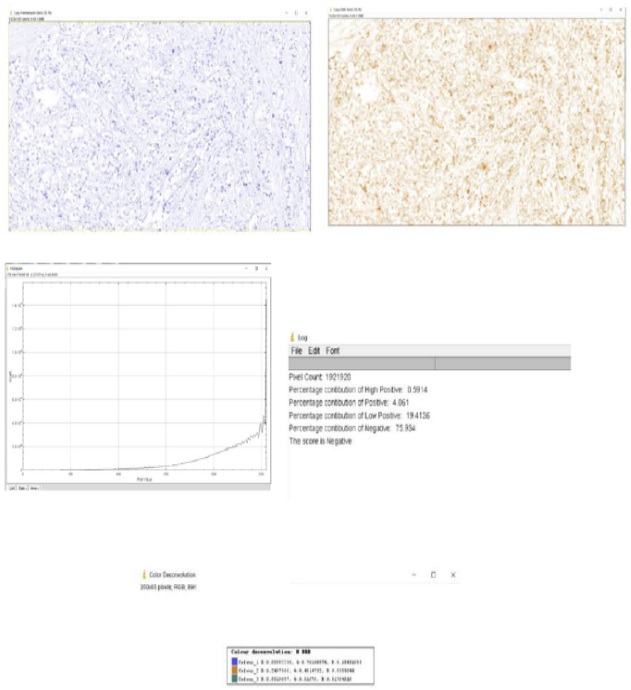


Figure 3. Analysis of immunohistochemical positivity for cytoplasmic VEGF-R1 using ImageJ programme

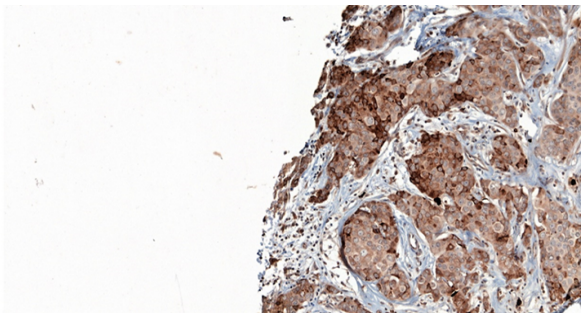


Figure 4. Examples of tumoral tissue staining with different percentages of cytoplasmic VEGF-R1 positivity
Figure 4.a. Tumoral tissue with 3.7% high positive cytoplasmic VEGF-R1 expression:

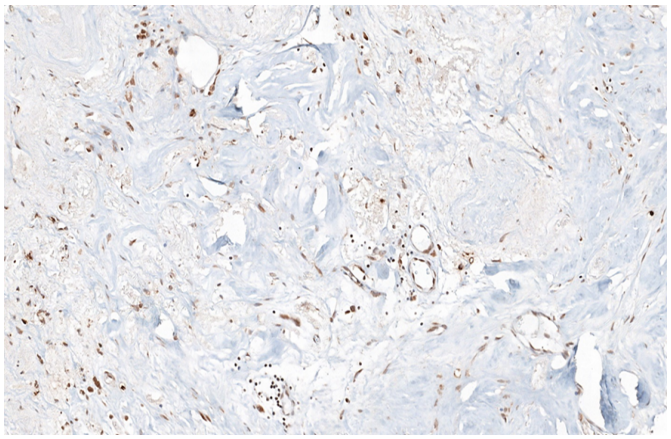


Figure 4.b. Tumoral tissue with a 0.04% high positive cytoplasmic VEGF-R1 expression

are added, resulting the Nottingham Score (Johns Hopkins Medicine Pathology 2021).
The statistical analysis was carried out using the MedCalc Statistical Software version 19.2.1 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2020). Quantitative data was expressed as median and 25-75 percentiles (non-normal distribution). Qualitative data was characterized as frequency and percentage. Comparisons between groups were carried out using Kruskal-Wallis tests. Correlations between variables were tested using the Spearman's rho. A p value <0.05 was considered statistically significant.

Results

Tumour histology was found to be similar throughout the study groups with the majority of cases beeing identified as invasive breast carcinoma NST (no specific type)(Table 2). Because of the inability to identify all patient samples in the pathology service (due to mislabeling / manipulation errors), VEGF-R1 expression was assessed in a smaller number of patients than the initial number of patients per study group (Figure 1 – Patient randomisation). Because all patients enrolled in the study had a breast cancer diagnostic, there were no control groups to compare VEGF-R1 immunohistochemical expression in healty tissue.
The results regarding the primary objective – determining if there is a correlation between VEGF-R1 immunohistochemical expression in the tumoral tissue and serum levels of preoperative proangiogenic factors (VEGF-A, MMP-3, MMP-9) are presented in Table 3. When analyzing these results, we come to the conclusion that there is no significant statistical correlation between the mentioned variables. Higher levels of VEGF-A, MMP-3 and MMP-9 were not associated with a higher cytoplasmic positivity for VEGF-R1 in the tumoral tissue. Because of the short duration of the surgical intervention in the enrolled patients (71.5 and 79 minutes) we did not consider that the type of anaesthesia or its duration can influence the cytoplasmic expression of VEGF-R1. We assumed that VEGF-R1 expression would remain unchanged and can be correlated with preoperative proangiogenic factors in order to evaluate the existence of any connection between their values. Due to financial constraints we restricted our research to VEGF-R1 determination and correlation to serum VEGF-A, MMP-3 and MMP-9, concentrating on the aspect of VEGF-R1's potential prognostic role in diagnostis, follow-up and possible target for new cancer therapies. We expected that the level of the preoperative proangiogenic factors would be correlated with a more stronger positivity of the tumoral tissue for VEGF-R1, given the connection between the action of MMPs, VEGF-A and its receptor. Instead we found no such correspondence, which may be indicative that the studied variables are exerting their effect through other molecular pathways involved in the proangiogenic process.
It would be of further interest to research to evaluate immunohistochemical expression of VEGF-A, MMP-3 and MMP-9 in order to determine if there is a connection between their serum values and tissue expression.
The second objective of the study was to evaluate if VEGF-R1 expression correlates with a higher aggressiveness of the disease and with a worse prognostic for the patients enrolled in the study. When trying to estimate the possibility of recurrence in breast cancer patients, Nottingham score can provide

Table 2. Postoperative tumor histology. Data is expresses as number and percentage of total

Trial groups	Sevoflurane (S) (n=17)	Sevoflurane+ Lidocaine (SL) (n=22)	Propofol (P) (n=18)	Propofol+Lidocaine (PL)(n=18)
Tumor histology				
Invasive breast carcinoma NST (no special type)	13 (76.4)	19(86.3)	13(72.2)	12(66.6)
Invasive lobular carcinoma	0	0		2(11.1)
Zero residual cancer burden	1(5.8)	2(9)	2(11.1)	2(11.1)
***In situ* ductal carcinoma**	2 (11.7)	0	0	0
Micropapillary invasive carcinoma NST	0	0	1(5.5)	0
Invasive cribriform carcinoma	0	1(4.5)	1(5.5)	0
Multifocal cribriform invasive carcinoma	0	0	0	1(5.5)
No available data	1(5.8)	0	1(5.5)	1(5.5)

Table 3. Correlation between VEGF-R1 positivity and seric levels of pro-angiogenic factors (VEGF-A, MMP-3, MMP-9)

	p-value		
VEGF-R1 expression	**Preoperative VEGF-A**	**Preoperative MMP-3**	**Preoperative MMP-9**
High positive	0.673	0.209	0.738
Positive	0.772	0.636	0.878
Low positive	0.353	0.882	0.573
Negative	0.443	0.999	0.636

Table 4. Comparison between VEGF-R1 expression throuought Nottingham scale. Results are expressed as mean, 25th percentile and 75th percentile of the percentage of positivity

VEGF-R1 expression (%)	Grade	% of VEGF-R1 positivity
High positive	Nottingham I	0.48 (0.19;0.75)
	Nottingham II	0.31 (0.23;0,63)
	Nottingham III	0.49 (0.27;0.84)
Positive	Nottingham I	2.67 (1.24;3.41)
	Nottingham II	2.16 (1.47;4.46)
	Nottingham III	3.97 (2.55;6.11)
Low positive	Nottingham I	20.27 (17.30;21.38)
	Nottingham II	14.64 (10.04;20.7)
	Nottingham III	23.33 (19.51;27.48)
Negative	Nottingham I	76.32 (74.58;80.31)
	Nottingham II	82.39 (72.86;88.41)
	Nottingham III	71.71 (67.41;77.04)

Table 5. Correlation between the percentage of VEGF-R1 expression and the rate relapse 1 year after surgery. Results are expressed as mean percentage, 25th and 75th percentile

	No relapse (%)	Relapse (%)	p-value
High positive (%)	0.32 (0.21;0.63)	0.53 (0.24;1.54)	0.364
Positive (%)	2.25 (1.39;3.97)	4.83 (1.40;9.12)	0.230
Low positive (%)	16.97 (10.74;21.71)	21.07 (8.27;31.74)	0.482
Negative (%)	80.08 (72.93;87.54)	73.16 (57.77;90.07)	0.429

an outlook for this process. Due to the fact that the score provides the least inter-observer variability when used for tumoral histological grading it is one of the most accepted systems for determining the risk factor for clinical outcome. Comprising the three pathological findings like the degree of tubular formation, nuclear pleomorphism and mitotic count, it estimates the risk of relapse in patients diagnosed with breast cancer, a higher score being associated with an early recurrence and a shorter survival. We presumed that a higher positivity for cytoplasmic VEGF-R1 in the tumoral tissue would be associated with a higher Nottingham score, thus validating our assupmtion that VEGF-R1 may represent a tumoral marker that can provide informations on the aggressiveness of the disease and possible on the patients' outcome.

The results for the correlation of VEGF-R1's immunohistochemical expression with the Nottingham score are presented in Table 4.

Our study did not find a difference in the distribution of high positive and postive VEGF-R1 expression across Nottingham grades, with the significance that tumors with higher Nottingham grades did not have a higher expression of cytoplasmic VEGF-R1, as expected (Table 4). This result may possibly indicate that VEGF-R1 cannot be used as a marker for estimating the severity of the disease. A worth mentioning result of this analisys is a statistically significant difference in low positive expression of VEGF-R1 observed between Nottingham grade II and III ($p=0.016$) (Table 4). The implication of this result is that a higher percentage of low positive expression is associated with a worse outcome for the patient. Also, the fact that we found a significant difference for negative VEGF-R1 expression between Nottingham grades III and II ($p=0.02$), may imply that the lack of VEGF-R1 expression is linked to a better outcome as expressed through the lower Nottingham scores (Table 4).

We believe that these results are extremly important in shaping other studies. The intricated results indicate that there may be other pathways through which VEGF-A exerts its actions and certainly, larger studies are needed in order to clarify the role of VEGF-R1.

The third aim of the study was to establish if cytoplasmic positivity for VEGF-R1 has a predictive value in patient's prognostic, evaluated through the rate of disease relapse 1 year after the surgical intervention. Our premise was that if higher cytoplasmic positivity for VEGF-R1 were associaterd with a greater rate of reccurence of the disease, this would also be a reflection of VEGF-R1's role in the tumoral progression. The results for this analysis are presented in Table 5. When analyzing the two variables (cytoplasmic positivity of the tumoral tissue for VEGF-R1 and the reate of relapse at 1 year) we did not find any correlations that would support our assumption, the data being in accordance to the ones already obtained through the previours comparisons. Certainly, comparing the expression of VEGF-R1 in normal healty tissue to the pathological one, could help clarify the role of VEGF-R1 and give more insight on the processes involved in tumoral progression and metastasis. Though VEGF-R1 is known for its positive role in regulating angiogenesis processes, our data shows no correlation between its cytiplasmic expression, proangiogenic factors and recurrence rate. When analyzing the Nottingham score and VEGF-R1 immunohistochemical expression, the lack of VEGF-R1 expression seems to be associated with a better prognostic, making the data difficult to interpret and emphasize the need for larger studies that can clarify the role of this receptor in the angiogenesis and tumoral development process.

Discussions

The first objective of the study was to assess the possibility of VEGF-R1 becoming a target for antiangiogenic therapies. As mentioned before, VEGF-R1's role is to mediate the action of VEGF-A, but also the action of other proangiogenic factors: Placental Growth Factor (PlGF), Vascular Endothelial Growth Factor B (VEGF-B) (Shaik et al 2020). Though during early development VEGF-R1 is a negative regulator of angiogenesis, the role of VEGF-R1 remains unclear regarding its subsequent proangiogenic activity, scientific evidence showing proof for both its negative and positive role in the angiogenic process (Silva et al 2021). Because anticancer therapies that target VEGF-A (like bevacizumab) did not show a clear survival benefit (Lebok et al 2016) attention has shifted towards other pathways involved in the process, including receptors VEGF-R2 and VEGF-R1. Therapies that directly target VEGF-R2 or all VEGF-A receptors by the use of TKI have been developed (Madu et al 2020) and their long-term results remain to be evaluated. But because tumoral tissues have acquired resistance to antiangiogenic therapies (Simon et al 2017) new targets are needed in order to efficiently combat proangiogenic processes that support tumoral development. Given its role in tumour associated angiogenesis, VEGF-R1 may prove to be such a target because of its expression in endothelial cells and a variety of tumours (Lacal et Graziani 2018). In order to evaluate the possibility that VEGF-R1 could represent such a target in breast cancer therapy, we determined its cytoplasmic expression in the excised tumoral tissue from the patients that underwent a surgical intervention for breast cancer removal and correlated it with the preoperative level of proangiogenic factors (VEGF-A, MMP-3, MMP-9). We assumed that a high level of proangiogenic factors would be associated with a high VEGF-R1 expression in the tumoral tissue. Our results did not confirm this assumption but the research in this direction proves to be of continuous interest, recent in vitro studies showing that there is a correlation between nuclear positivity for VEGF-R1 and the degree of malignancy of tumoral cells (Tyrsina et al 2018). A former study that researches immunohistochemical expression of VEGF-R1 and VEGF-A, and the correlation between them, states that there is a positive correlation of their expression in tumoral breast cancer tissue and also a correlation between their expression and node negative status (Schmidt et al 2008). Contrasting with this result, there is other evidence that correlate a high tumoral expression of VEGF-A, VEGF-R1 and VEGF-R2 with a worse prognostic in breast cancer patients (Ghosh et al 2008) and in vitro attestation on multiple breast cancer cell lines that VEGF-R1 supports growth and survival of human breast carcinoma (Wu et al 2006). Similar to our findings, Srabovici et al did not find a statistically significant connection between VEGF-R1 expression and the extent of the disease (Srabovic et al 2013), despite a correlation between VEGF-A and VEGF-R1 immunohistochemical expression in the tumoral tissue, stating that the prognostic value of VEGF-R1 cannot be confirmed. They also advocate for studies that explore the correlation between serum levels of VEGF-A

and immunohistochemical expression of VEGF-R1 which we attempted to undertake. A limitation to our study (due to financial restraints) may be represented by the fact that VEGF-R1 expression was not quantified in the surrounding tissue in order to compare its expression to the intratumoral findings. Also, determining the soluble form of VEGF-R1s receptor (in correlation with VEGF-A, MMP-3, MMP-9 and cytoplasmic expression of VEGF-R1) might have proved to bring more insight on the role played by VEGF-R1 in the angiogenetic process.

Because we did not find throughout the literature studies that investigate the association between Nottingham grade - which is a strong predictor of outcome in breast cancer patients (Rakha et al 2008)- and VEGF-R1 expression in the tumoral tissue, the second objective was to analyse if a worse prognostic is associated with a higher receptor expression. Our data showed mixed results: a higher percentage of low positive cytoplasmic VEGF-R1 expression is seen with Nottingham III score, and a lower percentage of negative expression is seen also with Nottingham III score. Bearing in mind that VEGF-R1 is described to have a role in positive regulation of angiogenesis (Shibuya 2006), a lower percentage of negative expression of VEGF-R1 is supportive of the already known data. Tissues that lack VEGF-R1 receptors (higher percentage of negative expression of VEGF-R1), have therefore a better prognostic. Consequently, we would assume that a higher low positive percentage would be associated to Nottingham grades I and II. The results provided by our comparison suggest the contrary, making data difficult to interpret. Possible explanations are that VEGF-A acts through different pathways in order to promote angiogenesis, VEGF-R1 may also play a role in limitation of angiogenetic processes as suggested by recent data (Silva et al 2021) and that there is a need for larger randomized- controlled studies conducted in this direction.

The third objective aimed to identify whether a connection between VEGF-R1 expression and the frequency of disease relapse exists. This would support our premise that VEGF-R1 might have the potential of being a prognostic marker used in the patients' follow-up and a possible target for future therapies. According to our findings, cytoplasmic expression of VEGF-R1 was not associated with a statistically different outcome throughout all study groups. Studies that look into a connection between breast cancer patient's outcome and immunohistochemical expression of VEGF-A receptors are weakly represented but there is some evidence that VEGF-R1 receptor expression is associated with a higher metastatic risk and local recurrence in breast cancer patients (Dales et al 2004). Other studies on VEGF-R1 cytoplasmic expression suggest that VEGF-R1 may indicate a poor prognostic in patients with invasive breast carcinoma (Mylona et al 2007) and some consider that VEGF-R1 detection can develop into a tool for future identification of patients who could benefit from antiangiogenic therapy (Meunier-Carpentier et al 2005). A limit to our study is the short observation time (of 1 year) which of course can be extended to identify more accurately if the possibility of using VEGF-R1 as a prognostic marker exists. New arising data may shed a light on the molecular pathways involved in angiogenesis, giving our results other possible interpretations.

Conclusions

Though there is a further need for studies that investigate VEGF-R1's role, it is noteworthy that the negative expression of VEGF-R1 is associated with a better Nottingham score. Also, the significant difference found between Nottingham scores for low positive expression of VEGF-R1, can imply that even a low percentage of tissue positivity for VEGF-R1 can influence the process of angiogenesis and the outcome of cancer patients. Despite the apparent absence of association between VEGF-R1's cytoplasmic expression and the serum levels of the studied proangiogenic factors (VEGF-A, MMP-3, MMP-9), and between its immunohistochemical expression and the aggressiveness of the disease, we consider that more studies should be developed in this direction. Angiogenesis is a complex process that needs to be further detailed and looked upon from different points of view in order to be fully comprehended.

Though we did not find a prognostic significance for cytoplasmic immunohistochemical expression of VEGF-R1 in breast cancer, further larger studies that imply the evaluation and correlation between the soluble form and immunohistochemical expression of VEGF receptors are necessary to clarify the existing data and to make a thorough assessment of their prognostic value.

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Authors

•Elena Galoș, Department of Anesthesia and Intensive Care, Clinical Hospital CF Cluj-Napoca, "Iuliu Haţieganu" University of Medicine and Pharma, 16-20 Republicii Street Cluj-Napoca, 400015, Romania; e-mail adress: draghiciuelena@yahoo.com

•Rareș Buiga, Department of Pathology, "Prof Dr Ion Chiricuță" Institute of Oncology, "Iuliu Haţieganu" University of Medicine and Pharmacy, 34-36 Republicii Street Cluj-Napoca, 400015, Romania; e-mail adress: raresbuiga@yahoo.fr

•Maja Laura, Department of Pathology, "Prof Dr Ion Chiricuță" Institute of Oncology, 34-36 Republicii Street, Cluj-Napoca, 400015, Romania, e-mail adress: laura_maja32@yahoo.com

•Tiberiu Tat, Department of Anesthesia and Intensive Care, "Prof Dr Ion Chiricuță Institute of Oncology", 34-36 Republicii Street Cluj-Napoca, 400015, Romania; e-mail adress: dr_tibi@yahoo.com

•Răzvan Popa : Department of Anesthesia and Intensive Care, "Prof Dr Ion Chiricuță Institute of Oncology", 34-36 Republicii Street Cluj-Napoca, 400015, Romania; e-mail adress: p.razvan@gmail.com

•Anca Ciurea, Department of Radiology, "Iuliu Hatieganu" University of Medicine and Pharmacy; Mammography Department, Emergency Clinical County Hospital Cluj, 3-5 Clinicilor Street, Cluj-Napoca, Romania; e-mail adress: ancaciurea@hotmail.com

•Ștefan Cristian Vesa, Department of Pharmacology and Toxicology, "Iuliu Haţieganu" University of Medicine and Pharmacy, 8 Babeș Street, 400012, Cluj-Napoca, Romania; e-mail adress: stefanvesa@gmail.com

•Daniela Ionescu, Department ofAnaesthesia and Intensive Care "Iuliu Haţieganu" University of Medicine and Pharmacy, 8 Babeș Street, 400012, Cluj-Napoca, Romania; e-mail adress: dionescuati@yahoo.com

•Carmen Mihaela Mihu, Department of Histology and Morphological Sciences , "Iuliu Haţieganu" University of Medicine and Pharmacy, 8 Babeș Street, 400012, Cluj-Napoca, Romania; e-mail adress: carmenmihu@umfcluj.ro

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