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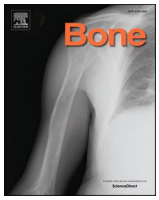

Full Length Article

Expression of RANKL in breast cancer tissue in patients with fibrous dysplasia/McCune-Albright syndrome

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ABSTRACT

Background: In fibrous dysplasia/McCune-Albright syndrome (FD/MAS), mosaic mutations in the *GNAS* gene lead to locally abnormal bone turnover. Additionally, patients with FD/MAS, particularly with thoracic lesions, have an increased risk for breast cancer. Development and progression of breast cancer has been associated with expression of Receptor Activator of NF-κB ligand (RANKL) in mammary tissue, and due to the *GNAS* mutation, RANKL is systemically increased in patients with FD/MAS. Yet it is unknown whether breast cancer in FD/MAS is also dependent on RANKL. We hypothesized that the *GNAS* mutation might induce RANKL overproduction and an oncogenic niche in mammary tissue, and examined RANKL expression in breast cancer tissue of patients with FD/MAS compared to controls.

Methods: Nine patients with FD/MAS and breast cancer were included and clinical data were retrieved. Patients were matched to controls with breast cancer without FD/MAS based on age and tumor type. Three pregnant breast cancer patients were included as positive controls. Immunohistochemical detection of RANKL was performed on formalin-fixed paraffin-embedded breast cancer specimens. Staining intensity was classified as weak, moderate or intense. The area of positive RANKL staining divided by the total ductal-lobular area was assessed (positive area percentage, PAP). Number of patients with RANKL expression was compared between FD/MAS and control group by chi-square (χ^2) test, the PAP by Mann-Whitney *U* test (MWU).

Results: RANKL expression was observed in 3 patients with FD/MAS (38 %), mainly in healthy tissue, and none of the control patients (χ^2 $p = 0.055$). The FD/MAS group demonstrated considerably more intense staining than the control group, comparable to positive controls. The median PAP was 0.64 % (range 0.14–2.04 %) in the 3 FD/MAS patients with RANKL expression, 0.01 % (Q1-Q3: 0.0003–0.514 %) in the entire FD/MAS group, 0.006 % (Q1-Q3: 0.001–0.012 %) in the control group (MWU = 0.574), and 0.19 % (0.08–0.32 %) in the pregnant patients. All patients with FD/MAS and RANKL expression had thoracic bone lesions, but no correlation was observed between RANKL expression and presence of the *GNAS* mutation or FD disease burden.

Conclusions: The triad of a higher number of patients, higher positive area percentage and stronger intensity in the FD/MAS compared to the control group indicates that RANKL may be upregulated in mammary tissue in a subset of patients with FD/MAS, which may explain the increased risk for breast cancer, although the clinical significance remains unclear. Further research is needed to establish risk profiles for the development of RANKL-positive breast cancer and to improve early screening and treatment.

1. Introduction

Fibrous dysplasia/McCune-Albright syndrome (FD/MAS) is a rare genetic bone disease with variability in phenotype and severity caused by a postzygotic, mosaic mutation in the *GNAS* gene [1]. The mutation can occur in each of the three germ lines and knows an extensive list of possibly affected tissues [2]. Most commonly affected is the skeleton, where dysplastic bone lesions may cause pain, nerve entrapment, fractures or malformations [1,2]. Next to bone lesions, extraskeletal manifestations of the *GNAS* mutation comprise endocrinopathies in MAS or

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intramuscular myxomas in Mazabraud's syndrome (MZB). Moreover, several benign and malignant tumors are more frequently observed in patients with FD/MAS compared to the general population [2,3]. A recent study from our group indicated that patients with FD/MAS have an increased risk for breast cancer at a younger age, especially if lesions affect the thoracic region [4]. In the investigated cohort 44 % of the breast cancer tissues showed a *GNAS* mutation, in contrast to 1 % of breast cancers in the general population. Since the mutation was not found in all cancer tissues, we aimed to explore other underlying mechanisms.

In patients with FD/MAS circulating levels of Receptor Activator of NF-κB ligand (RANKL) are 16-fold higher compared to controls [5]. A member of the tumor necrosis factor family, RANKL interacts with its receptor RANK on osteoclast progenitor cells and stimulates osteoclast differentiation and survival. RANKL may also bind to osteoprotegerin (OPG), a soluble decoy receptor competing with RANK [6]. In the last decade it has been discovered that the RANKL/RANK/OPG triad is not only involved in bone metabolism, but also in other physiological processes, such as the immune system, reproductive system and thermoregulation [7,8]. In addition, RANKL is detected in neoplastic tissues of the prostate, breast, bone marrow, kidney, bone and liver [7–9]. Excess levels of RANKL are thought to induce hyperplasia, chemoattraction of tumor cells, neovascularization and epithelial-mesenchymal transition, promoting tumor formation and metastasis [7–12]. Specifically, the female reproductive system and bone metabolism share RANKL-dependent features. Sex hormones are major regulators of RANKL expression in bone, by osteoblasts and bone marrow stromal cells resulting in bone resorption, as well as in the breast, by hormone receptor positive (HR+) mammary epithelial cells during pregnancy and estrous cycles, resulting in proliferation, differentiation, migration, expansion and survival of mammary stem cells in order to develop functional lobulo-alveolar structures [13–17]. However, RANKL (over) expression is also involved in mammary epithelial-mesenchymal transition and in cancer development [18,19]. OPG/RANKL/RANK expression has been demonstrated in luminal epithelial cells in lobules and ducts of normal breast tissue, in situ tumors and infiltrating tumors, both hormone positive (HR+) and negative (HR-) types [20,21]. In general, RANKL expression is higher in normal breast tissue compared to neoplastic tissue [21–23] and higher in pregnant women [24,25]. RANKL overexpression has also been associated with the presence of osteoclast-like giant cells (OGC) in breast cancer [26]. High serum levels of RANKL and high RANKL/OPG ratio seem to predispose for the development of HR+ [27,28] but not HR- cancer [28], particularly in women diagnosed at older ages [28], with progression of breast cancer [27], positive lymph nodes [29], presence of disseminating tumor cells [27,29], and osteolytic bone metastasis [29], the latter which can be attenuated by sequestering RANKL with Denosumab treatment [30–34].

These data highlight local RANKL expression as a risk factor for the development of breast cancer, next to previously known risk factors including advanced age, Caucasian race, genetic predisposition, or high exposure to progesterone/progestins in women with early menarche, late menopause, late childbearing, or hormone replacement therapy [35]. In addition high serum levels of RANKL may be associated with poor prognosis, next to young age, advanced stage, absence of hormone receptors, or presence of the HER2neu receptors [36]. We postulate that in patients with FD/MAS the *GNAS* mutation might create an oncogenic niche in mammary tissue under the influence of RANKL (over)production, and hypothesize that this interplay may link the skeletal consequences of FD/MAS to the increased breast cancer risk. To explore this relationship, we examined the expression of RANKL in breast cancer tissue of patients with FD/MAS compared to controls.

2. Methods

2.1. Subjects

10 Dutch patients with FD/MAS and breast cancer included in this study were described previously by Majoor et al. [4] Clinical patient data were collected on FD/MAS-related features including age of diagnosis of FD/MAS, type of FD/MAS, skeletal burden score (SBS) and localization of FD lesions, as well as on breast cancer-related characteristics including age at diagnosis, type of breast cancer, stage, type of therapy, evidence of progression and survival. Paraffin embedded pathological specimens were retrieved from the hospitals where the breast cancer surgery was performed. A control group consisted of female patients without FD/MAS, diagnosed with breast cancer in the Leiden University Medical Center (LUMC) and matched based on breast cancer type and age of diagnosis. Breast cancer tissues of 3 pregnant woman without FD/MAS were used as positive controls, since expression is increased during pregnancy [24]. The LUMC pathology department selected cases of both control groups anonymously and provided coded paraffin embedded pathological specimens. Hence no clinical data were available for both control groups, and no data on hormone receptor status were shared by the pathology department for the purpose of this study, although these data had been gathered for clinical purposes. All patients in the FD/MAS group had given consent as previously reported [4] and approval for the present analysis was obtained by the Medical Ethics Committee (protocol numbers G16–026 and P17–136).

2.2. Immunohistochemical detection of RANKL

Hematoxylin/Eosin stained sections were screened for the presence of tumor tissue by an experienced pathologist. Of subjects with multiple available paraffin blocks, both specimens with healthy breast tissue and with tumor tissue were selected. The blocks were sectioned (5 μm) and RANKL expression was detected by immunochemistry. Sections were rehydrated and endogenous peroxidase was quenched with 0.3 % H_20_2/dH_2O. Antigen retrieval was performed by incubation with 10 % citrate buffer/dH_2O for 30 min at 100 °C. After blocking non-specific binding sites with 5 % BSA/PBS for 30 min, the sections were incubated overnight at 4 °C with primary anti-RANKL antibody M366 (Amgen®), diluted 1:1000 in 1 % BSA/0.1 % Tween/PBS. Next, the sections were incubated 30 min. at room temperature with the secondary antibody DAKO EnVision™ anti-mouse with 5 % normal human serum. Finally, for color development the sections were incubated with DAB or with Nova Red and counterstained with Hematoxylin. Sections of both methods (DAB and with Nova Red) were scanned and analyzed in Qupath software, v.0.2.3 (Queen's University, Belfast, Northern Ireland) [37]. The staining was consistent across both methods using DAB or Nova Red.

2.3. Specialized analysis of RANKL expression

A pathologist estimated the percentage of lesional and of healthy tissue per section and within the RANKL positive areas. The total area of ducts and lobules (both healthy and lesional) was indicated as region of interest (ROI) using the polygon annotation tool. Similarly, the region of positive cytoplasmic RANKL staining was circled. RANKL positive area is expressed as RANKL positive cytoplasmic stained region/ROI in % and is further termed positive area percentage. Intensity of RANKL expression was assessed qualitatively and was for each polygon categorized as weak (barely positive), moderate and strongly positive (very clear and intense). Quality control of the positive area percentage of RANKL was performed by a second observer, confirming good to excellent interrater reliability with an intraclass correlation coefficient of 0.958 (95 % CI 0.709–0.995). In order to reduce false positivity a threshold of 0.02 % was set for the positive area percentage. Specimens with percentage area of expression below the threshold were considered negative. All

specimens with RANKL expression were screened for the presence of osteoclast-like giant cells (OGC).

2.4. Statistical analysis

Clinical characteristics were summarized as number and percentage or as median (Q1-Q3). The number of patients with RANKL expression was compared between the FD/MAS group and control group by Chi Square test. Interrater reliability of the positive area percentage was calculated by intraclass correlation coefficient based on two-way random effects model, absolute agreement, single rater. The positive area percentage was calculated for each subject, summarized as median (quartile 1 – quartile 3), and compared between the FD/MAS group and control group by Mann-Whitney *U* test. Characteristics of FD/MAS patients with RANKL tissue expression were compared with the entire FD/MAS group, including FD/MAS disease extent, presence of extraskeletal manifestations, tumor aggressiveness and prognosis, receptor status and additional mutations.

3. Results

3.1. Clinical characteristics

The FD/MAS group consisted of 10 patients (Table 1). Of 1 patient breast specimens were not available anymore (case 10) and this patient was excluded from the matching procedure. The remaining 9 patients were diagnosed with ductal carcinoma in situ (DCIS) ($n = 4$, 44 %), of which 1 within a papilloma, or with infiltrating ductal carcinoma ($n = 5$, 56 %) and were matched accordingly to controls. After tissue analyses (see below) one FD/MAS patient (case 9) and one control patient (case 19) appeared to have sections of insufficient quality. These patients were excluded from analyses and are shown in Tables 1 and 2 in gray. Of the remaining 8 patients with FD/MAS, all had polyostotic disease and in 6 patients the thoracic area was affected (75 %). 3 patients had additional myxomas (38 %) and 3 were diagnosed with MAS (38 %). Median age at diagnosis of breast cancer was 49.5 years (range 32–54 years). Positive controls were affected with infiltrating ductal carcinoma ($n = 1$, 33 %) or DCIS ($n = 2$, 67 %). These abnormalities will be further referred to as lesional tissue.

3.2. Tissue expression

RANKL expression with a positive area percentage above the threshold of 0.02 % was observed in 3/8 patients with FD/MAS (38 %) (pt. no. 1, 2 and 3), as opposed to none of the controls (χ^2 $p = 0.055$) (Table 2). The observed RANKL staining was mainly located in healthy tissue. In positive controls (pregnant women) all RANKL expression was observed in normal ductal or lobular cells (Fig. 1a). In the FD/MAS group, the ratio of healthy to lesional tissue within the RANKL-positive area was 60:40 for case 1, 85:15 for case 2 and 95:5 for case 3 (Figs. 1b). RANKL staining was absent in controls (patients with breast cancer without FD/MAS) (Fig. 1c), in both healthy and lesional tissue. The estimated percentages of lesional tissue on the total ROI for all patients are noted in Table 2. The RANKL staining in the FD/MAS group was considerably more intense than in the control group and was comparable to the positive controls: of the total area of RANKL expression in all patients with FD/MAS combined, 52 % was estimated to be of weak, 31 % intermediate and 17 % strong intensity, whereas in the control group, the expression (although minimal, with positive area percentage below the threshold of 0.02 %) was estimated as 93 % weak, 7 % intermediate and 1 % strong intensity, and in the positive controls 30 %, 43 % and 27 % respectively. In case 3, entire lobules were stained intensely positive (Fig. 1b). Such intense and focal expression was not observed in tissue of positive controls, where expression was more scattered through the healthy lobular tissue (Fig. 1a), nor in controls. In patients 1 and 2 with FD/MAS the expression was also scattered. In the 3 FD/MAS patients with RANKL expression the median positive area percentage was 0.64 % (range 0.14–2.04 %). In the entire FD/MAS group the median

Table 1
Patient characteristics.

Patient ID	Age diagnosis FD (y)	FD type and SBS	Thoracic localization FD	GNAS mutation FD	Age diagnosis breast cancer (y)	Breast cancer stage at diagnosis	Risk factors breast cancer	(history of) Additional tumors
1	<1	MAS+MZB 24.7	Yes	NA	32	DCIS	Menarche 0 y, 12 y OCP, late pregnancy 38 y	IPMN, FNH Thyroid cysts
2	49	PFD 3.3	Yes	NA	52	T2NmicM0	Late pregnancy 36 y, family +	Parathyroid adenoma, thyroid nodules
3	3	PFD NA, spine/femur	Yes	NA	37	T2N0M0	2x IVF, no pregnancy	-
4	24	PFD+MZB 16.8	Yes	R201H	54	DCIS Gr 3	Breast cysts	Multinodular goiter
5	58	PFD 16.1	Yes	R201C	50	DCIS Gr 3	30 y OCP	-
6	2	MAS 70.6	Yes	R201C	48	T2N1M0	Menarche 7 y, no children	-
7	50	MAS+MZB 15.8	No	NA	49	T2N1M0	Menarche 7 y, family +, no children, smoking	IPMN
8	56	PFD 3.6	No	NA	50	T2N1M0	NA	-
9	<1	MAS+MZB 61.0	Yes	R201C	37	DCIS Gr 2	Menarche 0 y, smoking, no children	IPMN, adrenal adenomas, ovarian cysts
10	16	PFD 27.1	Yes	R201H	52	T3N1M0	20 y OCP, Menarche 11 y	-

Abbreviations: FD: fibrous dysplasia, SBS: skeletal burden score, PFD: polyostotic fibrous dysplasia, MAS: McCune-Albright Syndrome, MZB: Mazabraud syndrome, DCIS: ductal carcinoma in situ, NA: not available, OCP: oral contraceptive pill, IVF: in vitro fertilization, IPMN: intraductal papillary mucinous neoplasm, FNH: focal nodular hyperplasia.

Table 2
Breast cancer tissue characteristics and RANKL expression.

Patient ID	Group	Tumor type	% region of RANKL expression on total ROI	% of lesional tissue on total ROI	Type of RANKL positive tissue (%)	Receptor status	GNAS mutation	Other genes and type of mutation
1	FD/MAS	DCIS in papilloma	0.64	70	Healthy 60% Lesional 40%	ER/PR +, Her2/neu NA	NA	NA
2	FD/MAS	Infiltrating ductal carcinoma	0.14	40	Healthy 85% Lesional 15%	ER/PR +, Her2/neu +	No	PIK3CA: H1047A
3	FD/MAS	Infiltrating ductal carcinoma	2.04	75	Healthy >95% Lesional <5%	ER/PR -, Her2/neu +	No	ERBB2: L755S PIK3CA: H1047A TP53: A248G
4	FD/MAS	DCIS	<0.02	80	-	ER/PR -, Her2/neu +	No	PIK3CA: G545G
5	FD/MAS	DCIS	<0.02	0	-	ER/PR +, Her2/neu -	Yes	GNAS: R201C
6	FD/MAS	Infiltrating ductal carcinoma	<0.02	50	-	ER/PR +, Her2/neu -	No	PIK3CA: G545L
7	FD/MAS	Infiltrating ductal carcinoma	<0.02	0	-	ER/PR +, Her2/neu -	Yes	GNAS: R201H PIK3CA: H1047A
8	FD/MAS	Infiltrating ductal carcinoma	<0.02	75	-	ER/PR +, Her2/neu NA	No	none
9	FD/MAS	DCIS	NA	NA	-	ER/PR +, Her2/neu -	Yes	GNAS: R201C AKT1: G17L
10	FD/MAS	Infiltrating ductal carcinoma	NA	NA	-	ER/PR +, Her2/neu -	NA	NA
11	Control	Infiltrating ductal carcinoma	<0.02	50	-			
12	Control	Infiltrating ductal carcinoma	<0.02	95	-			
13	Control	Infiltrating ductal carcinoma	<0.02	60	-			
14	Control	Infiltrating ductal carcinoma	<0.02	80	-			
15	Control	DCIS	<0.02	75	-			
16	Control	DCIS	<0.02	60	-			
17	Control	DCIS	<0.02	50	-			
18	Control	DCIS	<0.02	90	-			
19	Control	Infiltrating ductal carcinoma	NA	NA	-			
20	Positive control	Infiltrating ductal carcinoma	0.08	70	Healthy			
21	Positive control	DCIS	0.19	40	Healthy			
22	Positive control	DCIS	0.32	20	Healthy			

Abbreviations: ROI: region of interest. NA: not available. ER: estrogen receptor, PR: progesterone receptor, Her2/neu: Human Epidermal Growth factor Receptor.

percentage was 0.01 % (Q1-Q3 0.0003–0.514 %) and in the control group 0.006 % (0.001–0.012 %) ($p = 0.574$). All 3 positive controls showed convincing RANKL expression with median positive area percentage 0.19 %, range 0.08–0.32 % (Fig. 2). No OGC were found in any of the specimens with RANKL expression.

3.3. Clinical characteristics related to RANKL expression

3.3.1. Tumor stage/aggressiveness

Two out of 3 patients (67 %) with RANKL expression had breast cancer at young age (32 years and 37 years). Despite this young age, their RANKL-positive tumors were not larger than T2 and not disseminated to lymph nodes or bones. The remaining RANKL-positive patient was diagnosed with T2NmicM0 breast cancer at age 52. In the total FD/MAS group the median age at diagnosis of breast cancer was 49.5 years (Q1-Q3 40–52) and positive lymph nodes were present in 4 out of 5 patients (80 %) with infiltrative tumors (Table 1). Survival was 100 % and no recurrence or metastases were observed.

3.4. Receptor status

Hormone receptor status was positive for 2 (67 %) and negative for 1 patient (33 %) with RANKL expression. Her2/Neu receptor status was positive in 2 and missing in 1 patient. In the total FD/MAS group ER/PR positivity was observed in 6/8 patients (75 %) and Her2/neu positivity in 3 of the 6 analyzed patients (50 %) (Table 2).

3.5. Genetic mutations

2 of 3 RANKL-positive patients were screened for the presence of the mutations in the breast specimens. In both patients the *GNAS* mutation was absent, but PIK3CA mutations were observed (Table 2). The patient with the highest positive area percentage (pt. 3) had additional mutations in the TP53 gene and the ERBB2 gene along with Her2neu positivity. In the total FD/MAS group the *GNAS* mutation was detected in breast cancer tissue of 2/7 patients (29 %) and PIK3CA mutations in 5/7 patients (671 %).

3.6. FD disease extent

All 3 patients with mammary RANKL expression had FD bone lesions in the thoracic area, while in the total FD/MAS group 6/8 (75 %) had thoracic lesions. Disease burden was variable: pt. 1 had a skeletal burden score of 24.7 and pt. 2 of 3.3 (Table 1). In case 3 no bone scan was

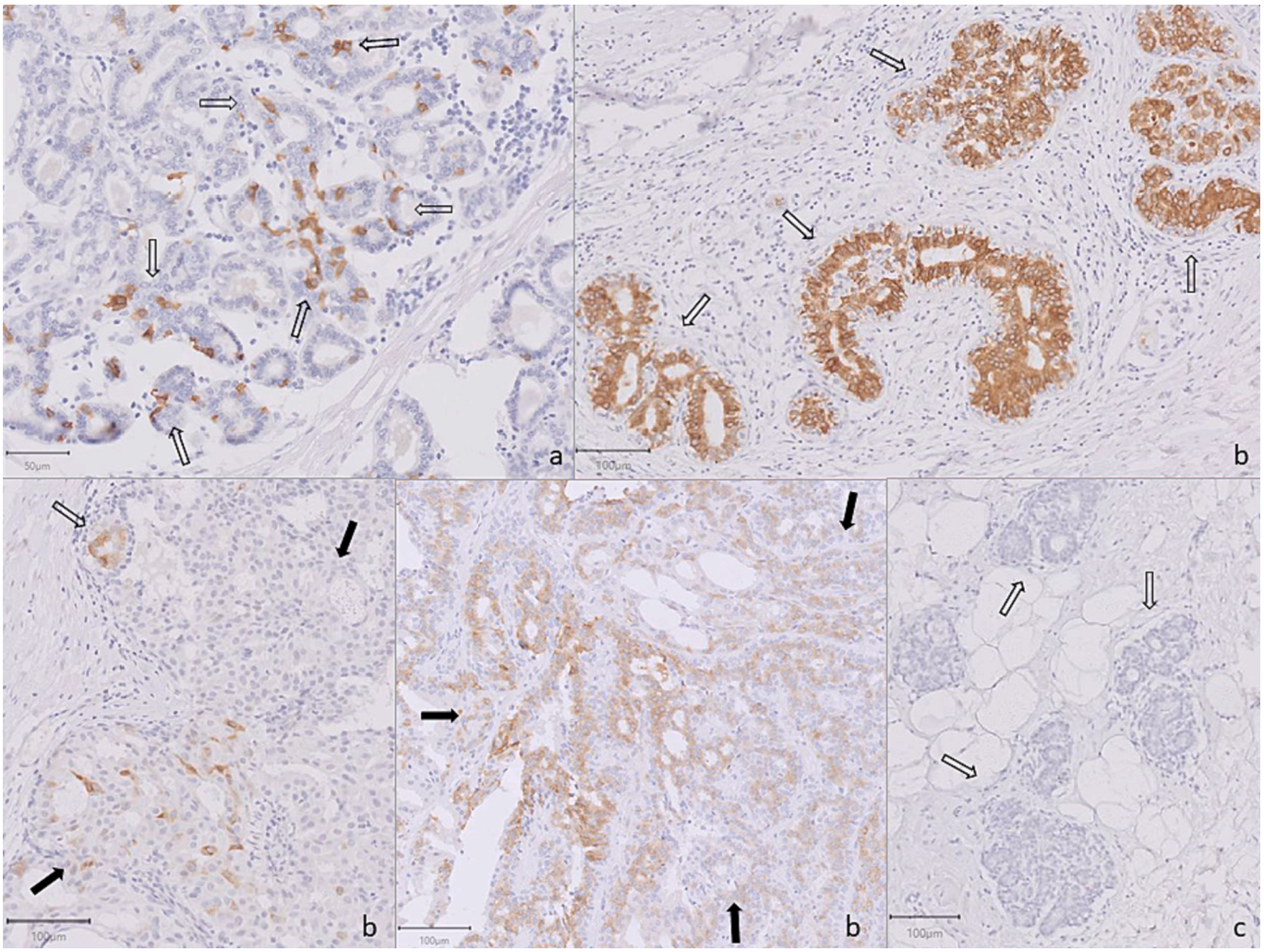

Fig. 1. Immunohistochemical detection of RANKL in breast cancer tissue from a. pregnant women, b. patients with FD/MAS and c. breast cancer patients without FD/MAS. Open arrows indicate normal breast lobules and closed arrows indicate lesional breast tissue. RANKL expression is shown in brown. All sections were counterstained with haematoxillin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

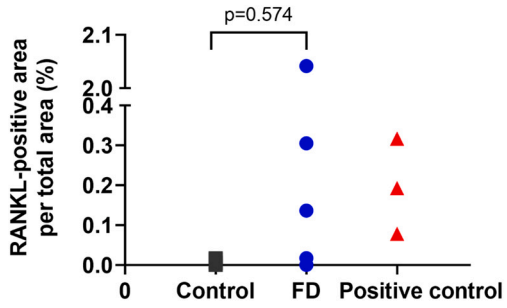

Fig. 2. Percentage of RANKL-positieve area per total area in breast cancer patients without FD/MAS (control), in breast cancer patients with FD/MAS (FD) and in pregnant breast cancer patients (positive control). Patients with FD/MAS tended to have a higher percentage of RANKL-positive area per total area.

performed, but apart from the clinically relevant and radiographically confirmed lesions in the femur and thoracic/lumbar spine, no signs of other lesions were present. In the total FD/MAS group the median SBS was 16.1 (Q1-Q3 3.6–24.7). 1 out of 3 patients (33 %) with RANKL expression was affected with extraskeletal manifestations of FD/MAS (MAS and MZB) versus 4 out of 8 patients (50 %) in the entire FD/MAS group (Table 1). None of the patients had additional malignant tumors. 2 of 3 (67 %) RANKL-positive patients were diagnosed with other benign or premalignant tumors: case 1 had intraductal papillary mucinous neoplasms (IPMN) of the pancreas, focal nodular hyperplasia (FNH) of the liver and thyroid cysts. Pt. 2 was diagnosed with parathyroid adenoma and thyroid nodules. Pt. 3 did not have additional tumors or cysts. In the total FD/MAS group 4 out of 8 patients (50 %) had benign or premalignant tumors, specified in Table 1.

4. Discussion

The aim of this study was to detect RANKL expression in breast cancer tissue of patients with and without FD/MAS, to explain the increased risk for breast cancer in FD/MAS. In our study, RANKL expression was detected in 38 % of the patients with both FD/MAS and ductal breast cancer but in none of the control breast cancer patients. These results indicate that RANKL may be expressed in a subset of patients with both FD/MAS and breast cancer. The proportion of RANKL-positive specimens in the FD/MAS group falls within the range reported in literature, that described RANKL expression in a range of 6–78 % of breast cancer tissues [14,20,22,24,26,38–41]. This wide range may be explained by differences in methodological aspects between studies or in patient characteristics. Expression of RANKL is in both healthy and malignant tissue dependent on serum progesterone levels [18,24,25]. In healthy tissue RANKL expression ranges from 0 to 100 % [18,22,24].

Surprisingly, no RANKL expression was detected in any of the mammary tissues from the breast cancer patients without FD/MAS, though in literature at least 6 % of breast cancer cases were classified as positive for RANKL [14,20,22,24,38–41]. This discrepancy between our findings and literature might be explained by different histomorphometric measurement protocols, although most studies did not describe these details. We did observe small areas of weak and non-convincing staining in several tissues. To increase the specificity (avoid false positive findings) we used a threshold and did not include these as positive in our data, explaining the observed differences. Furthermore, the limited number of included patients may prevent detection of controls with RANKL expression.

In patients with both FD/MAS and breast cancer, merely a small area of ductal lobular tissue expressed RANKL, with a remarkably low positive area percentage ranging from 0.14 to 2.04 %. This was similar to pregnant breast cancer patients and still higher than patients with breast cancer but not FD/MAS, indicating that only a subset of ductal or lobular cells express RANKL. Since this information was not mentioned in previous literature, we could not establish whether the positive area percentage measured in our patients was deviating from other studies. A recent study on OGC in breast cancer demonstrated a percentage of positive tumor cells in specimens with RANKL expression ranging from 10 % in the group with invasive cancer without OGC to 52 % in the group with OGC [26]. Although different methods were used to quantify RANKL expression, the results are in line with our study, with small areas of positive cells in absence of OGC. Besides a higher positive area percentage, also the intensity of the RANKL expression was much stronger in patients with FD/MAS compared to the incidental, single-cell expression observed in controls, and comparable to the intensity observed in the positive control group of pregnant women. The pattern of overexpression of RANKL is not homogenous across patients with FD/MAS and breast cancer, a finding which might be related to the mosaicism of the mutation, although this cannot be explained with our findings. Yet, this does not detract from the conclusion that RANKL may be expressed in a subset of patients with FD/MAS. The triad of a higher number of patients, higher positive area percentage and stronger intensity in patients with FD/MAS compared to controls indicates a higher number of RANKL-positive cells as well as an increased production of RANKL by these cells, thus supports the hypothesis of RANKL overexpression in FD/MAS.

In this study, all 3 RANKL-positive patients showed additional risk factors such as: early menarche, late or no pregnancy, positive family history, or mutations in the PIK3CA, ERBB2 or TP53 genes. These risk factors in combination with the upregulation of RANKL could trigger the creation of an oncogenic niche. *GNAS* mutated breast tissue may, through paracrine signaling, alter the breast microenvironment in favor of tumor formation. In mice it has been observed that RANKL overexpression caused impaired differentiation, hyperplasia of the mammary epithelia and decreased apoptosis [42–44]. These events contribute to tumorigenesis, both in absence of oncogenic stimuli as well as in the presence of carcinogenic agents or hormonal stimulation, for which RANKL acts as mediator [14,17,43]. Conversely, RANKL/RANK inhibition attenuated and delayed tumor development in mice [14,17,27,43,45]. Similarly in patients with FD/MAS this oncogenic niche may allow tumors to develop in presence of other triggers as second hit, including concurrent mutations or hormonal stimulation [46]. This hypothesis could however not be extended to tissues outside of the breast, as no other malignant tumors were observed in our cohort apart from breast cancer. In addition to this paracrine effect, an endocrine effect of circulating RANKL secreted from bone cannot be ruled out, which could similarly induce an oncogenic niche [47].

Notably, our results showed that RANKL expression was observed merely in healthy tissue and only a minor proportion in lesional tissue, in papilloma or DCIS tissue, but not in ductal infiltrating components. Similarly in mammary tissue from pregnant breast cancer patients all positive cells were located in healthy tissue. In control tissue of breast cancer patients without FD/MAS neither healthy nor lesional tissue expressed RANKL, while both types of tissue were present in all sections. We had expected to observe more RANKL expression in lesional tissue, as our hypothesis was that RANKL would play a role in the development of breast cancer in patients with FD/MAS, though the results seem to be consistent with literature, where in comparative studies RANKL expression was higher in normal tissue compared to lesional tissue [22–24]. The oncogenic niche appears to be initiated by healthy RANKL-positive tissue, and the paracrine effects including (pre)malignant change seems to occur in RANKL-negative ductal or lobular cells. In this way malignant crosstalk may occur, leading to increased initiation or survival of cancer cells.

In the patients with FD/MAS the clinical implications of RANKL expression in healthy tissue for prediction, prognosis or treatment success remain unclear. In this study, RANKL expression was only observed in patients with thoracic FD lesions, although not all patients with thoracic FD lesions expressed RANKL. Surprisingly no correlation was observed between RANKL expression and the magnitude of (extra) skeletal involvement in FD/MAS or with the presence of the *GNAS* mutation. From a developmental point of view patients with FD/MAS with a more severe subtype of FD, a higher skeletal burden score, and thus more *GNAS*-mutated tissue, are expected to have a higher chance for the *GNAS* mutation as well as RANKL expression to be located in breast tissue, but our results do not support this assumption. The theory of an oncogenic niche could not account for the absence of RANKL expression in *GNAS*-positive breast cancer tissues or vice versa. RANKL expression was observed locally and scattered through the tissue and *GNAS* mutations are also known to exhibit a mosaic pattern. Since different sections were used for both the RANKL and *GNAS* analyses, the detection of simultaneous expression could have been missed. Alternatively, currently unknown mechanisms independent from RANKL may be more important contributors to breast cancer in patients with FD/MAS. In addition, no correlation was observed between RANKL expression and other clinical parameters including breast tumor type, receptor status or tumor aggressiveness. This was remarkable, as in in patients with FD/MAS increased RANKL serum levels are observed [5] and increased RANKL are associated with poor prognosis in non-FD/MAS breast cancer patients. However the absence of this association is in line with our results demonstrating expression in healthy and not in malignant tissue and with our hypothesis that RANKL is responsible for an oncogenic niche, but for tumor progression or metastasizing.

A limitation of our study is the small sample size, a common difficulty in research into rare diseases. Moreover, data on mammary RANKL expression in patients with FD/MAS without breast cancer are lacking. Such healthy tissue was not available, but would provide valuable information. In addition, serum levels of RANKL and tissue expression of RANK or OPG were not determined. These were regarded as less relevant because of the known upregulation of skeletal RANKL in FD/MAS, but may be addressed in future research. Nonetheless, our observational study is the first to investigate possible links between FD/MAS and the development of breast cancer. Other strengths include the quantitative aspect of the measurements, high intraclass correlation coefficient, staining of both lesional and healthy breast tissue, and the use of specimens from pregnant women as positive controls, which confirmed the reliability and validity of our staining method.

Yet several questions remain unanswered. The clinical significance of mammary RANKL expression once the diagnosis of breast cancer has been established needs to be elucidated. More research is needed to establish a risk profile for the development of RANKL-positive breast cancer, which may benefit screening and treatment in an early stage. The potential role of the RANKL-inhibitor Denosumab may be addressed, a drug available for treatment of the skeletal consequences of FD/MAS [48,49], as this might be prescribed instead of bisphosphonates for symptomatic patients with FD/MAS at risk for or diagnosed with breast cancer. Lastly, in vitro studies may elucidate the pathophysiological mechanisms underlying the development of RANKL-driven

breast cancer in FD/MAS. Nevertheless, this explorative study comprises an important contribution to the understanding of the extraskeletal manifestations of FD/MAS. We conclude that RANKL expression is upregulated in healthy mammary tissue in a subset of patients with FD/MAS and breast cancer, particularly in patients with thoracic lesions, which may contribute to breast cancer formation.

CRediT authorship contribution statement

M.E. Meier: Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **M. Hagelstein-Rotman:** Investigation, Resources, Writing – review & editing. **B.C.J. Majoor:** Investigation, Resources, Writing – review & editing. **R.E.S. Geels:** Investigation, Validation, Writing – review & editing. **N.M. Appelman-Dijkstra:** Conceptualization, Methodology, Resources, Writing – review & editing, Visualization, Supervision, Funding acquisition, Project administration. **N. Bravenboer:** Conceptualization, Methodology, Validation, Writing – review & editing, Visualization, Supervision.

Declaration of competing interest

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Data availability

The authors do not have permission to share data.

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