Pathology - Research and Practice 216 (2020) 152859

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Breast cancer cutaneous metastases are associated to uMUC1 and sialyl Lewis x and to highly malignant primary tumors

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ARTICLE INFO

Keywords:
Breast cancer
Cutaneous metastasis
uMUC1
sLex

ABSTRACT

Breast cancer spreading to different organs have been related to different molecules and mechanisms, but cutaneous metastasis remains unexplored. Increasing evidence showed that MUC1 and some of its carbohydrate associated antigens may be implicated in breast cancer metastasis. In this study we analyzed these tumor markers in order to identify breast cancer cutaneous metastatic profiles. A cohort of 26 primary tumors from breast cancer patients with cutaneous metastases were included; also, cutaneous and lymphatic node metastatic samples and primary tumors from breast cancer patients without metastases were analysed. Immunohistochemical (IHC) studies demonstrated that both underglycosylated MUC1 (uMUC1) and sialyl Lewis x (sLex) to be positively associated with cutaneous metastatic primary tumors ($p < 0.05$). Notably, a high percentage of tumors with cutaneous metastases were characterized as triple negative and Her2+ tumors (37.5 % and 29 %, respectively). Some discordant results were found between primary tumors and their matched cutaneous metastases. To determine if MUC1 variants may be carriers of carbohydrate antigens, subcellular fractions from a cutaneous metastatic lesion were obtained, immunoprecipitated and analyzed by Western blot. We found that the isolated uMUC1 with a molecular weight of > 200 kDa was also the site for binding of anti-sLex MAb; in coincidence, a high correlation of positive IHC expression of both markers was observed. Our findings confirm that breast cancer cutaneous metastases were associated to highly malignant primary tumors and sustain the hypothesis that u-MUC1 and sLe x may drive breast cancer cutaneous metastases.

1. Introduction

Breast cancer is the leading cause of death among solid tumors in women. It affects 12 % of all women and about 40 % of patients will die from metastatic disease, with a 5-year overall survival of patients with distant metastasis of 23.4 % [1]. Cutaneous metastasis of internal malignancies has an overall incidence of 5.3 % being breast cancer the most common tumor to metastasize to the skin with an incidence of 24 % [2].

Increasing evidence shows that MUC1 and its carbohydrate associated antigens may be implicated in breast cancer metastasis [3–5]. This large transmembrane glycoprotein is expressed by normal and neoplastic benign and malignant breast epithelial cells [6,7]and it is a frequently expressed surface marker in metastatic breast cancer [8]. MUC1 is translated as a single polypeptide that undergoes autocleavage into two subunits: MUC1 ectodomain (MUC1-N) and MUC1 cytoplasmic tail (MUC1-C) in the endoplasmic reticulum [9] and form a stable heterodimer at the apical membrane of normal epithelial cells [10,11]. Following malignant transformation, MUC1 often becomes highly overexpressed, displays aberrant glycosylation and altered mRNA splicing variants, and may be found over the entire plasmatic membrane as well as at the cytosol losing its apical polarization [7,10,12–14].

MUC1 is involved in metastatic progression through both MUC1-N ectodomain as well as MUC1-C. This last was shown to be sufficient to induce transformation and it is targeted to the mitochondria and nucleus where it activates the Wnt/b-catenin, signal transducer and activator of transcription (STAT) and NF (nuclear factor)-kB RelA pathways [15]. MUC1-C also interacts with EGFR (epidermal growth factor receptor), ErbB2 and other receptor tyrosine kinases at the cell membrane and contributes to activation of the PI3K-AKT and mitogen-activated protein kinase (MEK)-extracellular signal-regulated kinase (ERK)

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https://doi.org/10.1016/j.prp.2020.152859
Received 22 October 2019; Received in revised form 10 January 2020; Accepted 10 February 2020

Table 1
Antibodies assayed.

Antigen	Epitope structure	Ab	Isotype and source	Reference
MUC1VNTR	Pro-Asp-Thr-Arg	HMFG1	IgG1-mouse MAb	Taylor-Papadimitriou J et al [23]
MUC1VNTR	Pro-Asp-Thr-Arg-Pro	SM3	IgG1-mouse MAb	Girling et al [24]
Sialyl Lewis x (sLex)	NeuAc2-3Galβ1-4GlcNAcβ1-3Gal-R	KM93	IgM-mouse MAb	Hanai et al [25]
Lewis x (Lex)	3Galβ1-4GlcNAcβ1-3Gal-R	KM380	IgM-mouse MAb	Hanai et al [25]
Tn	Gal*N*Acα-R	Anti-Tn	IgM- mouse MAb	Dako
Thomsen-Friedenreich (TF, T)	Galβ1-3Gal*N*Acα1-R	Anti-TF	IgM-mouse MAb	Dako
Cytokeratin 5/6	Cytokeratins 5 and 6	anti-Cytokeratin 5/6 (D5/16B4)	IgG-mouse MAb	Dako
D240	O-linked sialoglycoprotein (MW 40 K)	Anti-D240 (ab77854)	IgG1-mouse MAb	ABCAM
CD31	C-terminus of CD-31	Anti CD-31 (ab28364)	IgG3-rabbit polyclonal	ABCAM

Notes: Dakopatts, Dako Corporation, Copenhagen, Denmark.
Abbreviations: Ig: immunoglobulin; Ab: antibody; MAb: monoclonal antibody.

pathways [16]. These interactions promote an invasive phenotype in breast tumorigenesis.

The MUC1-N terminal subunit has a protein core mainly composed by a variable number of tandem repeats (VNTR). Each tandem repeat consists of a sequence of 20 aminoacids which contains five potential *O*-glycosylation sites in Ser and Thr [17,18]. Normal epithelia present long carbohydrate chains but subtle changes as a consequence of neoplastic transformation leads to an aberrantly underglycosylated MUC1 (uMUC1). In tumors, *O*-glycosylation is initiated by one of the several ppGalNActransferases and, if no further glycans are added, results in the expression of the Tn antigen. In some tumors, short glycans might be generated by early sialylation (sialyl Tn, *s*Tn), while in others, core structures are developed by core synthases. Core synthesis chain extension results in T antigen; sialylation and fucosylation of the outermost part might give rise to antigens of the Lewis group (in type 2 chains, Ley or x); finally, some tumors are capable of sialyl Lewis x (sLex) synthesis [19–22].

The molecular mechanisms by which breast cancer cells metastasize to different sites such as bone, lung, liver, and brain are being elucidated but spread to skin remains unexplored; we hypothesize that this process likely may involve uMUC1 and its shortened glycans.

In the present study, we assessed the expression of MUC1, uMUC1 and carbohydrate related antigens in breast cancer cutaneous metastasis. We investigated whether this expression is related to hormonal receptors (HR), HER2, and histopathological features.

2. Materials and methods

2.1. Samples

The samples used in the study were provided by the Breast Clinic, La Plata (Buenos Aires), the Grupo Oncologico del Sur, Neuquen, and the Centro Privado de Patología, Citopatología e Inmunohistoquímica, Neuquén; samples were obtained between 1995 and 2005, and collected in 2014. This research was approved by the Medical Bioethics Committee, Faculty of Medical Sciences, National University of La Plata, Argentina, reference N°0800-017399/13-000. All procedures followed the World Medical Association Declaration of Helsinki (Finland, 1964) and further modifications.

A total of 148 formalin-fixed paraffin-embedded malignant tissues were studied from female patients with invasive breast cancer: 69 corresponding to patients with cutaneous metastases (CM) and 71 to patients without distant metastases and 5 years of follow up (controls).

The 69 specimens from patients with cutaneous metastases (CM) included 26 primary tumors (named in this study: primary tumors with cutaneous metastases), 30 cutaneous metastatic lesions and 13 axillary nodes. For 21 patients, a sample of the primary tumor and matched cutaneous metastases were obtained. The mean age of the patients was 55.18 years (range: 30–71 years). The pathological staging at diagnosis was established according to UICC TNM classification system as follows: 70,6% had stage III, 23 % stage II, and 8,3% stage IV. No patients with direct cutaneous invasion were included. All tumors were invasive ductal carcinoma.

On the other hand, controls included: 71 samples of primary tumors (named in this study: primary tumors without cutaneous metastases) and 8 lymphatic nodes. The mean age of the patients was 55 years (range: 31–85 years). Breast cancer type distribution was: 80 % invasive ductal carcinoma, 9 % lobular carcinoma, 6 % ducto-lobular carcinoma, and 5 % other types. The pathological staging was 36 %, 39 %, 25 % corresponding to stages I, II, and III, respectively. No patients with direct cutaneous invasion were included. The histological differentiation grade was as follows 20 % corresponding to grade 1, 50 % to grade 2, and 30 % to grade 3 while the nuclear grade was 34 %, 40 % and 26 % for grade 1, 2 and 3, respectively.

2.2. Cell line

Breast cancer ZR-75-1 cells were cultured in complete RPMI-1640 Medium (SIGMA, USA) at 37 °C, 5% CO_2 until sub confluence.

2.3. Antibodies (Abs)

Abs employed are summarized in Table 1. Two anti-VNTR MUC1 were assayed: HMFG1 which reacts with full glycosylated MUC1 (MUC1) and SM3 with underglycosylated MUC1 (uMUC1) [24,26], both gifted by Prof. *J*. Taylor -Papadimitriou and Prof. *J*. Burchell. Anti-Tn and anti-T MAbs were a gift of Prof. U. Mandel.

Vascular and lymph invasion were analysed employing CD31 and D2-40 MAbs, respectively.

2.4. Methods

2.4.1. Immunohistochemical analysis

The technique was performed following previous reports [27]: paraffin embedded specimens were treated with 10 mM sodium citrate buffer pH: 6.0 employing microwave oven heating (potence: 650 W) for 15 min for antigenic retrieval and incubated overnight at 4 °C with MAb. No antigenic retrieval was employed for anti-Tn and T MAbs. Reaction was developed with the LSAB + biotin-avidin peroxidase-conjugated anti-mouse immunoglobulins kit (DAKO, USA) following manufacturer's instructions. The chromogen employed was 3,3′-diaminodiazobenzidine (DAKO, USA).

Sections were examined by light microscopy and the antibody staining patterns were scored in a semiquantitative manner by two independent observers (AL and MVC) in a blinded way following previous reports [7]. Staining intensity was graded as negative (–), low (+), moderate (++), or strong (+++). The number of optical fields in a specimen that were positively stained was expressed as a percentage of the total number of optical fields containing tissue. The proportion of cell staining was divided on < 5% (negative), 6–50 %, 51–80

Table 2
Characteristics of Primary Tumors with cutaneous metastases.

	Histological grade	Nuclear grade	Lympho-vascular invasion	ER	PR	HER2	Cytokeratin 5/6
MC 1	**1**	**3**	**ND**	–	–	–	–
MC 6	**3**	**3**	**no**	–	–	–	–
MC14	**2**	**3**	**yes**	–	–	–	+
MC19	**3**	**3**	**no**	+	–	–	–
MC22	**3**	**3**	**no**	+	+	–	–
MC24	**3**	**3**	**yes**	+	+	–	+
MC27	**3**	**3**	**yes**	+	+	–	–
MC35	**3**	**3**	**no**	+	+	+	–
MC41b	**3**	**3**	**no**	–	+	–	–
MC49	**3**	**3**	**no**	+	+	+	–
MC50	**3**	**3**	**no**	+	+	–	–
MC57	**3**	**3**	**yes**	–	–	–	–
MC62	**3**	**3**	**yes**	–	–	+	–
MC17	**3**	**2**	**no**	–	–	+	+
MC11	**1**	**3**	**no**	+	+	–	–
MC59	**3**	**2**	**yes**	–	–	–	–
MC55	**3**	**3**	**yes**	–	–	+	–
MC69	**3**	**3**	**yes**	+	+	+	–
MC77	**1**	**2**	**ND**	–	–	–	–
MC43	**3**	**3**	**no**	+	–	+	–
MC73	**2**	**3**	**no**	+	+	–	+
MC 8	**3**	**3**	**no**	–	–	–	+
MC10	**3**	**3**	**no**	+	+	–	+
MC40	**3**	**3**	**yes**	–	–	–	–
MC 9b	**3**	**3**	**no**	–	–	–	+
MC65	**3**	**1**	**no**	–	–	–	–

% and > 81 %. In lymph nodes, any fraction of reactive cells was considered positive. The reaction at the cytoplasm, plasma membrane, and nucleus was evaluated; cells were considered positive when at least one of these components was stained; apical and non-apical reactivity was also recorded. The pattern of reaction was classified as membrane (reaction at the plasmatic membrane), cytoplasmic, or mixed (cytoplasmic and membrane) and the positive reaction in gland lumen content was identified as cellular debris or secretion. A positive and a negative control were run for all immunohistochemical assays; negative controls included a specimen of all samples incubated with PBS instead of MAbs and a known negative specimen for each MAb. Positive controls were specimens of breast cancer containing the target molecules recognized by the MAbs in their known location by IHC as well as employing other techniques [11,19,23,27].

2.4.2. Preparation of homogenates, subcellular fraction, immunoprecipitation, and immunoblot

Immunoprecipitation was performed on cultured cells and tumor tissue biopsies. Subconfluent ZR-75-1 cells were washed three times with ice-cold PBS, scraped and lysed with lysis buffer (20 mM Tris HCl, 137 mM NaCl, 1% Nonidet P-40, 2 mM EDTA), pH 8, supplemented with a protease inhibitor cocktail (SIGMA, USA) at 4 °C for 30 min, in gentle agitation. Cell lysates were forced through a 23 G syringe 12 times and centrifuged at 15,000 xg for 15 min at 4 °C, then supernatants were collected, aliquoted and stored at −70 °C.

Tumor tissue was rinsed in ice-cold PBS and minced onto small 1 mm side pieces and homogenized with an electric homogenizer (Kinematica AG, Switzerland) in lysis buffer. Tissue lysates were left 5 min to decant and supernatant was centrifuged at 15,000 xg for 15 min at 4 °C, then supernatants were collected, aliquoted, and stored at −70 °C.

Before immunoprecipitation 450 µl of lysates (50 µg of protein) were pre-cleared with 50 µl of protein A-Sepharose CL4B (SIGMA, USA) beads, incubated for 30 min at 4 °C with gentle agitation and then centrifuged at 15,000 x g at 4 °C for 10 min. Immunoprecipitation was performed on 450 µl cleared lysates incubated with 5 µl of monoclonal antibody (SM3 anti-uMUC1 or anti-carbohydrate antigens MAbs: KM93 anti-sLex, KM380 anti-Lex, anti-Tn) overnight at 4 °C with gentle agitation. Then, 50 µl of protein A-Sepharose CL4B (SIGMA, USA) beads were added and incubated for 4 h at 4 °C with gentle agitation. Lysates were centrifuged at 2000 xg at 4 °C for 10 min and washed twice with PBS. Final pellet was eluted with 50 µl 2x SDS loading buffer, heated at 100 °C for 5 min, and centrifuged at 2000 xg at 4 °C for 10 min. Final supernatant was loaded onto SDS-PAGE gels.

Immunoprecipitated subcellular fractions, after SDS-PAGE, were transferred to a nitrocellulose sheet (Whatman, Germany), which were subsequently blocked with 5% skim milk in 0.05 % PBS-Tween 20 (PBST). Afterwards, membranes were washed with PBST and incubated overnight at 4 °C with anti-uMUC1 (SM3 MAb) or anti sLe x MAb, diluted in blocking buffer, washed with PBST and then incubated with peroxidase-conjugated secondary antibody and were developed by enhanced chemoluminiscence (ECL).

2.4.3. Statistical analysis

All statistical analysis was performed with SPSS statistics (v24) software. Univariate statistical correlation analysis was performed employing Kendall's tau coefficient, while differences between paired samples were assessed by McNemar's Sign test. Multivariate analysis by principal component was performed; tumor variables included in the analysis were: patient's age at diagnosis, stage, histological grade, nuclear grade, and ER, PR and HER2-neu status. In all cases, null hypothesis rejection and statistically significant differences were considered when $p < 0.05$. IHC heatmap visualization was performed with MeV 4.9 software.

3. Results

The sample cohort of 26 primary tumors with cutaneous metastases was characterized in terms of data according to the parameters listed in Table 2. Most tumors corresponded to histological grade 3 (21/26, 81 %) as well as nuclear grade 3 (23/26, 88 %). It is remarkable that 10 out of 26 (38,5%) tumors were ER-, PR-, and HER2- (triple negative, TNBC) while in 3/26 the three receptors were positive, and 4/26 were ER-, PR-, and HER2+; furthermore, only 7/26 were ER+, PR + and HER2-.

By microscopic observation in samples stained with Hematoxylin-Eosin, tumoral vascular and lymphatic invasion was detected in 9 tumors; we highlighted these observations employing CD31MAb for blood vessels (Fig. 1a) and D2-40 for lymphatic ones (Fig. 1b). These findings were also observed at the metastatic cutaneous lesions.

We found that cytokeratins 5/6 (CK5/6) were present in 7/26 primary tumors: 3 of them corresponded to TNBC while 3 were ER+, PR +, HER2-, and the last one was ER-, PR-HER2 + .

3.1. Immunohistochemical expression of MUC1, uMUC1 and associated carbohydrate antigens

Fig. 2a–c shows a summary of the antigenic expression of primary tumors without cutaneous metastasis (in Fig. 2, group A: Nonmetastatic primary tumors) and with cutaneous metastasis (group B: Cutaneous metastatic primary tumors), and cutaneous metastases (group C).

Statistical analysis of antigenic expression showed that uMUC1 and sLex were overexpressed in cutaneous metastatic primary tumors and cutaneous metastatic samples; in this last group, Tn showed its highest expression while Lex, its lowest.

3.2. MUC1, uMUC1, and carbohydrate associated antigens at primary tumors with cutaneous metastases

Anti-MUC1 HMFG1 MAb stained most tumors with a strong reaction (20/25, 80 %) and a mixed (17/25, 68 %) and cytoplasmic pattern (7/25, 28 %); a non-apical staining was observed in 22/25 (88 %) and most samples (18/25) showed a high percentage of staining (80–100

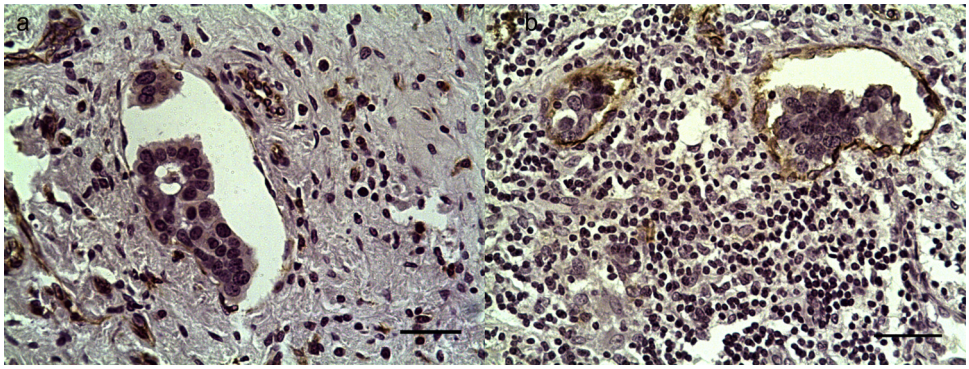

Fig. 1. Representative images of tumoral blood and lymph vessels with metastatic invasion: staining with (a) CD31 pw and (b) D2-40 MAb.

%). Fig. 3ashows a strong mixed reactivity of a primary breast cancer sample with HMFG1 MAb.

In the case of SM3 MAb (anti-uMUC1 MAb), most samples (18/24) showed a strong intensity (Fig. 3b) while a mixed non-apical pattern of expression was mainly found. The percentage of positive tumor cells showed the following results: in 14 samples, less than 50 %; 2 specimens between 50–100 %, and in 8 samples the staining reached between 80–100%.

Eleven out of 26 samples (42.3 %) showed a positive sLex reaction, mainly with a strong intensity and a varied pattern. Anti-sLex KM93 MAb stained well-defined areas; the reactivity was heterogeneous regarding the distribution of some positive cells of several acini in 10 samples with an expression of less than 50 % of tumor cells while in only one specimen the reaction got up to more than 50 %. Fig. 3c illustrated the strong, mixed reactivity of an area of a well differentiated primary tumor with cutaneous metastases.

Lex antigen, as detected by KM380 MAb, was expressed in 14/26 specimens (Fig. 3d); most of them (85.6 %) showed a strong reactivity which was mainly detected at the cytoplasm and the plasmatic membrane (8/14, 57 %); 12/14 showed a uniform non-apical reaction; the percentage of positive cells varied among specimens.

Expression of Tn antigen was found in 10/26 primary tumors with a strong intensity in half of them and a weak one in the other half. The reaction was mainly found at the cytoplasm (9/10, 90 %) (Fig.3e). All samples showed a non-apical reactivity. In three specimens the percentage of positive cells reached to 80 %–100 %, in another 3, 50 %–80

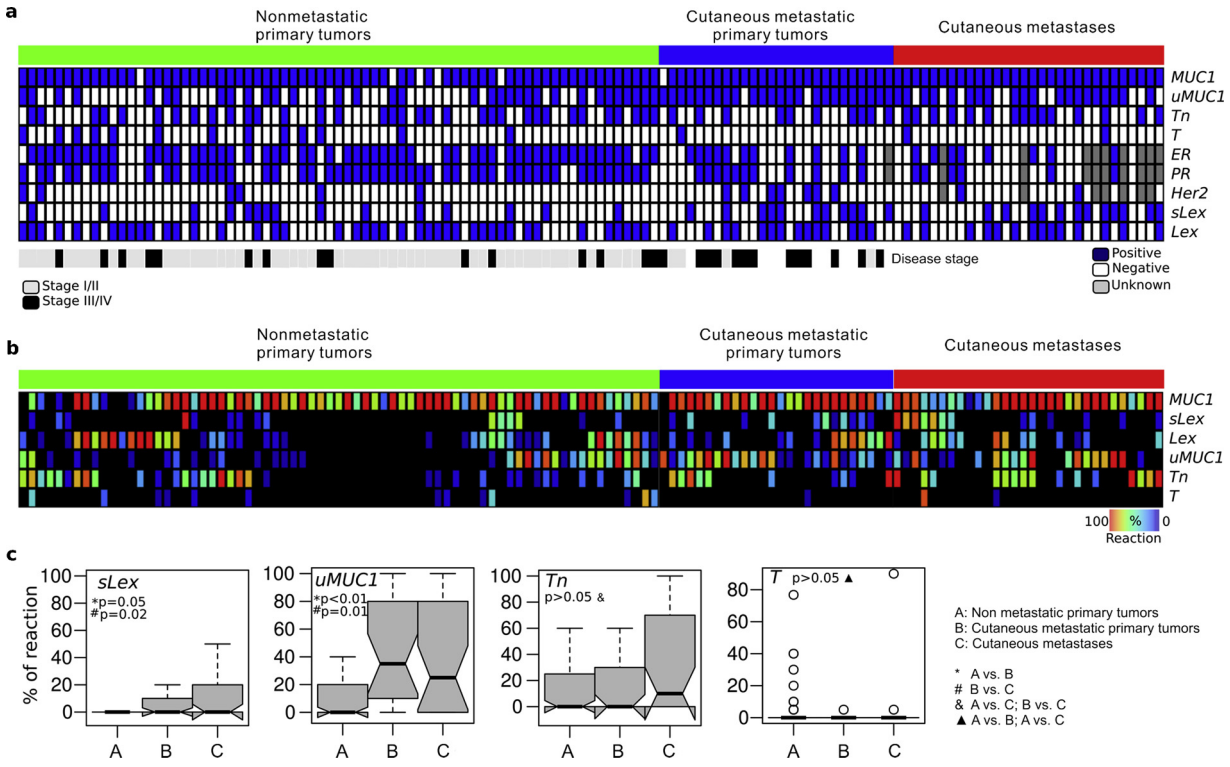

Fig. 2. Schematic representation of immunohistochemical antigenic expression of the three groups considered: A- Nonmetastatic primary tumors, B- Cutaneous metastatic primary tumors, and C- Cutaneous metastases.

a and b correspond to Heatmap visualizations. Fig. 2 **a** at the top shows results of immunohistochemical intensity. Expression of MUC1, uMUC1, Tn, T, sLex and Lex as well as ER, PR and Her2 are shown. Full colored corresponds to positive results, white corresponds to negative results while grey to unknown (not determined). **a** (bottom) shows disease stage at diagnosis; samples were grouped in two: grey corresponds to I and II (I/II) disease stages, and black to III and IV (III/IV) disease stages. Group A were more frequently disease stage I/II than Group B which were mainly III/IV.

b shows percentage of immunohistochemical reaction of MUC1, uMUC1, Tn, T, sLex, and Lex in the three groups (A, B, and C).

c depicts Boxplots of sLex, uMUC1, Tn, and T antigenic expression; in the x-axis, the three sample groups (A, B, and C) are shown; y-axis represents the percentage of reaction by IHC. Statistic differences among groups are shown.

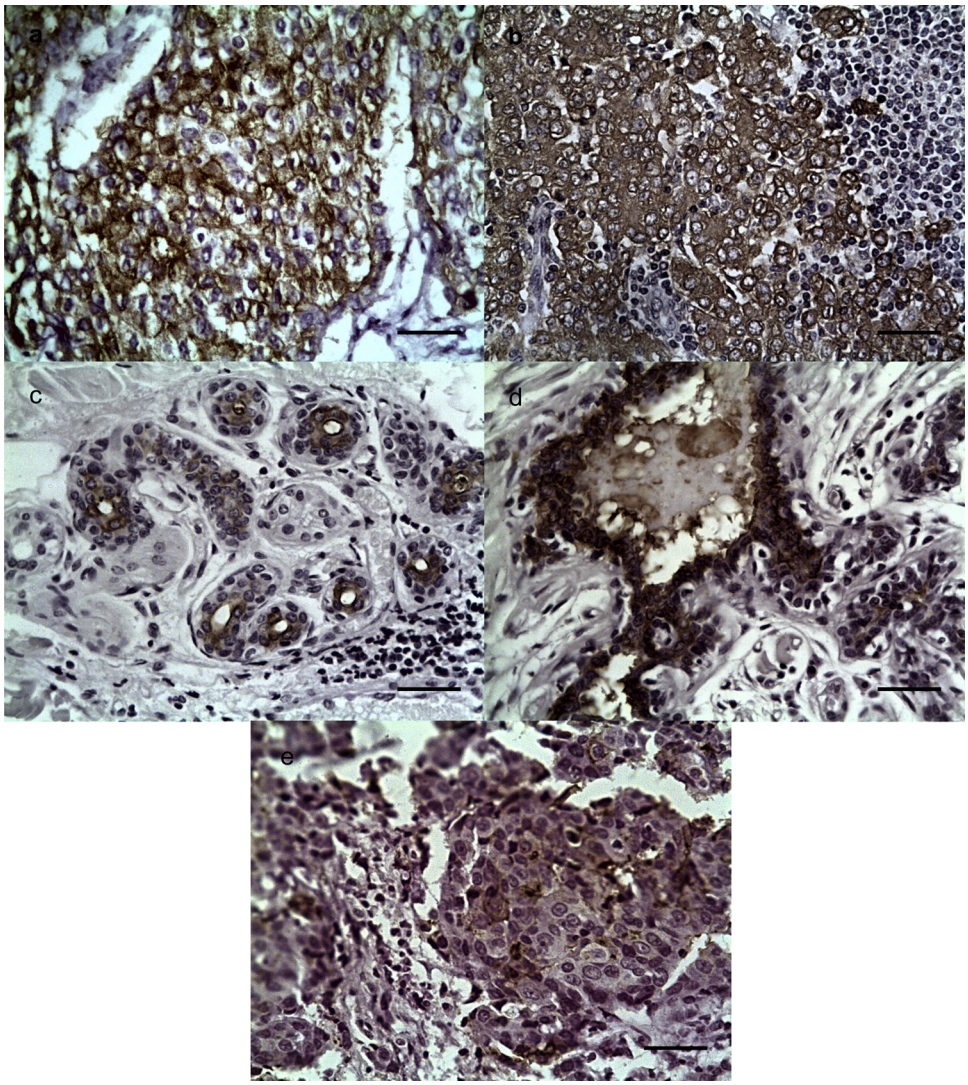

Fig. 3. Immunohistochemical analysis of breast primary tumors with cutaneous metastasis: (a) a positive strong and mixed reactivity with anti-MUC1 MAb is shown. (b) The section depicts an intense uMUC1 staining at the cytoplasm and plasmatic membrane of tumor cells. (c) A strong sLex is shown with a mixed pattern throughout the cytoplasm and membranes; lumen content is also stained. (d) Well differentiated cancer section with Lex expression showing reactivity a mixed pattern of reactivity. (e) The staining of a non- differentiated primary tumor incubated with anti-Tn MAb is depicted; most cells show a mainly cytoplasmic expression.

% while in 4, less than 50 %.

Finally, only one tumor expressed T antigen (3.8 %); reactivity was detected in a low percentage of neoplastic cells with a mild reactivity and a mixed non-apical localization.

3.3. MUC1, uMUC1, and carbohydrate associated antigens at metastatic cutaneous lesions (Fig. 4)

A total of 30 cutaneous metastatic specimens were included. All samples were reactive with anti-MUC1 HMFG1; staining was located at the cytoplasm with a non-apical pattern (Fig. 4a); in 17/30 samples, reactivity was also observed along the plasmatic membrane (mixed pattern); in most specimens, the reaction was strong with a high percentage of positive cells. One specimen showed a metastatic lesion with conserved ducts which reacted at the apical part of the cell.

uMUC1 was detected in 20 specimens (66.6 %); most of them showed a strong reactivity (17/20, 85 %) located at the cytoplasm (18/20, 90 %) with a non-apical pattern (16/20, 80 %) (Fig. 4b). In 12 specimens, a high percentage of reactive tumor cells was found while the other samples showed lower percentages.

Staining with anti-sLex antigen was detected in 13/30 (43.3 %) samples; most of them (10/13, 77 %) showed reactivity at the cytoplasm; in 3/13, the plasmatic membrane was also stained while in only 3/13 samples the expression was restricted to the membrane. In most specimens, the reaction was strong and non-apical (10/13, 77 %). In general, positive specimens showed reactivity in some areas of the specimen with a high percentage of positive cells on nearly half of the samples. Fig.4c illustrates the reactivity of one sample with a mixed reaction.

Lex antigen was detected in 14 out of 30 (46.6 %) specimens; most of them depicted a non-apical pattern with a strong intensity. Seven out of 14 specimens showed a mixed pattern (Fig. 4d) while 6 showed reaction only at the cytoplasm and in only one specimen, Lex antigen was expressed at the plasmatic membrane. Lex expression was limited to some metastatic cells which, in general, showed a low percentage of positivity.

Fifteen out of thirty samples were positively reactive with anti-Tn MAb; most of them (11/15, 73 %) showed a non-apical cytoplasmic reactivity (Fig. 4e) while the percentage of positive cells varied among samples.

Finally, T antigen was only detected in 2/30 (6.6 %) specimens; in one sample, the staining was weak, non-apical at the cytoplasm, and a high percentage of positive tumor cells showed reactivity (80–100 %). The other positive sample also showed a weak staining but with a mixed pattern and a lower percentage of reactive cells (< 50 %).

3.4. MUC1 and carbohydrate associated antigens at lymph nodes of patients with cutaneous metastases

A total of 13 specimens of lymph nodes belonging to patients with

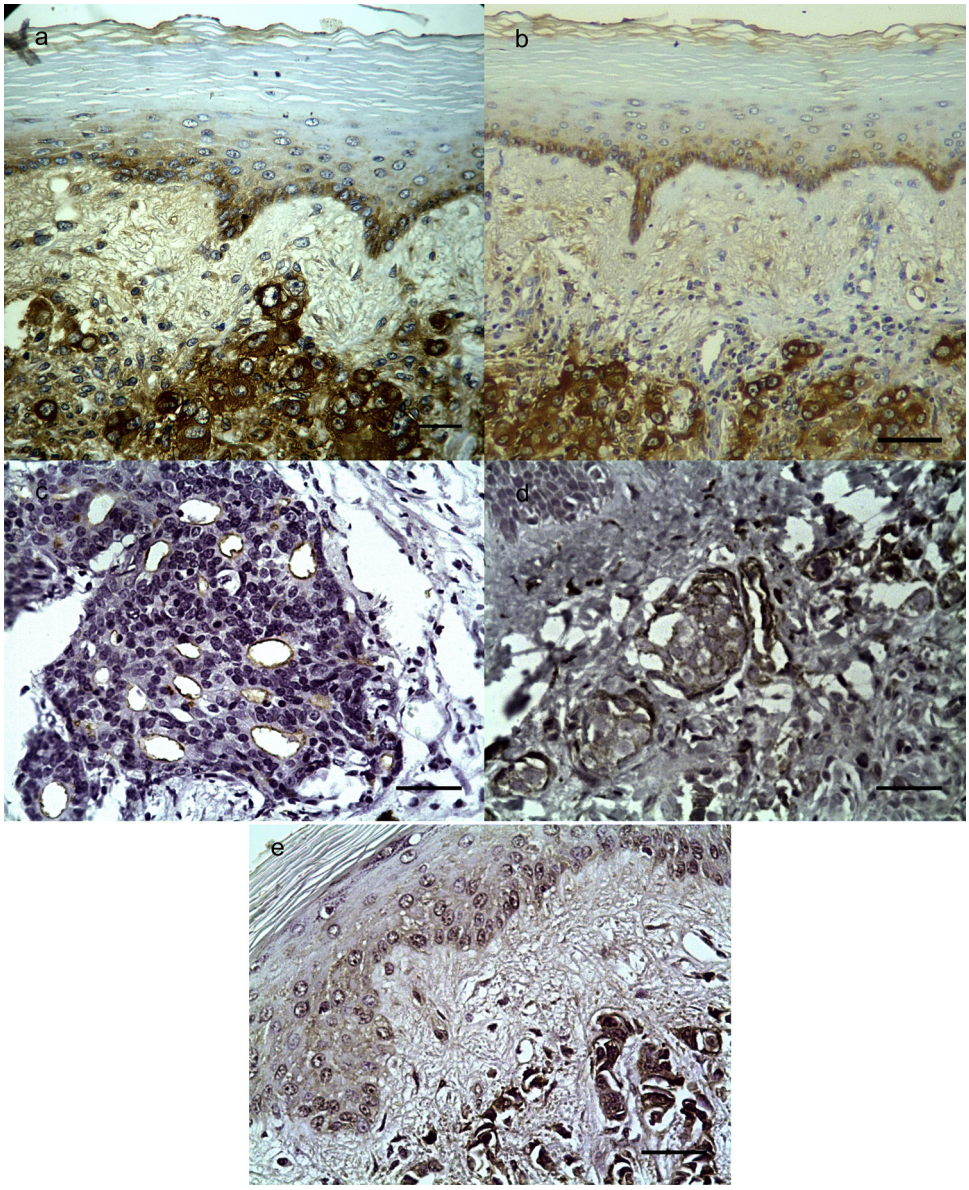

Fig. 4. Immunohistochemical analysis of cutaneous metastatic sections: (a) a positive intense and mixed reactivity with anti-MUC1 MAb is depicted. (b) Similar reactivity is shown with anti-uMUC1 MAb. (c) sLex staining shows a heterogenous expression with a mixed pattern. (d) Sample incubated with anti-Lex antigen; a mainly apical staining at plasmatic membranes is observed while some cells show a cytoplasmic non-apical staining. (e) Section incubated with anti-Tn MAb shows a strong cytoplasmic reactivity.

cutaneous metastases were studied. MUC1 was expressed in 11/13 samples; all of them showed a non-apical pattern at the cytoplasm although many specimens also showed a reaction at the plasmatic membrane; most specimens showed a high percentage of stained tumor cells.

On the other hand, 9/13 (69 %) samples expressed uMUC1; the reaction was diffuse, staining mainly at the cytoplasm (5 specimens) while in 4 samples the staining also involved the plasmatic membrane; in all cases a non-apical pattern was detected. A high percentage of stained metastatic cells was found in 5 samples while in the other four, a lower percentage was detected.

Considering the carbohydrate associated antigens, most samples showed a strong cytoplasmic non-apical pattern; in general, staining was restricted to well-defined areas although some samples showed a diffuse reaction. In the case of sLex, 3 out of 13 nodes were positively stained; 2 of them showed only a few stained acini while in the other one, 90 % of metastatic cells were positive. Lex was detected in 5/13 nodes; 3 of them presented a high percentage of reactive tumor cells. In the case of Tn, 2 samples were positive; in one of them, the staining involved most metastatic cells and, in the other one, a lower proportion (30 %) was reactive. Finally, T antigen was weakly expressed in 3 nodes showing a cytoplasmic or mixed reaction comprising less than 51 % of metastatic cells.

3.5. Comparative analysis between paired lesions of primary tumors with cutaneous metastases and cutaneous metastatic samples

To further clarify the question of the possible disparities between the primary tumor and its metastases, in a cohort of 21 patients the comparison between the matched samples was performed (Table 3); considering all the antigens analyzed, conversion results were detected.

It is interesting to note that detection of sLex showed the largest disagreement (9 cases) between the primary tumor sample and the corresponding CM; four cases showed a negative conversion (sLex + to sLex-) while five cases, the contrary. A different situation was found on Tn expression which showed only one negative conversion while in 7

Table 3
IHC results of the 21 primary tumors and their cutaneous metastasis paired cases.

Cases	ER	PR	Her2	Muc1	uMuc1	sLex	Lex	Tn	T	Cases	ER	PR	Her2	Muc1	uMuc1	sLex	Lex	Tn	T
P1	0	0	0	0	+	0	0	0	0	CM1	0	0	0	+	+	0	0	0	0
P2	0	0	+	+	+	+	+	+	0	CM2	0	0	0	+	+	0	+	+	0
P3	0	0	0	+	+	0	+	+	0	CM3	0	0	0	+	+	0	+	+	+
P4	+	+	0	+	+	0	0	+	0	CM4	+	+	0	+	+	0	0	+	0
P5	0	0	0	+	+	0	0	0	+	CM5	+	+	0	+	0	0	0	+	0
P6	+	0	0	+	+	+	0	0	0	CM6	0	+	0	+	+	+	+	0	0
P7	+	+	0	+	+	0	0	+	0	CM7	+	0	0	+	+	0	0	+	0
P8	+	+	0	+	+	0	+	0	0	CM8	+	+	0	+	0	0	0	0	0
P9	+	+	0	+	+	0	0	0	0	CM9	+	+	0	+	+	0	0	+	0
P10	+	+	+	+	+	+	+	+	0	CM10	+	+	+	+	0	0	0	0	0
P11	0	+	0	+	+	0	+	0	0	CM11	0	0	0	+	+	0	+	+	0
P12	0	0	+	+	+	0	0	0	0	CM12	0	0	+	+	+	+	+	+	0
P13	+	+	+	+	0	0	0	0	0	CM13	0	0	0	+	+	0	0	0	0
P14	+	+	0	+	+	0	0	0	0	CM14	+	0	0	+	+	+	+	+	0
P15	0	0	+	+	0	0	+	0	0	CM15	0	0	0	+	0	+	+	+	0
P16	0	0	0	+	+	+	+	0	0	CM16	0	0	0	+	0	0	+	0	0
P17	0	0	0	+	+	0	+	0	0	CM17	+	0	0	+	+	+	+	+	0
P18	0	0	+	+	+	+	+	0	0	CM18	0	0	0	+	0	+	0	0	0
P19	+	+	+	+	+	+	+	+	0	CM19	+	+	0	+	0	0	+	+	0
P20	+	+	0	+	+	+	0	0	0	CM20	0	0	0	+	+	0	0	0	0
P21	0	0	0	+	+	+	+	0	0	CM21	0	0	0	+	+	+	+	0	0

P: primary tumors, CM: cutaneous metastases.

CM a positive one was detected (McNemar's test, $p = 0.07$). In the case of uMUC1, a negative conversion was found in 6 cases while only one showed the contrary (McNemar's test, $p = 0.13$).

Considering all the samples and taking into account ER, PR, and HER2 expression, primary tumors showed a higher expression respect to cutaneous metastatic lesions.

In 10 patients, paired samples of the primary tumor, the cutaneous lesion and a lymphatic node were obtained. In some metastatic lesions (cutaneous and lymphatic), some antigens were not expressed although in some others they were gained. In all samples MUC1 and uMUC1 were the most frequently detected.

3.6. Comparison between primary tumors with cutaneous metastases and primary tumors without cutaneous metastases

In order to characterize the antigenic profile of breast primary tumors with cutaneous metastases, a comparison with a cohort of 71 primary tumors without distant metastases was performed (Fig. 2**).** It is remarkable the higher expression of uMUC1 and sLex ($p < 0.05$) in primary tumors with cutaneous metastasis respect to those without while Lex also showed a higher reactivity. In the case of Tn and T antigens, they were more expressed in primary tumors without cutaneous metastases, although without statistical significance.

Respect to the pattern of expression, in primary tumors with cutaneous metastases, MUC1 and uMUC1 showed a higher mixed pattern and a non-apical expression than those without. In a similar comparison, sLex showed a high cytoplasmic and mixed reactivity in both primary tumors while Lex showed a higher cytoplasmic and also a mixed pattern in primary tumors with cutaneous metastases respect to the other group.

The comparison of ER, PR, and HER2 expression between the two groups showed strong differences. In tumors with cutaneous metastases, a lower expression of ER and/or PR was found (12/26, 46 %) while a higher reactivity of HER2 was detected (7/26, 27 %); in the case of tumors without metastases, ER was expressed in 77,5% (55/71), PR in 71,7% (51/71) and HER2 in 6 out of 71 (8.45 %).

It was also found a significant increase of ER-/PR- among cutaneous metastatic primary tumors and cutaneous metastatic lesions compared with non-metastatic primary tumors (Fig.2a). In addition, high histological grades were more frequently detected among primary tumors with cutaneous metastases than without (Table 2).

3.7. Correlation of variables in primary tumors with cutaneous metastasis

We first analyzed the association of each antigen with the histological and clinical features. uMUC1 and sLex high expression correlated with ER and PR negativity and with high grade.

Then, we analyzed the correlation among antigens and we found that uMUC1 correlated with sLex, Lex and Tn.

3.8. Determination of uMUC1-sLex glycoform in a cutaneous metastatic lesion by immunoprecipitation followed by Western blot

Since the interaction between sLex expressed by cancer cells and selectins of endothelium has been related with metastatic dissemination, we decided to determine if uMUC1 may be a carrier of sLex in cutaneous metastases. From a sample of a cutaneous metastatic lesion, subcellular fractions were obtained and immunoprecipitated with SM3 MAb (anti-uMUC1). Afterwards, the immunoprecipitated fraction was subjected to electrophoretic separation and Western blot analysis. The incubation with anti uMUC1 (SM3) and anti-sLex revealed a band at the same molecular weight (> 200 kDa); results are shown in Fig. 5, including ZR-75 cell line employed as a control.

Same experiments were also performed using anti-Lex and anti-Tn MAbs with negative results.

4. Discussion

In this study, we retrospectively found that the expression of uMUC1and sLex in primary tumors with cutaneous metastases not only was high, but also had a statistically significant correlation. Coincidentally, it was observed that the cellular pattern of expression of both markers also correlated. In addition, we found that the isolated uMUC1 from a cutaneous metastatic lesion, with an approximate molecular weight of > 200 kDa, was also the site for binding of anti-sLex MAb (KM93). These results indicate that uMUC1 may be a carrier of sLex. This mechanism may be implicated in breast cancer cutaneous metastasis, as tumor cells expressing uMUC1 as a carrier of sLex may gain the circulation and disseminate to the skin.

In contrast, regardless of having found in our series a high immunohistochemical expression of carbohydrate antigens other than sLex, it was not possible to isolate them using Western blot with SM3 MAb. The fact that carbohydrate antigens may also be carried by other mucins/glycoproteins/glycolipids [22,28–30] may serve as plausible

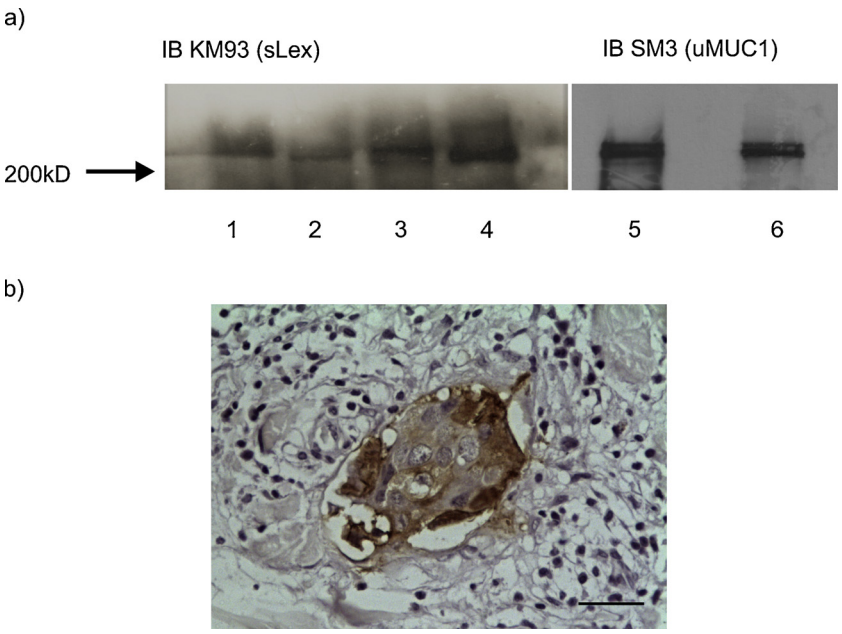

Fig. 5. Determination of uMUC1as a carrier of sLex in a cutaneous metastatic lesion. (a) Western blot analysis: in lines 1 and 3 results obtained with the specimen are depicted: in line 1 the homogenate was immunoprecipated with anti-uMUC1 and immunoblotted with anti-sLex; line 3 shows the homogenate directly immunoblotted with anti-sLex (input control). Lines 2 and 4 show the results obtained with ZR-75 metastatic breast cancer cell line used as a control and similarly assayed as the specimen. In line 2 ZR75 homogenate was immunoprecipated with anti-uMUC1 and immunoblotted with anti-sLex; line 4 shows the ZR75 homogenate directly immunoblotted with anti-sLex (input control). In all lines a band at > 200 kDa is shown.
To further show that ZR-75 cell line expresses uMUC1, the controls shown were performed. In lines 5 and 6 results obtained with anti-uMUC1 MAb (SM3) are shown: in line 5 the homogenate was immunoprecipated and immunoblotted with the same MAb while line 6 shows the homogenate directly immunoblotted with uMUC1 MAb (input control).
(b) Immunostaining of the cutaneous metastatic lesion employed for in (a) with anti- sLex MAb shows a strong non-apical mixed pattern.

explanation. However, it may have simply been the result of carbohydrate concentrations under the technical detectable ranges [31].

Lex was highly expressed in primary tumors with cutaneous metastases; its significance at neoplastic samples has not been already defined. Our present data showed that Lex expression was lower at cutaneous metastases respect to primary tumors with or without metastasis. Other authors [32] found Lex expression to be associated with invading tumor areas, while Elola et al. [33] confirmed that MCF-7 cancer cells binding to HUVEC partially depended on Lex epitopes. In contrast, in previous reports, we found high Lex expression in non-disseminated breast cancer samples as well as in normal and benign breast lesions [34,35]. Also, we found Lex associated to a better prognosis in HNSCC [36].

On the contrary to Lex, we found the highest levels of Tn hapten at cutaneous metastatic lesions, being lower at primary tumors.

Since early, Tn and T [37] were found to be expressed in 90 % of carcinomas including breast and, furthermore, these antigens were more detected in metastatic lesions respect to primary tumors and frequently correlated with carcinoma´s aggressiveness [38], which is in accordance with our present and previous findings [39]. Kanitakis et al. [38] showed that Tn was associated to poorly differentiated breast carcinomas and more advanced stages, while T to well differentiated tumors; on the other hand, a recent report suggested that Tn may be involved in anti-tumor immune response. Zizzari et al. [40] found that Tn + breast cancer cells may be recognized by macrophage galactose type C-type lectin (MGL). This lectin was expressed by dendritic cells (DCs) and activated macrophages which, modifying Tn-antigens, can result in generating immunogens that can efficiently bind to MGL, strongly activate DCs and achieve an efficient presentation in HLA classes I and II compartments. On the other hand, it has been found that the interaction of T hapten on cancer associated MUC1 with the circulating galectin-3 promotes metastasis by enhancing tumor cell heterotypic adhesion to the vascular endothelium and by increasing tumor cell homotypic aggregation [41]. It seems that more data are necessary to elucidate the significance of these carbohydrate antigens in tumor dissemination.

It is known that cancer disseminated cells from the primary tumor transiently adhere to blood vessels activated endothelium, which leads to extravasation and the formation of secondary tumor sites. One such mechanism is the interaction between sLex antigen expressed by cancer cells and the selectins expressed by endothelial or circulating immune cells [42]. In a large meta-analysis, Liang et al. [43] showed that a high level of sLex expression was significantly associated with lymphatic invasion, venous invasion, deep invasion, lymph node metastasis, distant metastasis, tumor stage, tumor recurrence, and overall survival in cancer. They considered that sLex might be a new prognostic biomarker, and it might become a new diagnostic and therapeutic target for cancer.

Julien et al. [44] have reported that sLex was over-expressed in estrogen receptor ER- breast tumors compared to ER + ones and, furthermore, they have also reported that rolling on endothelial cells was more efficient when sLex was carried by glycoproteins rather than glycolipids. Preference for metastatic sites is determined by many factors including breast cancer subtype [45–47]. Hormone Receptor-positive (HR +) patients are more likely to have bone metastases, whereas HR − /HER2 + and HR − /HER2 − patients present more visceral metastases, including to the liver and lung. Moreover, liver only metastasis was most frequent in HR − /HER2 + patients, while lung only metastasis was most common in HR − /HER2 − patients [48,49]. Also, the HR − /HER2- and HR − /HER2 + subtypes exhibited more metastasis to the brain alone [48,50,51]. We observed that a high percentage (37.5 %) of tumors with cutaneous metastases were characterized as HR − / HER2 − ; Woodman et al. [52] reported that ER- tumors colonized distant organs such as distant lymph nodes (contra-lateral or mediastinal), pleura or skin at a frequency of 23.2 %. In our series, 29 % were Her2 + tumors; it is interesting that in a previous report we found that Her2 + status showed a significant association with sLex expression [39]. Also, uMUC1 and sLex high expression correlated with ER negativity and high grade.

We found that CK5/6 was present in 7/26 primary tumors: 3 of them corresponded to TNBC while 3 were luminal A subtype and the last one was HER2 + . It has been found that CK5/6 is an adverse prognostic marker in TNBC [53] while in a recent large pooled analysis including more than 10 000 invasive breast cancer cases, Blows et al. [54] showed that luminal A tumors expressing basal markers (CK5 or CK5/6 or EGFR), known as luminal A basal-positive, had worse prognosis than luminal A tumors that were negative for basal markers (luminal A basal-negative).

Analysis of sLex expression correlated with disease stage as well as with high nuclear and histological grade [39] which has been previously considered as an accurate predictor of tumor behavior [55]. We demonstrated invasion of sLex + cancer cells in lymphatic as well as

blood vessels both in primary tumors as well as cutaneous metastatic lesions (also, MUC1 + and uMUC1 + cancer cells, data not shown).

Differences in HR and HER2 receptor expression seem to be a frequent phenomenon in breast cancer primary tumors and metastases [56]. Addressing this issue, we found discordance on receptor status and on MUC1, uMUC1 and carbohydrate antigens which may be explained either by early dissemination before any apparent primary tumor masses were detected [57,58] or by the selective pressures provided by treatment. Lindstrom et al. [59] reported that the proportion of patients loosing ER was highest in the group of patients treated with endocrine therapy alone or in combination with chemotherapy. Also, the degree of genomic instability of the cancer genome and the time span in which genomic alterations can take place may account for changes on molecular discrepancies [56] with impact on the effectiveness of systemic treatment [60]. Our study demonstrated changes of molecular subtypes on advanced breast cancer patients during disease evolution. As it has been above stated, each molecular subtype has its own biological characteristics and exerts different activities in promoting metastatic breast cancer.

We propose that: 1- uMUC1 is a carrier of sLex in cutaneous breast cancer dissemination, 2- breast cancer cutaneous metastases are associated to highly malignant primary tumors, and 3- a differential expression of MUC1 and carbohydrate associated antigens would be related to the complexity of the dissemination behavior that these antigens modulate in breast cancer, and may play a role in cutaneous metastases.

To our knowledge, this is the first detailed analysis on breast cancer cutaneous metastasis which may contribute to improve prognostic and therapeutic strategies. Future studies will provide molecular explanations for the clinical phenomenon in which women diagnosed with similarly staged or treated breast cancer can have widely divergent outcomes with regard to future metastases.

Compliance with ethical standards

Fundings: This study was financed by the Instituto Nacional del Cancer (RM 493/14), the Universidad Nacional de La Plata (M153 and M185) and the Comision de Investigaciones Cientificas de la provincia de Buenos Aires.

Studies involving human samples were in accordance with the World Medical Association Declaration of Helsinki (Finland, 1964) and further modifications. Informed consent was obtained from all individual participants included in the study. This research was approved by the Medical Bioethics Committee, Faculty of Medical Sciences, National University of La Plata, Argentina, reference N°0800-017399/13-000.

This research does not contain studies with animals performed by any of the authors.

CRediT authorship contribution statement

A. Luna: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - review & editing. **M.E. Rabassa:** Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - review & editing. **M. Isla Larrain:** Data curation, Validation, Investigation, Methodology. **P. Cabaleiro:** Data curation, Investigation, Methodology, Validation, Visualization, Writing - review & editing. **A. Zwenger:** Data curation, Investigation, Methodology, Validation, Visualization, Writing - review & editing. **R. Canzoneri:** Investigation, Methodology, Software, Validation, Visualization, Writing - review & editing. **A. Segal-Eiras:** Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - review & editing. **M.C. Abba:** Conceptualization, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - review & editing. **M.V. Croce:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the Instituto Nacional del Cancer (RM 493/14), the Universidad Nacional de La Plata (M153 and M185) and the Comision de Investigaciones Cientificas de la provincia de Buenos Aires for financial support.

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