ORIGINAL RESEARCH

Invasive breast cancer in Argentine women: association between risk and prognostic factors with antigens of a peptidic and carbohydrate nature

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Objective: In breast cancer, several tumor markers have been identified. The marker most extensively associated with breast cancer is MUC1. The objective of the study was to analyze prognostic and risk factors in relation to tumor markers in order to clarify breast cancer biology. A total of 349 primary tumor samples and lymph nodes from breast cancer patients were studied. Risk and prognostic factors were considered. An immunohistochemical approach was applied and an extensive statistical analysis was performed, including frequency analysis and analysis of variance. Correlation among variables was performed with principal component analysis.

Results: All the antigens showed an increased expression according to tumor size increment; moreover, sialyl Lewis x expression showed a significant increase in relation to disease stage, whereas Tn and TF presented a positive tendency. Vascular invasion was related to sialyl Lewis x expression and number of metastatic lymph nodes. Taking into account risk factors, when a patient had at least one child, Lewis antigens diminished their expression. In relation to breastfeeding, sialyl Lewis x expression diminished, although its apical expression increased.

Conclusion: Associations between MUC1 and carbohydrate antigens and risk and prognostic factors show the complexity of the cellular biological behavior that these antigens modulate in breast cancer.

Keywords: breast cancer, Argentine women, risk factors, prognostic factors, antigenic expression

Introduction

In Argentina, it has been estimated that 17,000 new cases of breast cancer are diagnosed each year. This tumor localization is the commonest cause of cancer death in women, reaching 5400 deaths per year,[1] which is the second rate of mortality in Latin America.[1] Although there is a strong public health system that covers all the expenditure in relation to cancer treatments, disadvantaged groups have high mortality rates, probably because of a lack of preventive actions.

Because MUC1 has been shown to be overexpressed and aberrantly glycosylated in breast and other carcinomas, in this report we studied this mucin and carbohydrate associate antigens.

MUC1 is a high molecular transmembrane glycoprotein expressed as a stable heterodimer after translation of a single polypeptide and cleavage into two subunits in the endoplasmic reticulum.[2] The MUC1 N-terminal subunit contains variable numbers of 20 amino acid tandem repeats that are extensively modified by *O*-linked glycans.[3]

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The MUC1 C-terminal subunit consists of a 58 amino acid extracellular domain, a 28 amino acid transmembrane domain, and a 72 amino acid cytoplasmic chain.[4]

MUC1 expression in normal epithelia can be quite dynamic, varying in response to steroid hormone or cytokine influences.[5] Following malignant transformation, MUC1 often becomes highly overexpressed, loses its apical polarization, and displays aberrant glycosylation as well as altered mRNA splicing variants.[6] In addition, post-translational regulation of cell surface expression occurs via the activity of cell surface proteases that release soluble forms of MUC1 large ectodomain. In cancer, mucin *O*-glycosylation is characterized by aberrant expression of immature carbohydrate structures such as Tn, T, and sialyl Tn antigens[7] and Lewis blood group antigens (Lewis x, Lewis y, Lewis b, Lewis a, and their sialylated derivatives).[8]

Some reproductive factors, including age at menarche, age at first full-term pregnancy, number of live births, and breastfeeding, have been related to risk of breast cancer,[9] because their impact on lifetime number of ovulatory cycles modifies exposure to endogenous ovarian hormones.[8] Additionally, both pregnancy and breastfeeding induce the differentiation of breast epithelial cells, making them less susceptible to carcinogenic insults.[10]

MUC1 expression in breast tissue increases during pregnancy and lactation;[11] also, MUC1 serum levels augment according to pregnancy development.[12,13] In relation to this, it has been hypothesized that, during pregnancy, MUC1 may stimulate the immune system, which may play a protective role against breast cancer.[14] MUC1 has been detected to be bound to immunoglobulins (Igs), forming circulating immune complexes (MUC1CIC) in pregnant women.[15]

Many authors have described associations between MUC1 expression and prognostic factors in breast cancer. In general, a significant positive correlation has been found between estrogen receptor (ER) status and MUC1 expression.[16] At the same time, between increased MUC1 staining and histological grade, a significant, inverse relationship was detected,[16–18] as well as with a nuclear grade.[19]

This study selected MUC1 and glycan epitopes to ascertain their associations with different risk and prognostic factors in breast carcinoma in Argentine women.

Materials and methods
Patients and tumors
From January 2007 to December 2010, 349 patients underwent surgery for invasive breast carcinoma at institutions related to the Faculty of Medical Sciences of the National University of La Plata, La Plata, Argentina. All these patients were included in the present study. Primary tumor samples were obtained and typed as 81% not otherwise specified ductal type (NOS ductal), 16% lobular type (L), and 3% medular type. Tumors corresponding to NOS ductal presented different grades of differentiation: 17.2% were well differentiated, 55.2% moderately differentiated, and 27.6% poorly differentiated. Staging according to the Union for International Cancer Control's TNM classification system was established: 105 of 349 (30%) corresponded to Stage I, 142 of 349 (41%) to Stage II, 87 of 349 (25%) to Stage III, and 15 of 349 (4.3%) to Stage IV. In some cases, metastatic lymphatic nodes were also obtained.

Patients' clinical data and cancer characteristics included some risk and prognostic factors. Risk factors were age at diagnosis (56 ± 14, range 29–85 years); age at menarche (12.9 ± 1.5, range 10–16 years); menopausal status: premenopausal included 43% of patients while postmenopausal, 57%; breastfeeding: positive 51.7% and negative 48.3%; and children: positive 74.3% and negative 25.7%. Prognostic factors included disease stage; axillary nodal status: negative (1) 38.7% of patients, positive: ≤ 3 (2) 84.6% while >3 (3) 15.4%, not assessed: 1.9% of patients; tumor size: T1 38.1% of patients, T2 54.3%, and T3 7.6%; histological differentiation: I 31.5%, II 45.7%, and III 22.8%; nuclear grade: Grade 1 32.7%, Grade 2 40.4%, and Grade 3 26.9%; mitotic figure counts; Nottingham Prognostic Grade:[20] good (I) 44.6%, moderate (II) 42.9%, and poor (III) 12.5%; vascular invasion: positive 29.8% and negative 70.2%; ER status: positive 66.7%, negative 18.1%, unknown 15.2%; and progesterone receptor (PR) status: positive 63.8%, negative 18.1%, unknown 18.1%.

Controls
A total of 148 tissue samples belonging to patients with benign breast disease (BBD) and normals were included. BBDs were classified as follows: (1) simple fibroadenoma (FA), (2) nonproliferative lesions (NPF) comprised fibrocystic changes/apocrine metaplasia and fibrosis, (3) proliferative lesions without atypia (usual epithelial hyperplasia [UEH]), and (4) proliferative lesions of ductal type with atypial ductal hyperplasia (ADH).

A total of 116 samples corresponded to BBD being 50 FA, 28 NPF, 30 UEH, and eight ADH. Also, 32 normal specimens obtained during breast cosmetic surgery were included. The mean age of patients with BBD and normal females was 42.2 ± 13.6 years.

Procedures followed the World Medical Association Declaration of Helsinki (Finland, 1964) and further modifications. Informed consent was obtained from all patients included in this study. This research was approved by the local Human Investigation Committee, Faculty of Medical Sciences, National University of La Plata.

Tumors and tissues were collected fresh and delivered to the laboratory with minimal delay. For histopathological diagnosis and immunohistochemical analysis, a sample fraction was fixed in methacarn (methanol: chloroform: acetic acid 60:30:10) for 2 hours and transferred into 70% ethanol until processing in paraffin.

Monoclonal antibodies (Table 1)

Three anti-MUC1 variable number of tandem repeats (VNTR) monoclonal antibodies (MAbs) were assayed: HMFG1,[21] C595,[22] and SM3.[11] Also, five anticarbohydrate epitopes: anti-sialyl Lewis x (KM93),[23] anti-Lewis x (KM380),[23] anti-Lewis y (C14),[24] anti-Tn Mabs, and anti-TF MAbs (DAKO, Copenhagen, Denmark) were used in these studies.

Methods

Immunohistochemical analysis

The technique was performed following previous reports.[25] Dewaxed sections were placed in methanol with hydrogen peroxide (3%) for 15 minutes to block endogenous peroxidase activity. After three washes in phosphate buffered saline (PBS), sections were blocked for nonspecific binding with normal horse serum diluted 1:10 in 1% bovine serum albumin/PBS for 15 minutes and rinsed. Before samples were immunostained with MAbs, tissues were treated with 10 mM sodium citrate buffer at 100°C for 5 minutes for antigenic retrieval. Then, sections were incubated overnight at 4°C with MAbs, which were diluted as follows: C595, KM93, KM380, and C14, 1/1000; anti-Tn; and TF 1/100. HMFG1 and SM3 were not diluted (tissue culture supernatants). After three washes with PBS, peroxidase-conjugated antimouse Igs (Sigma-Aldrich Co, St Louis, MO) were added and incubated for 60 minutes. After being washed, slides were counterstained with hematoxylin and coverslipped with mounting media. In every assay, we included a positive control sample, and the same tissue section was incubated with PBS instead of the primary Ab as a negative control.

Specimens were examined with a light microscope and the antibody staining patterns were scored in a semiquantitative manner. Staining intensity was graded as negative, low, moderate, and strong. The number of low power ($\times$10) optical fields in a specimen with a positive reaction was expressed as a percentage of the total number of optical fields containing tissue. The pattern of reaction was classified as linear (membrane), cytoplasmic, and mixed pattern (cytoplasmic with plasma membrane staining), and apical and nonapical staining was recorded.[25] The positive reaction of the nucleus was considered, as well as the staining of gland lumen content, which was identified as cellular debris or secretion.

Statistical analysis

Patients were grouped, taking into account risk and prognostic factors, and positive responses were evaluated by means of frequency analysis (χ^2 test, $P < 0.05$).

A reactivity index (RI) was calculated as intensity (I) $\times$ 100 + percentage of positive area (A).[26]

Normality of variables was tested by Shapiro Wilk's, and data were normalized when necessary.

Correlation among variables was performed with principal component analysis (PCA). Immunohistochemical pattern, percentage of positive area, and RI were analyzed employing analysis of variance ($P < 0.05$) after data standardization.

Also, logistic regression analysis was performed. In this sense, a multiple logistic regression analysis was conducted, subgrouping breast cancer patients according to ER, PR,

Table 1 Antibodies assayed

Antigen	Epitope structure	MAb	Isotype and source	Reference
MUC1VNTR	Pro-Asp-Thr-Arg	HMFG1	IgG1 – mouse MAb	Taylor-Papadimitriou J et al[21]
MUC1VNTR	Arg-Pro-Ala-Pro	C595	IgG3 – mouse MAb	Price et al[22]
MUC1 VNTR	Pro-Asp-Thr-Arg-Pro	SM3	IgG1	Burchell et al[11]
Sialyl Lewis x	NeuAc2-3Galβ1-4GlcNAcβ1-3Gal-R	KM93	IgM – mouse MAb	Hanai et al[23]
Lewis x	Galβ1-4GlcNAcβ1-3Gal-R	KM380	IgM – mouse MAb	Hanai et al[23]
Lewis y	Gal(α1-2Fuc)β1-4GlcNAc(α1-3Fuc)β1-3Gal-R	C14	IgM – mouse MAb	Brown et al[24]
Tn	GalNAcα-R	Anti-Tn	IgM – mouse MAb	Dako[a]
Thomsen-Friedenreich	Galβ1-3GalNAcα1-R	Anti-TF	IgM – mouse MAb	Dako[a]

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

Table 2 Percentage of positive immunohistochemical results obtained in normal, benign, and cancer breast samples

Specimens	Anti-VNTR MUC1			Anti-carbohydrate antigens MAbs				
	C595	HMFG1	SM3	Lewis x	sialyl Lewis x	Lewis y	Tn	TF
Normal	40	100	5	46	10	7	2	0
FA	57	92	0	50	12	3	21	2
NPF	58	100	2	58	15	25	10	3
UEH	56	100	16	92	24	28	36	14
ADH	57	ND	14	71	14	43	14	29
I	63	92	40	54	24	22	25	3
II	59	92	38	57	25	31	31	22
III	58	93	41	44	35	20	35	28
IV	86	98	47	60	33	40	20	49

Abbreviations: ADH, atypial ductal hyperplasia; FA, fibroadenoma; MAbs, monoclonal antibodies; NFP, nonproliferative lesions, UEH, usual epithelial hyperplasia; VNTR, variable number of tandem repeats.

and Her2-neu positive-negative responses using patients' prognostic and risk factors as independent variables. Missing data cases were deleted (minimum n = 332).

Results

Immunohistochemical reactivity of the different antigens assayed obtained in malignant samples according to disease stage are summarized in Table 2; results obtained in control samples (benign and normal) are also included. Results are expressed as percentage of positive samples in respect of the total.

Table 3 shows antigenic expression results of normal breast, BBD, and breast cancer samples expressed as RI.

Breast cancer

MUC1 expression in primary tumor samples

MUC1 expression was detected employing three anti-MUC1 VNTR MAbs. Most samples were stained with at least one anti-MUC1 MAb; the most reactive was HMFG1 MAb, because 92.7% of samples were positive, whereas C595 MAb reacted with 61.4% of samples and SM3 MAb with 39.5% of samples.

As has been previously observed,[17,18,27] the cellular localization as well as the intensity of the staining varied among specimens. Most samples showed a nonapical, cytoplasmic pattern with a strong staining (Figure 1A, B, and C), and several samples showed an apical pattern with a linear, cytoplasmic, or mixed reaction.

Carbohydrate antigenic expression in primary tumor samples

Seventy-three percent of samples expressed at least one glycan antigen. Lewis x was the most frequently found, and 50.7% of tumors showed reactivity with either a linear or a mixed staining, mainly restricted to the apical part of the ductal cells. In most cases, a strong reaction was detected.

In respect of sialyl Lewis x, it was found that 27.5% of samples were reactive. Staining was mainly linear and apical, although some samples depicted a cytoplasmic nonapical reaction (Figure 1D). Twenty-six percent of specimens were positive for Lewis y, showing different patterns of staining: linear and apical in some samples and cytoplasmic and nonapical in some others, whereas several specimens showed a mixed and apical or even nonapical staining. Tn hapten was detected in 29.6% of specimens. Reaction was mainly found in a few cells. In some tumors, expression was cytoplasmic, whereas others stained the plasmatic membranes, usually

Table 3 Antigenic expression of normal breast, BBD, and breast cancer samples expressed as reactivity indexes

Antigen	Normal samples	BBD samples	Breast cancer
MUC1 (HMFG1MAb)	110 ± 164	162 ± 162	376 ± 112
MUC1 (C595 MAb)	125 ± 155	185 ± 174	299 ± 101
MUC1 (SM3 MAb)	20 ± 70	17+/68	261 ± 146
Sialyl Lewis x	27 ± 65	50 ± 110	219 ± 109
Lewis x	181 ± 133	234 ± 155	307 ± 77
Lewis y	24 ± 68	84 ± 127	269 ± 65
Tn	12 ± 62	46 ± 92	277 ± 118
TF	0	19 ± 66	223 ± 129

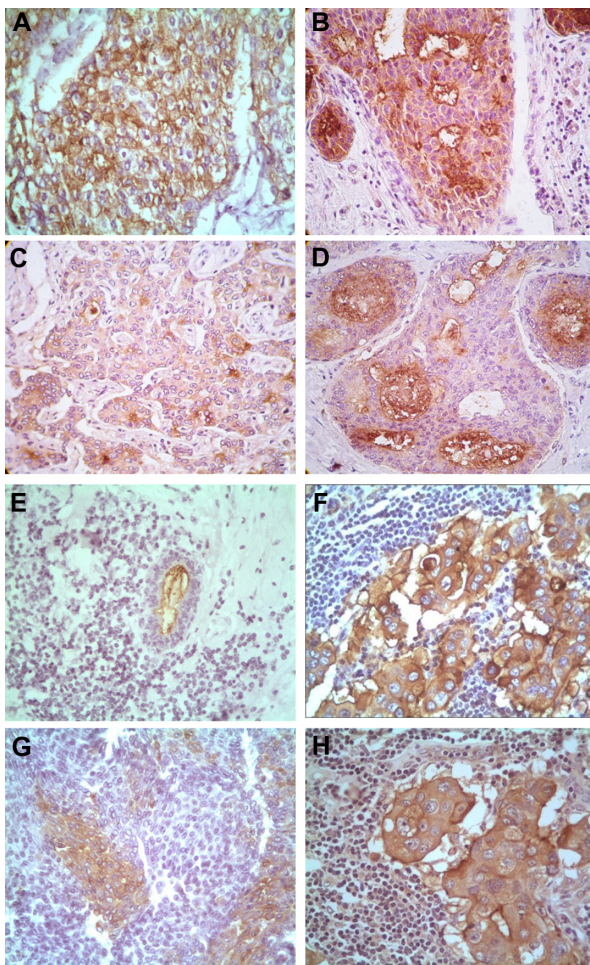**Abbreviations:** BBD, benign breast disease; MAb, monoclonal antibody.

Figure 1 Photomicrographs of immunoperoxidase staining of primary ductal breast carcinoma samples (**A–E**) are shown. Staining with anti-MUC1 HMFG1 monoclonal antibodies (MAbs) (**A**), a mixed nonapical pattern of reaction with a strong intensity is observed; (**B**) a mixed reaction is shown (note the strong apical pattern); (**C**) staining with anti-MUC1 C595 MAb, a cytoplasmic and moderate reaction is depicted; (**D**) reaction obtained with anti-sialyl Lewis x MAb, a mixed reaction is shown; lumen content appears strongly stained; (**E**) the TF antigenic reaction shows a linear and apical pattern. Immunohistochemical reactions of metastatic lymphatic nodes (**F–H**) are depicted: (**F**) staining with anti-MUC1 HMFG1 MAb, a strong cytoplasmic reaction is shown; (**G**) the sialyl Lewis x cytoplasmic reaction is clearly visible in many metastatic cells and (**H**) staining obtained with anti-TF MAb, a cytoplasmic strong reaction is shown.

with a mild reaction. Finally, TF was found in 25% of samples with either linear or apical (Figure 1E) or mixed nonapical patterns.

Antigenic expression in metastatic lymphatic nodes

In a group of 20 patients, a metastatic node sample was analyzed. Fifty percent of lymphatic nodes were positive for MUC1. In these samples, metastatic malignant cells showed a cytoplasmic staining with a strong intensity (Figure 1F). The most reactive anti-MUC1 MAb was HMFG1. Sialyl Lewis x showed reactivity in eight of 20 samples (Figure 1G) and

Lewis x in six of 20 samples with the same pattern. Lewis y, Tn, and TF (Figure 1H) were detected in four of 20 samples with a cytoplamic reaction and low intensity.

Correlation among antigens

Sialyl Lewis x showed a positive correlation with Tn antigen ($r = 0.8147$; $P < 0.034$), whereas Lewis x and Lewis y showed a strong and positive correlation ($r = 0.92$; $P < 0.016$).

A positive tendency between MUC1 and Lewis x apical expression was detected. Taking into account the RI, a positive correlation between MUC1 (detected with SM3 MAb) versus Lewis x was found ($r = 0.8830$; $P < 0.022$).

Relationship between MUC1 expression and prognostic factors

MUC1 detection with the three different anti-MUC1 MAbs (HMFG1, C595, and SM3) showed some differences when they were correlated with prognostic factors.

In order to analyze in more depth the antigenic expression, RI was calculated as intensity (I) $\times$ 100 + percentage of positive area (A).[26] According to anti-MUC1 MAb in relation to disease stage, for HMFG1, MAb RI and Stage I results were 355 ± 155, Stage II 386 ± 113, Stage III 389 ± 98, and Stage IV 480 ± 71. A raise tendency was detected but with no statistical significance (Tukey HSD; $P = 0.067$) (Figure 3). For C595, MAb and Stage I results were 299 ± 110, Stage II 287 ± 109, Stage III 311 ± 84, and Stage IV 289 ± 122. Finally, for SM3, MAb and Stage I results were 281 ± 156, Stage II 267 ± 137, Stage III 236 ± 147, and Stage IV 272 ± 112.

Percentage of positive responses detected with all MAbs increased according to tumor size increment (Figure 2), with statistically significant C595 and SM3 MAbs reactivity ($P < 0.040$).

It was not possible to find a relationship between the pattern of expression of MUC1 and disease stages.

Furthermore, MUC1 apical expression frequency detected with HMFG1 MAb showed a significant decrease according to histological grade increment ($P < 0.031$) (Figure 3). The percentage of samples with apical expression for Grade 1 tumors was 37.5%, for Grade 2 tumors was 25.5%, and for Grade 3 tumors was 16.7%; a similar observation was found with nuclear Grade I 41%, Grade II 19%, and Grade III 16%.

Finally, considering the percentage of MUC1 reactive area, tumors that presented a high percentage showed a low nuclear grade.
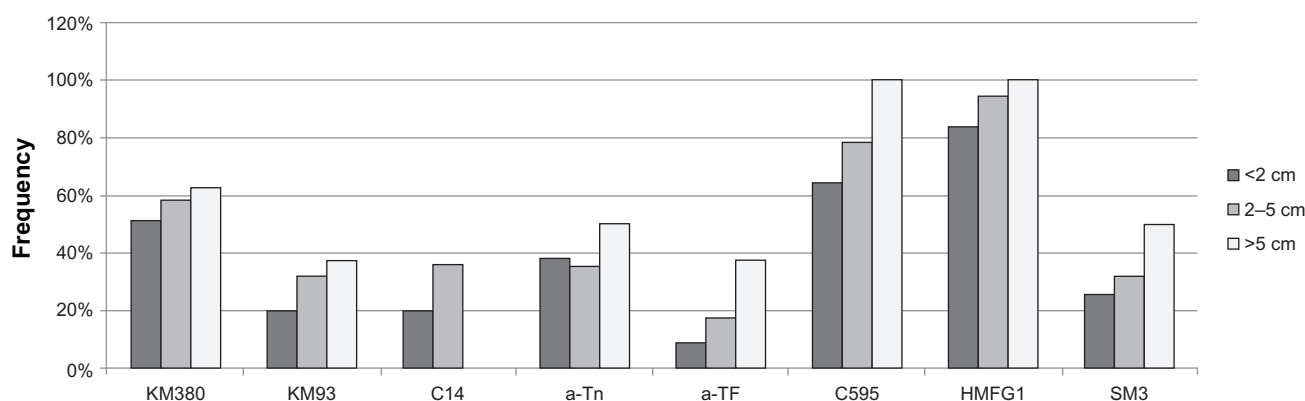
Figure 2 Relative percentage frequencies of staining obtained with the different anticarbohydrate antigens and MUC1 monoclonal antibodies according to tumor size. In all cases, a rising tendency is observed.

A significant mild correlation was found between MUC1 RI detected with HMFG1 MAb and ER ($r = 0.23609$, $P < 0.033$), whereas with PR status a similar, though not significant, tendency was observed.

Relationship between carbohydrate antigenic expression and prognostic factors

TF percentage of positive samples increased with disease stage; sialyl Lewis x as well as Tn expression also augmented with disease stage, although Stage IV showed a decrease, whereas Lewis y and Lewis x did not vary with stage (Table 2).

Stage I patients showed a lower percentage of sialyl Lewis x, Lewis y, and TF reactive area in respect of the other stages ($P < 0.011$). On the contrary, Lewis x showed a higher percentage of reactive area in Stage I patients and also in more differentiated tumors ($P < 0.045$).

Considering RIs, sialyl Lewis x and TF RIs increased with disease stage (Figure 4A and B, respectively), showing

a positive tendency, whereas Tn RI augmented with the presence of metastatic nodes, histological grade, and mitotic index.

The percentage of glycan-positive responses increased according to tumor size increment, being statistically significant for Tn and Lewis y antigens (Figure 2) ($P < 0.0043$).

Vascular invasion and antigenic expression

Ejlertsen et al[28] defined low-risk patients as those patients who were older than 35 years at diagnosis, with LN-negative disease, tumor size ≤ 2 cm, low or moderate histologic grade, and positive hormonal status. Taking into account this classification, in our study most of the low-risk patients (87%) did not present vascular invasion (VI), whereas MUC1 apical expression was found in 25% of them and VI-positive tumors did not show MUC1 apical reaction.

Percentage of positive MUC1 tumor area was higher in VI-negative tumors than in positive ones, being statistically significant when MUC1 was measured with C595 MAb.
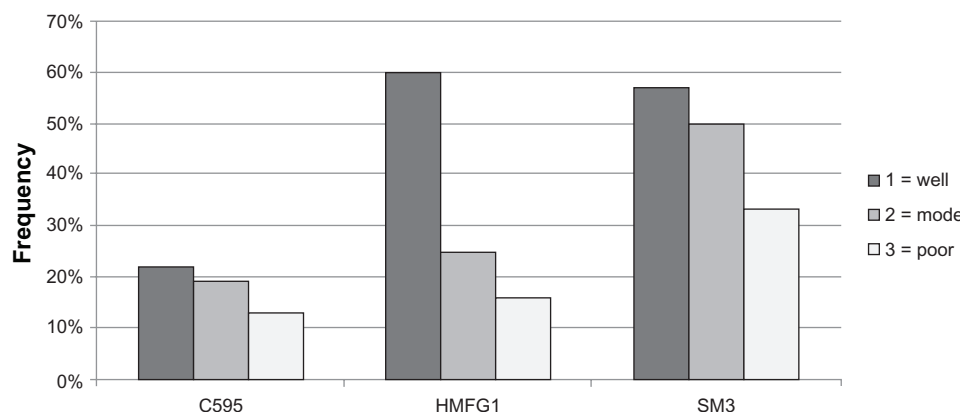

Figure 3 Apical expression frequencies of anti-MUC1 monoclonal antibodies according to histological grade. In all cases, apical expression decreases when histological differentiation grades increase.
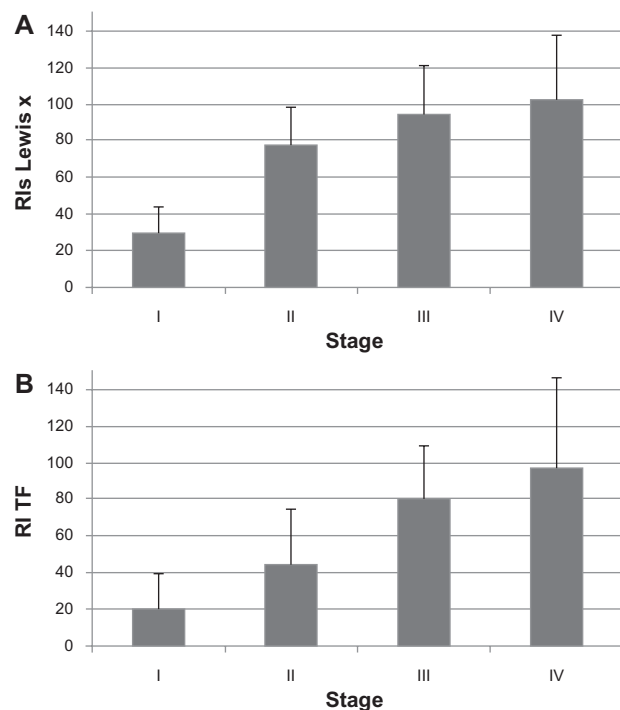
Figure 4 Reactivity index according to disease stage. An increasing reactivity is observed in higher stages: (**A**) results obtained with anti-sialyl Lewis x monoclonal antibodies (MAbs) and (**B**) anti-TF MAbs.

Furthermore, poorly differentiated tumors had higher rates of VI than well and moderately differentiated tumors ($P < 0.0012$); in large tumors, VI was detected more frequently. VI was significantly higher in patients aged < 50 years than in those aged > 50 years ($P < 0.036$).

Positive tumors for sialyl Lewis x showed more VI than negative ones: 23% versus 12% ($P < 0.0073$). Employing frequency analysis it was possible to demonstrate an association between sialyl Lewis x expression and the number of metastatic lymph nodes. This analysis showed that patients with sialyl Lewis x reactive primary tumors presented an increment of the frequency on the number of metastatic lymph nodes ($P < 0.0249$). Finally, a positive relationship between VI and Nottingham Prognostic Index was demonstrated ($P < 0.033$).

Relationship among risk factors and antigenic expression

In relation to the number of children and breastfeeding, the percentage of positive tumor samples in respect of the total showed some variations. When a patient had at least one child, a significant increment on MUC1 reactive area ($P < 0.037$) and MUC1 expression increased significantly from 75% to 92% ($P < 0.021$). On the contrary, Lewis antigens diminished their expression in patients

who had children, being significant for sialyl Lewis x (from 46% to 27%, $P < 0.05$) and Lewis y (from 43% to 22%, $P < 0.025$). Considering the percentage of MUC1 (detected with HMFG1) reactive area and breastfeeding, a significant increment was found in patients who had breastfed ($P < 0.018$). Although no differences were found between breastfeeding and frequency of positive response, when RI was considered; C595MAb showed a positive tendency, whereas Lewis x showed a low expression in those patients who had lactated.

In respect of sialyl Lewis x, although its expression decreased according to increase on breastfeeding, its apical expression showed a positive tendency with lactation, whereas Tn and TF antigens showed an increment on apical expression in relation to breastfeeding and number of children ($P < 0.018$).

ER, PR, and Her2-neu subgroups

In addition, patients were classified according to hormone receptor status (ER and PR) and Her2-neu expression, and analysis in relation to risk and prognostic factors (Table 4) was performed.

Number of positive nodes, tumor size, tumor differentiation, and nuclear grade showed significant differences between ER-positive versus ER-negative subgroups and also between PR-positive versus PR-negative status, whereas age and number of mitotic counts showed significant differences only between ER-positive and -negative subgroups. Furthermore, Her2-neu-positive and Her2-neu-negative patients showed significant differences with number of positive lymph nodes, tumor differentiation, and nuclear grade.

Multivariate analysis

A multivariate comparative study by PCA was performed, taking into account RI values and risk and prognostic factors (Figure 5). RI corresponding to MUC1 detected with HMFG1 and SM3 MAbs and also to anti-sialyl Lewis x, Lewis x, Lewis y, Tn, and TF explained 59% of data variability.

When the age was considered, a clear tendency of patients aged <44 years to be grouped was observed in PC1(−) and PC2(±) patients, whereas patients aged between 44 years and 70 years showed a spread distribution mainly in PC1(+) and elderly patients (>70 years old) and remained exclusively in PC1(+) and PC2(−) patients. A similar tendency was observed when menopause status was analyzed. In general, premenopause patients showed less differentiated tumors. In these patients, C595 and HMFG1 anti-MUC1 MAbs showed a very high correlation, HMFG1 being the variable that makes

Table 4 Logistic regression analysis for patients grouped according to ER, PR, and Her2-neu expression considering risk and prognostic factors

Risk and prognostic factors	Patient subgoups		
	ER P; OR (ORR CL)	PR P; OR (ORR CL)	Her2-neu P; OR (ORR CL)
Age (years)	0.017; 3.53 (0.916–12.5)[a]	0.094; 2.01 (0.070–6.35)	0.921; 0.257 (0.086–1.09)
Number of children	0.224; 1.76 (0.080–1.88)	0.726; 0.527 (0.071–6.35)	0.123;1.43 (0.471–11.2)
Breastfeeding	0.573; 0.415 (0.095–3.68)	0.995; 0.549 (0.375–2.65)	0.848;0.63 (0.031–3.16)
Menopausal age (years)	0.099; 1.31 (0.008–1.531)	0.007; 0.126 (0.035–0.416)[a]	0.951; 0.890 (0.071–8.25)
Menarche age (years)	0.685; 0.161 (0.177–13.8)	0.897; 1.13 (0.122–6.34)	0.927; 0.458 (0.066–1.98)
Disease stage	0.214; 1.531 (0.700–1.69)	0.901; 0.849 (0.279–4.24)	0.454; 2.10 (0.028–5.06)
Number of positive lymph nodes	0.002; 0.157 (0.047–0.519)[a]	0.016; 0.219 (0.064–0.750)[a]	0.002; 15.49 (2.78–86.1)[a]
Tumor size	0.004; 0.309 (0.138–0.688)[a]	0.008; 0.349 (0.159–0.764)[a]	0.210;1.89 (0.695–5.19)
Tumor differentiation	0,000; 0.274 (0.143–0.525)[a]	0.001; 0.338 (0.181–0.633)[a]	0.042; 2.95 (1.04–8.36)[a]
Nuclear grade	0.000; 0.237 (0.120–0.467)[a]	0.001; 0.353 (0.187–0.665)[a]	0.000; 7.83 (1.04–8.36)[a]
Number of mitotic counts	0.004; 0.093 (0.019–0.462)[a]	0.156; 0.320 (0.066–1.55)	0.434;2.75 (0.216–34.9)
Vascular invasion	0.890; 0.964 (0.571–1.63)	0.359; 0,790 (0.477–1.308)	0.161; 1.70 (0.808–3.59)

Note: [a]Indicates significant regression *P* < 0.05.
Abbreviations: ER, estrogen receptor; OR, odds ratio; ORR CL, odds ratio range confidence level for ± 95% range; PR, progesterone receptor.
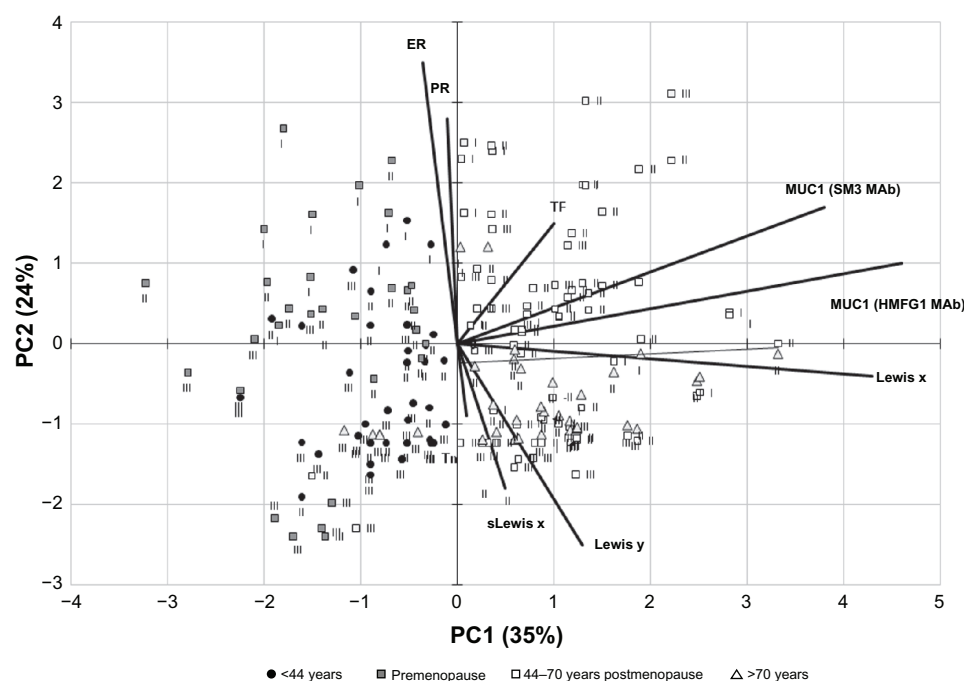

Figure 5 Principal component analysis of reactivity indexes. Length of rays shows variable weights. Points represent each sample data. Patients aged <44 years appear grouped on the left of the graphic PC1(−), whereas middle-age groups remain mainly spread in PC2(+). Postmenopause and elder patient groups predominate on the right of the graphic (PC1(+),PC2(+) and PC1(+),PC2(−), respectively); I, II, and III indicate histological grades.

the highest contribution; as a consequence, RI obtained with C595 MAb was excluded in the final analysis.

PCA analysis including RI of MUC1 and glycan antigenic expression and also ER and PR separated a group of patients who were characterized by their low age, premenopause status, less differentiated tumors, and negative ER and PR status, PC1(−) and PC2(−).

Employing logistic regression analysis we compared Stage I versus advanced stages using independent variables risk factors. Significant regressions were not found (Table 5).

Benign breast diseases

Positive results obtained are summarized in Table 2 and depicted in Figure 6A–D. In general, a decrease tendency of positive expression was found from ADH and UEH to NPF and FA specimens.

Anti-MUC1 C595 and SM3 MAbs exhibited a very similar pattern of reaction. Apical and linear staining was mainly found.

Lewis x epitope and sialyl Lewis x were expressed at apical membranes and cytoplasm. The luminal content was stained. Although the former was found very frequently, the latter was expressed in a low percentage of specimens. Lewis y showed a cytoplasmic mild reaction in several samples, whereas Tn was reactive in only a few cells in some sections. TF was expressed with either a linear or cytoplasmic pattern, mainly apical and restricted to some areas.

Normal samples

MUC1 showed a moderate to strong expression. In most samples, an apical pattern mainly restricted to the plasma membrane was found (Figure 6E–F). Lumen content was also stained.
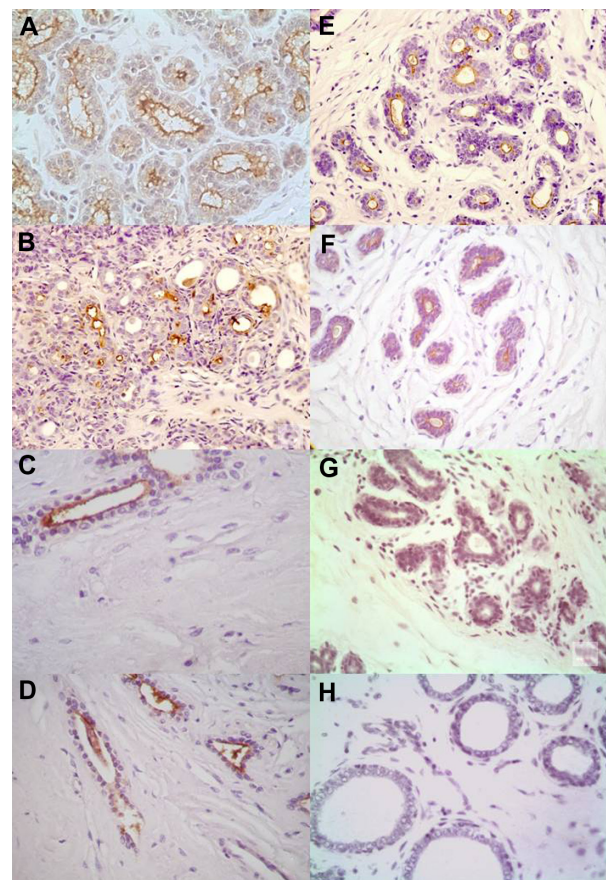

Figure 6 Photomicrographs of immunoperoxidase staining of benign breast diseases (**A–D**) and normal (**E–H**) are shown. Staining with anti-MUC1 HMFG1 monoclonal antibody (MAb), (**A**) an apical pattern of reaction with a strong intensity is observed in a non proliferative lesion; (**E**) normal mammary ducts are shown with an apical reactivity. Staining with anti-MUC1 C595 MAb, (**B**) a sample of an apocrine metaplasia is depicted; an apical mainly linear reaction is observed; (**F**) normal mammary gland sample showing a linear apical reaction. Expression of sialyl Lewis x (**C**) a strong linear staining in a fibroadenoma sample is shown and (**G**) a negative normal mammary gland sample is depicted. The TF antigenic reaction in (**D**) a fibroadenoma specimen is shown with a linear and apical pattern and in a (**H**) normal negative sample.

Table 5 Logistic regression analysis for patients grouped according to disease stage using Stage I as a reference and considering risk factors

Risk and prognostic factors	Stage I vs advanced stages
Age (years)	0.077; 0.396 (0.142–1.10)
Number of children	0.056; 3.71 (0.963–14.4)
Breastfeeding	0.363; 2.70 (0.314–23.5)
Menopausal age (years)	0.102; 0.261 (0.052–1.31)
Menarche age (years)	0.441; 1.47 (0.545–3.99)

Lewis x was expressed with a high intensity in the apical part of cell ducts; this pattern was found in all positive samples. Sialyl Lewis x (Figure 6G), Lewis y, and Tn epitopes were detected at the cytoplamic level and with a low intensity, whereas TF was not detected in any sample (Figure 6H).

To compare risk factors between normal group and malignant patients and also between benign versus malignant patients, a logistic regression analysis was performed. Results are summarized in Table 6; significant differences were found only between normal and malignant ages.

In our research, in BBD and normal women, risk factors such as age of menarche, parity, and breastfeeding, were similar to those for breast cancer patients. Significant differences were found between normal and malignant ages (Table 5).

Table 6 Logistic regression analysis for patients and controls (normal and BBD) considering risk factors.

Risk and prognostic factors	Normal vs malignant P (ORR CL)	BBD vs malignant P (ORR CL)
Age (years)	0.020; 128 (2.80–61873)[a]	0.054; 3.49 (0.947–362)
Number of children	0.973; 0.956 (0.069–13.1)	0.246; 20.3 (0.200–526)
Breastfeeding	0.656; 0.392 (0.006–24.2)	0.389; 0.199 (0.015–5.11)
Menopausal age (years)	0.115; 10.1 (0.567–179)	0.276; 0.527 (0.381–29.1)
Menarche age (years)	0.553; 2.98 (0.081–110)	0.742; 0.697 (0.098–26.2)

Note: [a]Indicates significant regression $P < 0.05$.
Abbreviations: BBD, benign breast disease; OR, odds ratio; ORR CL, odds ratio range confidence level for ±95% range.

This result was expected because normal samples were obtained from women who had had plastic surgery.

Discussion

Breast cancer studies by area can provide important clues to the underlying factors that influence the biological behavior of this disease. This knowledge would be useful to plan interventions and may serve to define indicators for preventive strategies.

It has been largely described that MUC1 may behave as an oncoprotein. Almost all breast cancer expresses this mucin, although normal breast cells express MUC1 as well. Due to the structural complexity of the MUC1 glycoprotein, multiple epitopes can be detected by monoclonal antibodies.[29] In this study we found a direct relationship between the percentage of MUC1-positive responses in respect of tumor size, between RI with disease stage, and also between the percentage of positive area and nuclear grade. Coincident with these results we found a significant decrease on MUC1 apical expression frequency according to histological as well as nuclear grade increment. Rakha et al[30] pointed out that histological grade can accurately predict tumor behavior. Rahn et al[19] reported that aberrantly localized MUC1 in the tumor cell cytoplasm or nonapical membrane is associated with a worse prognosis. The presence of apical membrane staining would indicate that MUC1 targeting pathways are intact, and it has been associated with better prognosis possibly related to functional differentiation of the tumor.[30] Rahn et al[19] found a significantly lower mean nuclear grade in tumors with high MUC1 expression (>50%). Our results showed a positive relationship between vascular invasion and Nottingham Prognostic Index ($P < 0.05$), and poorly differentiated tumors had higher rates of vascular invasion

than well and moderately differentiated tumors ($P < 0.005$). In large tumors, vascular invasion was detected more frequently.

Some authors argue that the glycosylation pattern of MUC1 expressed by normal mammary epithelium is based on elongated Core 2 structures, whereas in breast carcinomas the glycans expressed on MUC1 are often shorter and Core 1 based.[31] In contrast, Müller and Hanisch[32] found that no general pattern could be regarded as breast cancer associated and demonstrated a preponderance of Core 2-based structures. These structures have been associated with sialyl Lewis x determinant,[33] which acts as a ligand for binding carcinoma cells to endothelium in different tumor localizations, including breast cancer.[34,35] In this sense, we found that tumors with sialyl Lewis x expression frequently showed more vascular invasion. This parameter was evaluated by conventional histological assessment, which is considered an adequate method of examination.[36] Furthermore, we found that patients whose tumors expressed more sialyl Lewis x had a large number of metastatic lymph nodes ($P < 0.01$) and that patients with sialyl Lewis x reactive primary tumors presented an increment of the frequency on the number of metastatic lymph nodes ($P > 0.05$). However, we found that sialyl Lewis x expression was reduced in lymph nodes compared with the primary tumor. This finding coincides with that of Jeschke et al.[37]

In a study that comprised more than 16,000 patients, Ejlertsen et al[28] defined low-risk patients as those patients who were older than 35 years at diagnosis, with LN-negative disease, a tumor size ≤ 2 cm, and with a positive hormonal status. Taking into account this classification, in our study most of the low-risk patients (87%) did not present vascular invasion, whereas MUC1 apical expression was found in 25% of them. Vascular invasion-positive tumors did not show MUC1 apical reaction at all. This would be related to a defective MUC1 pathway that is associated with lack of functional differentiation and worse prognosis.[19]

It is considered that MUC1 is one of the molecules bearing the epitope sialyl Lewis x in cancer cells,[38] as well as other carbohydrate epitopes such as Lewis x and Lewis y.[39] In this report, a positive correlation between MUC1 versus Lewis x ($r = 0.8830$; $P < 0.05$) was found and also a tendency between MUC1 and Lewis x apical expression. These results indicate that MUC1 and Lewis x would be coexpressed, which would suggest that this mucin may be a Lewis x carrier. In accordance with Brooks and Leathem,[40] we have detected a higher Lewis x expression in normal and benign samples than in breast cancer samples.

In all cases, we observed a strong, mainly apical, staining, although no association with histological grade was found. However, Lewis x showed a higher percentage of reactive area in more differentiated tumors ($P < 0.05$). Brooks and Leathem[40] found Lewis x expression to be associated with invading tumor areas, whereas Elola et al[41] confirmed that MCF-7 cancer cells binding to HUVEC partially depended on Lewis x epitopes.

Overexpression of Lewis y antigen has frequently been found in human cancers[42] and has been observed to be associated with poor prognosis.[43] Our results showed that the percentage of Lewis y-positive responses increased according to tumor size increment ($P < 0.05$) with different patterns, and many positive samples showed a linear expression. Basu et al[44] observed Lewis y at the plasma membrane either as a glycolipid or glycoprotein or linked to surface receptors, such as epidermal growth factor receptors. On the other hand, Klinger et al[45] showed that two anti-Lewis y antibodies inhibited the ErbB-induced stimulation of downstream signalling by intracellular rerouting of internalized EGF receptors.

Tn and TF are early stage-specific differentiation and carcinoma-associated antigens that are related to alterations on mucin type *O*-linked glycosylation and often result in shorter glycan chains.[31] In accordance to other authors,[46] our results showed that TF and Tn antigens were expressed in hyperplastic benign diseases and also that, in cancer, their expression showed a positive association with disease stage. Furthermore, the percentage of positive responses augmented in relation to increased tumor size. Employing multivariate analysis, Tsuchiya et al[7] found that Tn expression correlated significantly with tumor size, nodal status, and overall survival. Although it has been suggested that Tn and TF antigens may constitute cancer prognostic factors and that they may play significant roles in cancer development, their implications need further investigation.

In this study we analyzed several parameters related to hormonal aspects and antigenic expression. In this sense, we found a significant correlation between MUC1 RI and ER status. This positive correlation was found in the majority of studies previously published, although considering other parameters such as the percentage of tumor cells.[16] Furthermore, subgroup analysis according to hormone receptor (ER and PR) and antigenic expression showed that ER-positive and PR-positive groups presented higher MUC1 RI than ER-negative and PR-negative groups (statistically different). It is known that ER-positive tumors have better prognosis than negative ones, and

almost all tumors expressed MUC1. There is evidence that steroid hormones play a role in MUC1 gene and protein expression. In this sense, McGuckin et al[47] found that progesterone caused significant increases in cells and secreted MUC1 in breast cancer cell lines. Moreover, in MCF7 cells, Ren et al[48] concluded that estradiol treatment resulted in a gradual increase in MUC1 mRNA expression. Our results showed that with breastfeeding and when a patient had at least one child, MUC1 expression increased. Also, with breastfeeding, antigens involved in tumor dissemination here studied diminished their expression, although their apical pattern increased, suggesting a more differentiated phenotype. It is very interesting that our PCA analysis including RI of MUC1 and glycan antigenic expression and ER and PR separated a group of patients who were characterized by their low age, premenopause status, less differentiated tumors, and more advanced disease stages. In this analysis, which explained 59% of data variability, menopause status appeared as a relevant factor, because premenopause patients showed less differentiated tumors. When ER and PR were included in this study, premenopause patients with the lowest values were separated. It is considered that exposure of ovarian hormones throughout life affects the risk of developing breast cancer. A meta-analysis of 47 epidemiological studies conducted in 30 countries concluded that the relative risk of breast cancer decreased by 4.3% (95% confidence interval 2.9–5.8; $P < 0.00001$) for every 12 months of breastfeeding in addition to a decrease of 7.0% (5.0–9.0; $P < 0.0001$) for each birth.[49]

Tumor cell membrane immunoreactivity for c-erbB2 (Her2-neu) is associated with poorer survival and is more frequently seen in Grade III tumors.[50] In our analysis, the Her2-neu+ subgroup presented a large number of positive nodes, less differentiated tumors, and higher nuclear grades than in the Her2-neu negative subgroup. Furthermore, it is interesting that Her2-neu+ status showed a significant association with sialyl Lewis x expression.

The interpretation of breast cancer incidence and mortality patterns is complex in view of the numerous and interactive known and putative risk determinants. In this sense, our results emphasize that the carbohydrate antigenic relationship with risk and prognostic factors contributes to explain breast cancer biological heterogeneity.

The relationship between the carbohydrate antigens selected and risk factors such as breastfeeding and number of children in this malignancy is of special significance, as these factors have been associated with breast protection. The

panel of antigens studied has been related to tumor progression and dissemination.

Disclosure

Professor María Virginia Croce and Dr Marina Isla-Larrain are members of the research team of the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC/PBA). Professor Sandra O Demichelis is a scientific researcher for the Faculty of Natural Sciences and Museum, the National University of La Plata, and the Department of Biology, Kennedy University of Argentina, Buenos Aires, Argentina.

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