Clinical and morphological factors associated with two groups of concordant breast cancer immunophenotypes: a cross-sectional study of 24-year historical series

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Abstract

This study aimed to describe the prevalence of invasive breast cancer (IBC) in women assisted in a public hospital in Brazil and to establish a correlation between two models of classification by immunophenotypes, one of them based on the 13th St. Gallen Conference classification and the other on biomarker-defined subtypes based on HER2 and oestrogen receptor status, as described in World Health Organization (WHO). We selected IBC of 1335 cases between 1994 and 2018. Univariate frequencies and associations were estimated using chi-square tests. The concordance between the two immunohistochemical analysis models above mentioned using Cohen's kappa coefficients. The most prevalent subtype was luminal B/HER2, and the frequency of tumours with a worse prognosis was 62.7%. Has been identified an association between histological grade 3 (G3) and the worst prognostic subtypes: non-luminal A, Triple Negative Breast Cancer (TNBC), non-ER+/HER2- and ER-/HER2-. A similar association was found in nuclear G3 tumours. The results showed agreement between 99.48% and 100% when we compared the two immunohistochemical analysis models. Furthermore, there was an absolute agreement

between the two models of immunohistochemical analysis. These results can contribute to institutions that do not have a molecular investigation, enabling accessible routine practice tools. Among the most important questions addressed in this work is the association between histological and nuclear G3 with the worst prognostic subtypes. Using the St. Gallen Conference classification and HER2 and ER status based on subtypes verified the feasibility of selecting IBC with different prognoses and correlated them with recognized predictive and prognostic factors.

Keywords: Invasive Breast Cancer; Immunohistochemical; Immunophenotypes; HER2; Oestrogen Receptor.

1. Introduction

Breast cancer is the most incident cancer among women worldwide [1]. In 2018, 2.1 million new cases were reported, with an estimated death toll of 627,000 patients [1]. The widespread implementation of breast screening programs, as well as advances in molecular biology and the development of new chemotherapeutic drugs, has contributed to a recent improvement in survival rates in high-income countries [1-3]. Furthermore, the cancer genome study led to the elucidation of intrinsic subtypes of invasive breast cancer (IBC), improving targeted therapies' success rate [1-4].

Cancer is a multifactorial disease with a strong relationship between genetic and non-genetic factors [1]. The accumulation of numerous molecular alterations leads to cell proliferation, genetic instability, and acquisition of resistant and invasive phenotypes [5]. Tumour progression to different phenotypes results from gene activity changes affecting the internal environment and tumour cells' vicinity, a combination of exogenous factors, and the individual's intrinsic genetic variability [6, 7].

Protein profile, DNA, RNA, and genome distribution studies have been conducted to portray tumour phenotypes more accurately [8]. A systematic and detailed characterization of tumours on a genomic scale can be correlated with clinical information, contributing to an increased understanding of the causes of cancer and its progression [8]. As our knowledge of tumour phenotypes evolves, new molecular markers with the potential to improve therapeutic interventions may be discovered [8-10]. For instance, gene expression profiling allowed identifying five intrinsic subgroups of invasive breast cancer [9] that were later validated by immunohistochemical analysis of protein expression [8, 10-12]. Thus, protein characterization may provide valuable prognostic and predictive markers for invasive breast cancer [12, 13]. The importance of St. Gallen consensus positioned the luminal A as an indolent tumour with a better prognosis, and luminal B (HER2- and HER2+) was the most frequent subtype, with a high proliferative index, worse prognosis, and less sensitivity to endocrine therapy [14,23,30].

This study aimed to (i) evaluate the prevalence of invasive breast cancer in women, (ii) investigate the correlation between two immunohistochemical classification systems, one according to the classification of 13th St Gallen Conference [14] and the other based on WHO, HER2 and estrogen receptor (ER) status [1], and (iii) examine the clinical and morphological characteristics of these cases. The most important issue addressed in this work is to verify the possible association between the morphological degrees (histological and nuclear) and the subtypes immunohistochemical with the worst prognosis, using only two

immunohistochemical biomarkers (ER and HER2). It will allow the attending physician to identify women with high-risk breast carcinomas, making it possible in some centers around the world, where financial resources are scarce, a standardized follow-up improving the chances of specific therapies.

2. Materials and Methods

This study is a descriptive observational non-randomized cross-sectional analytical study. The studies were approved by the Human Research Ethics Committee of the Federal University of Santa Catarina (the first part of the project filed under protocol no. 141/2005 and the second under protocol no. 52861715 0 0000 0121). Criteria adopted at the time of diagnosis were reviewed by an unblinded senior pathologist, in 2019, from February to November.

2.1 Patient selection

We performed a retrospective database analysis. Out of 145014 biopsies received between January 1994 and December 2018 in a public hospital of Federal University of Santa Catarina (Brazil), samples from 1510 women with a histological diagnosis of invasive breast cancer were referred to selected. Of these, 1335 were included in the study, and 175 samples were lost for the following reasons: (i) material not found in the file, (ii) lack of immunohistochemical results, (iii) absence of clinical data in medical records, and (iv) samples from male patients.

2.2 Data collection

Were selected from the 1335 samples: age, histological type, histological grade, nuclear grade, and immunohistochemistry data were transcribed from archived records. The hospital did not perform immunohistochemical examinations between 1994 and 2004. Researchers from the Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Portugal, performed the immunohistochemical tests of this period. As of 2005, immunohistochemical studies were conducted by the Department of Pathology of the Federal University of Santa Catarina.

2.3 Clinical and morphological criteria

Patients aged 18 years or older were selected, regardless of weight or ethnic group. Age was collected in full years at the time of diagnosis and divided into three groups: <50 years, 50 – 69 years, and ⩾70 years. The 50 – 69 years age group was selected as the reference population, as defined by World Health Organization (WHO) screening recommendation [1]. The tumours were stratified according to WHO [1] guidelines for histological evaluation into invasive breast carcinoma of no special type (NST) or invasive lobular carcinoma (ILC) (e-cadherin-negative cases) [1, 15]. Histological grading was performed by independently assessing tubule formation, nuclear pleomorphism, and mitotic count. These data were combined to obtain the histological Nottingham grade (G), which includes the following categories: G1, well-differentiated; G2, moderately differentiated; and G3, poorly differentiated [16]. The nuclear grade was assessed by size and pleomorphism into G1, low grade; G2, intermediary; and G3, high grade [16].

2.4 Criteria used for immunohistochemical evaluation

Information on oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and proliferative index (Ki-67) were gathered. Tumours were considered ER- or PR-positive if at least 1% of nuclei demonstrated positivity [1, 15]. HER2 status was categorized as positive when at least 10% of tumour cells exhibited a membrane staining score of 3+, as equivocal when more than 10% of tumour cells had a weakly/moderately positive membrane staining score (2+), and as negative when neither of these criteria applies [1, 15, 17]. Equivocal cases were not categorized subtype, as the required data to obtain in situ hybridization (ISH) results were not available. For Ki-67, the mean percentage of nuclear positivity was evaluated between two cut-off points, the lower 15% and the higher 15% of results, and, in parallel, mitotic counts were performed [1, 14, 15, 18, 19]. Two scorers reviewed any discordant scores until consensus was reached.

2.5 Determination of breast cancer subtypes by immunohistochemistry

Two classification systems based on immunohistochemistry biomarkers were used, the 13th St Gallen Consensus Conference classification [14] and the ER/HER2-based subtype classification (see Supplementary Table 1; Appendix 1).

2.6 Statistical analysis

Frequencies and their respective percentages were estimated for each variable of interest. Univariate associations were assessed using chi-square tests. Odds ratios (OR) and 95% confidence intervals (95%CI) were calculated. Cohen's kappa coefficient measured agreement between two immunohistochemical classification systems (κ). Calculations were performed using Stata/SE v. 14.0 (Stata Soft, College Station, Texas, USA).

Sample size calculation: The study's primary outcome was the agreement between the 13th St Gallen Consensus Conference classification and ER/HER2-based subtype classification. A minimum Cohen's kappa coefficient of 0.95 was deemed clinically significant. As the estimated percentage of female breast cancer samples in the sample pool at our institution was 1% and considering a type I error (α) equal to 5%, a type II (β) error probability equal to 10%, and losses equal to 15%, a sample size of 1150 was estimated (https://wnarifin.github.io/ssc/sskappa.html).

3. Results.

The predominant histological type of invasive breast cancer was NST (92.5%, n = 1.235), followed by ILC (7.5%, n = 100) (data not shown). Tumors were more common in the 50-69 age group (48.8%, n = 652) (Table 1). As evidenced by morphological characteristics, histological and nuclear grade 2 tumors were the most prevalent (Table 1). Most of the cases were ER+ (75.3%, n = 1005) and 16.6% were HER2+ (n = 221).

Table 1. Demographic data, frequency of IBC morphological characteristics and IHC biomarkers.

Age group	**Cases % (*n*)**		
		Media ± DP	Median (lower - higher value)
<50 years	35.0 (467)	42.4 ± 5.5	44 (24 – 49)
50-69 years	48.8 (651)	58.9 ± 5.5	59 (50 – 69)
≥70 years	16.2 (217)	77.4 ± 5.8	77 (70 – 96)
Total	100 (1335)	56.1 ± 13.2	55 (24 – 96)
Morphological characteristics		**Histological grade % (n)**	**Nuclear grade % (n)**
	G1	24.3 (325)	13.4 (179)
	G2	46.2 (617)	50.8 (678)
	G3	29.5 (393)	35.8 (478)
	Total	100 (1335)	100 (1335)
IHC biomarkers		**Positive**	**Negative**
ER % (*n*)		75.3 (1005)	24.7 (330)
PR % (*n*)		62.0 (828)	38.0 (507)
HER2 % (*n*)		16.6 (221)	70.7 (944) *

n: number of cases; IBC: invasive breast cancer; *n*: number of cases; variable histological grade (G1: Grade 1/ well-differentiated; G2: Grade 2/moderately differentiated; G3: Grade 3/poorly differentiated) and variable nuclear grade (G1: Grade 1/low grade; G2: Grade 2/intermediary grade; G3: Grade 3/high grade). ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2, based on immunohistochemical results. *Equivocal: 12.7% (170).

Among subtypes defined by 13th St Gallen Conference [14] criteria, luminal B/HER2− showed the highest prevalence (32%, *n* = 427) (Table 2). ER, positivity was observed in 64.8% (*n* = 865) of cases, of which 55.4% were $ER^+/HER2^-$ and 9.4% were $ER^+/HER2^+$.

Table 2. Prevalence of IHC subtypes according to the St Gallen Conference14 (group 1) and distribution of subtypes on basis of ER and HER2 status IHC1 (group 2).

IHC subtypes group 1	**ER**	**PR**	**HER2+**	**Ki-67**	**% (*n*)**
Luminal A	+	+/-	-	Low	23.4 (313)
Luminal B/HER2-	+	+/-	-	High	32.0 (427)
Luminal B/HER2+	+	+/-	+(3+)	Any	9.9 (132)
HER2+	-	-	+(3+)	Any	6.7 (89)
TNBC	-	-	-	Any	14.1 (188)
Equivocal	+/-	+/-	+(2+)	Any	12.7 (170)
Unclassified	-	+	Any	Any	1.2 (16)
Total					100 (1335)
IHC subtypes group 2					

ER+/HER2-	+		n/a	-	n/a	55.4 (740)
ER+/HER2+	+		n/a	+(3+)	n/a	9.4 (125)
ER-/HER2+	-		n/a	+(3+)	n/a	7.2 (96)
ER-/HER2-	-		n/a	-	n/a	15.3 (204)
Equivocal		+/-	n/a	+(2+)	n/a	12.7 (170)
Total						100 (1335)

IHC: immunohistochemical; ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; Ki67: Proliferation marker; TNBC: triple negative breast cancer; by analyses into the: +: positive; -: negative; +/-: positive or negative; +(3+): positive if at least 10% of tumor cell exhibited a cell membrane staining score of 3+; n: number of cases; n/a: not applicable.

Table 3 shows the relationship between age group and morphological characteristics with IHC subtypes according to the St Gallen Conference14 (group 1) and distribution of subtypes based on ER and HER2 status IHC1 (group 2) frequency. The ER+/HER2+ was the most prevalent subtype in younger patients (<50 years age group) (52.0%); ER−/HER2+, in the 50–69 years age group (58.3%); and ER−/HER2−, in the ≥70 years age group (17.7%). In the 51.7% of luminal A tumours were histological grade 1, 53.9% of luminal B/HER2− were histological grade 2, and 71.8% of TNBC were histological grade 3. Among histological grade 1 and 2 tumours, ER+/HER2− and ER+/HER2+ subtypes were the most frequent. ER−/HER− was the most common subtype among histological grade 3 tumours. Regarding nuclear grade, 29.1% of luminal A tumours were grade 1, and 69.1% of TNBC corresponded to grade 3. Luminal A and luminal B/HER2− tumours were more frequently nuclear grade 2, whereas luminal B/HER2+, HER2+, and TNBC subtypes were predominantly nuclear grade 3. Table 3 also shows that the most frequent nuclear grade among ER+/HER2− cases was grade 2. A higher frequency of nuclear grade 2 and 3 tumours in ER+/HER2+ cases. However, among ER−/HER2+ and ER−/HER2− cases, nuclear grade 3 tumours were more frequent.

Table 3. Frequency of St Gallen Conference[14] subtypes and of ER and HER2 biomarkers[1] by histological types (IBC NST and ILC), age groups and morphological characteristics.

	Luminal A	Luminal B / HER2-	Luminal B / HER2+	HER2+	TNBC	ER+ / HER2-	ER+ / HER2+	ER-/HER2+	ER-/HER2-
	% (*n*)	% (*n*)	% (*n*)	% (*n*)	% (*n*)	% (*n*)	% (*n*)	% (*n*)	% (*n*)
Histological type									
IBC NST	86.9 (272)	91.8 (392)	96.2 (127)	100 (89)	96.8 (182)	89.7 (664)	96.8 (121)	99.0 (95)	97.1 (198)
ILC	13.1 (41)	8.2 (35)	3.8 (5)	0.0 (0)	3.2 (6)	10.3 (76)	3.2 (4)	1.0 (1)	2.9 (6)
Total	100 (313)	100 (427)	100 (132)	100 (89)	100 (188)	100 (740)	100 (125)	100 (96)	100 (204)
Age group (years)									
<50	30.7 (96)	33.7 (144)	50.8 (67)	24.7 (22)	38.8 (73)	32.4 (240)	52.0 (65)	25.0 (24)	38.2 (78)

50–69	48.2 (151)	52.5 (224)	40.9 (54)	57.3 (51)	42.6 (80)	50.7 (375)	39.2 (49)	58.3 (56)	44.1 (90)
≥70	21.1 (66)	13.8 (59)	8.3 (11)	18.0 (16)	18.6 (35)	16.9 (125)	8.8 (11)	16.7 (16)	17.7 (36)
Total	100 (313)	100 (427)	100 (132)	100 (89)	100 (188)	100 (740)	100 (125)	100 (96)	100 (204)
Histological grade									
G1	51.7 (162)	22.2 (95)	12.9 (17)	7.9 (7)	5.3 (10)	34.7 (257)	13.6 (17)	7.3 (7)	5.4 (11)
G2	46.3 (145)	53.9 (230)	53.0 (70)	42.7 (38)	22.9 (43)	50.7 (375)	52.8 (66)	43.7 (42)	24.0 (49)
G3	1.9 (6)	23.9 (102)	34.1 (45)	49.4 (44)	71.8(135)	14.6 (108)	33.6 (42)	49.0 (47)	70.6 (144)
Total	100 (313)	100 (427)	100 (132)	100 (89)	100 (188)	100 (740)	100 (125)	100 (96)	100 (204)
Nuclear grade									
G1	29.1 (91)	11.9 (51)	4.6 (6)	3.4 (3)	3.2 (3)	19.2 (142)	4.8 (6)	3.1 (3)	2.9 (6)
G2	60.1 (188)	60.9 (260)	44.7 (59)	37.1 (33)	27.7 (52)	60.5 (448)	44.8 (56)	37.5 (36)	27.5 (56)
G3	10.8 (34)	27.2 (116)	50.7 (67)	59.5 (53)	69.1 (130)	20.3 (150)	50.4 (63)	59.4 (57)	69.6 (142)
Total	100 (313)	100 (427)	100 (132)	100 (89)	100 (188)	100 (740)	100 (125)	100 (96)	100 (204)

n: number of cases; HER2: human epidermal growth factor receptor 2; TNBC: triple negative breast cancer; variable group: histological type: IBC NST: invasive breast carcinoma of no especial type and ILC = invasive lobular carcinoma; variable group: Age group; histological grade (G1: Grade 1/ well-differentiated; G2: Grade 2/moderately differentiated; G3: Grade 3/poorly differentiated); nuclear grade (G1: Grade 1/low grade; G2: Grade2/intermediary grade; G3 Grade 3/high grade) and the five molecular subtypes and the four molecular subtypes.

As demonstrated in Table 4, the chance of developing a non-luminal A tumour in the ≥70 years age group was 1.56 times lower than in control, luminal A (OR = 0.64, 95%CI = 0.47–0.90, p = 0.0084). Comparison between outcomes of TNBC and non-TNBC cancers revealed no associations between age groups and these subtypes. However, the chances of having a non-$ER^+/HER2^-$ subtype in the <50 years age group was 1.28 times higher than in control, $ER^+/HER2^-$ (OR = 1.28, 95 %CI = 1.02–1.61, p = 0.0296).

Table 4. Pattern of association between the prevalence of subtypes by the Consensus of St Gallen[14] and the biomarkers ER/HER2[1] by age groups.

Age Group			**OR**	**95% CI**	***p***
	Non-Luminal A	Luminal A			
<50 years	371	96	1.29	0.98 – 1.70	0.0680†
	651	217			
50–69 years	500	151	1.02	0.80 – 1.32	0.8330†
	522	162			
≥70 years	151	66	0.64	0.47 – 0.90	0.0084**
	871	247			
	TBNC	Non TBNC			
<50 years	73	394	1.21	0.88 – 1.66	0.2331†

	115	753			
50–69 years	80	571	0.74	0.54 – 1.02	0.0667†
	108	576			
≥70 years	35	182	1.21	0.81 – 1.81	0.3442†
	153	965			
	Non-ER+/HER2-	ER+/HER2-			
<50 years	227	240	1.28	1.02 – 1.61	0.0296*
	368	500			
50–69 years	276	375	0.84	0.68 – 1.64	0.1193†
	319	365			
≥70 years	92	125	0.90	0.67 – 1.20	0.4817†
	503	615			
	ER-/HER2-	Non-ER-/HER2-			
<50 years	78	389	1.18	0.87 – 1.60	0.2901†
	126	742			
50–69 years	90	561	0.80	0.59 – 1.08	0.1497†
	114	570			
≥70 years	36	181	1.12	0.75 – 1.66	0.5583†
	168	950			

OR: odds ratio; 95% CI: 95% confidence interval. Variables: Age group in years and the worst prognostic immunophenotypes by molecular subtypes (Non-Luminal A, TNBC: triple negative breast cancer, non-ER+/HER2-, ER-/HER2-: ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2, by analyses into the: +: positive; -: negative). †$p < 0.1$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

An association was found between histological grade 3 and the poor-prognosis subtypes non-luminal A (OR = 31.18, 95%CI = 13.76–70.64), TNBC (OR = 8.77, 95%CI = 6.20–12.41), non-$ER^{+}/HER2^{-}$ (OR = 5.37, 95%CI = 4.11–7.04), and $ER^{-}/HER2^{-}$ (OR = 8.50, 95%CI = 6.10–11.85) (Table 5).

Table 5. Pattern of association between the subtypes by the Consensus of St Gallen[14] and the biomarkers ER/HER2[1] by histological degree.

Histologicalgrade			OR	95% CI	*p*
	Non-Luminal A	**Luminal A**			
G1	163	162			
			0.17	0.13 - 0.23	<0.001***
	859	151			
G2	472	145			
			1.0	0.77 - 1.28	0.9649†
	550	168			
G3	387	6			
			31.18	13.76 - 70.64	<0.001***
	635	307			
	TNBC	**Non TNBC**			
G1	10	315			
			0.14	0.07 - 0.28	<0.001***
	178	832			
G2	43	574			
			0.30	0.20 - 0.42	<0.001***
	145	573			
G3	135	258			
			8.77	6.20 - 12.41	<0.001***
	53	889			
	Non-ER+/HER2-	**ER+/HER2-**			
G1	68	527			
			0.24	0.17 - 0.32	<0.001***
	257	483			
G2	242	353			
			0.66	0.53 - 0.83	0.003**
	375	365			
G3	285	310			
			5.37	4.11 - 7.04	<0.001***
	108	632			
	ER-HER2-	**Non-ER-HER2-**			
G1	11	314	0.14	0.08 - 0.27	<0.001***
	193	817			
G2	49	568			
			0.31	0.22 - 0.44	<0.001***
	155	563			

G3	144	249			
	60	882	8.50	6.10 - 11.85	<0.001***

OR: odds ratio; 95% CI: 95% confidence interval. Variable group: histological grade (G1: Grade 1/well-differentiated; G2: Grade 2/moderately differentiated; G3: Grade 3/poorly differentiated) and the worst prognostic immunophenotypes by molecular subtypes (Non-Luminal A, TNBC: triple negative breast cancer, non-ER+/HER2-, ER-/HER2-: ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2, by analyses into the: +: positive; -: negative). †p < 0.1, *p < 0.05, **p < 0.01, ***p < 0.001

A similar correlation was observed between nuclear grade 3 and non-luminal A (OR = 6.3, 95%CI = 4.29–9.47), TNBC (OR = 5.14, 95%CI = 3.64–7.31), non-ER$^+$/HER2$^-$ (OR = 4.83; 95%CI = 3.80–6.15), and ER$^-$/HER2$^-$ (OR = 5.41, 95%CI = 3.92–7.50) (Table 6).

Table 6. Pattern of association between the prevalence of subtypes by the Consensus of St Gallen[14] andthe biomarkers ER/HER2[1] by nuclear degree.

Nuclear grade			OR	95% CI	*p*
	Non-Luminal A	**Luminal A**			
G1	88	934			
			0.22	0.16 - 0.32	<0.001***
	91	222			
G2	490	532			
			0.61	0.47 - 0.80	0.002**
	188	125			
G3	444	578			
			6.3	4.29 - 9.47	<0.001***
	34	279			
	TNBC	**Non TNBC**			
G1	6	182			
			0.18	0.07 - 0.42	<0.001***
	173	974			
G2	52	136			
			0.31	0.22 - 0.45	<0.001***
	626	521			
G3	130	58			
			5.14	3.64 - 7.31	<0.001***
	348	799			
	Non-ER+/HER2-	**ER+/HER2-**			
G1	37	142			
			0.27	0.19 - 0.40	<0.001***
	558	598			

G2	230	448			
			0.41	0.32 - 0.51	<0.001***
	365	292			
G3	328	150			
			4.83	3.80 - 6.15	<0.001***
	267	590			
	ER-HER2-	**Non-ER-HER2-**			
G1	6	173			
			0.16	0.07 - 0.38	<0.001***
	198	958			
G2	56	622			
			0.31	0.22 - 0.43	<0.001***
	148	509			
G3	142	336			
			5.41	3.92 - 7.50	<0.001**
	62	795			

OR: odds ratio; 95% CI: 95% confidence interval. Variable group: nuclear grade category (G1: Grade 1/low grade;G2: Grade 2/intermediary grade; G3: Grade 3/high grade) and the worst prognostic immunophenotypes by molecular subtypes (Non-Luminal A; TNBC: triple negative breast cancer; non-ER+/HER2-; ER-/HER2-: ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2, by analyses intothe: +: positive; -: negative). †p < 0.1, *p < 0.05, **p < 0.01, ***p < 0.001.

The percentage of agreement between luminal A and luminal B/HER2$^-$ subtypes and ER$^+$/HER2$^-$ was 100% (κ = 1; standard error, SE = 0.03; p < 0.001). The agreement percentage between luminal B/HER2$^+$ and ER$^+$/HER2$^+$ was 99.48% (κ = 0.97, SE = 0.02, p < 0.001). In comparing HER2$^+$ and ER-/HER2$^+$, we found an agreement of 99.48% (κ = 0.96, SE = 0.03, p < 0.001). TNBC and ER$^-$/HER2$^-$ showed an agreement of 100% (κ = 1, SE = 0.03, p < 0.001).

4. Discussion

Breast cancer is a major public health problem and the most common cancer among women worldwide [1]. Although there are various screening programs, biomarkers' laboratory diagnosis is essential to define tumour subtype, treatment, prognosis, and treatment and post-treatment surveillance schemes [1]. In this study, 1335 cases of women referred to a public hospital in Brazil between 1994 and 2018 were assessed. Immunohistochemical profiles were examined, and the correlation between two immunohistochemical classification methods was determined.

Invasive breast cancer was more frequent in the 50–69 years age group (48.8%, Table 1), and the predominant histological type was NST (92.5%). The most frequent histological and nuclear grades were grades 2 (Table 1). These results are in line with literature data [1] (Table 3); women aged <50 years were 1.28 times more likely to develop non-ER$^+$/HER2$^-$ tumours than women aged 50–69, those aged ≥70 years

were 1.56 times less likely to develop non-luminal A. Nevertheless, there was no difference between the OR of developing poor-prognosis subtypes among the three age groups.

Histological grading is a major prognostic factor for determining adjuvant therapy in invasive breast cancer [1]. Grading criteria can also be combined with gene expression profiles assessed by immunohistochemistry for prognosis determination [1, 15, 20]. Histological grade 3 tumours are associated with poor differentiation and high aggressiveness [1, 20, 21]. This study found a correlation between luminal A and histological grade 1 tumours (Table 2). Histological grade 3 tumours, on the other hand, were frequently identified as $HER2^+$ and TNBC (Table 2).

ER, PR and HER2 are widely used as biomarkers of invasive breast cancer [1, 11, 22-26]. ER is an important prognostic and predictive factor, and PR is greatly associated with overall survival [10, 23]. According to the literature, ER and PR are positive in 60–75% and 65% of invasive breast cancer cases, respectively [21, 27]. A similar result was found in this study, were ER and PR positivity frequencies were 75.3% and 62%, respectively (Table 1). In such cases, tumour growth can be inhibited by anti-oestrogenic therapy [26]. HER2/*neu* gene amplification or overexpression of its oncoprotein occurs in 20–30% of invasive breast cancer cases [1, 17, 27, 28, 29]. In the current study, HER2 overexpression was observed in 16.6% of tumours (Table 1), of which 92.1% were histological grades 2 or 3 (Table 2). $HER2^+$ subtypes are associated with poor prognosis, high histological grade, reduced survival rate, increased recurrence, and mortality [17, 27, 29]. Its amplification is used as a predictive factor of response to target therapy [10, 13].

The St Gallen consensus defined luminal A as an indolent tumour with a better prognosis. Luminal B ($HER2^-$ and $HER2^+$) was reported as the most frequent subtype, with a high proliferative index, worse prognosis, and low sensitivity to endocrine therapy [14, 23, 30]. In this study, 41.9% of cases were luminal B (Table 2). However, the agreement of luminal A and luminal B/$HER2^-$ subtypes with $ER^+/HER2^-$ was 100%, indicating the need for tools to differentiate between luminal A and luminal B/$HER2^-$, as the proliferation signature is more expressed in luminal B [19, 31]. The Ki-67 index can be used to define and stratify tumours between luminal subtypes, especially luminal B/$HER2^-$ [23]. However, there are many controversies regarding Ki-67, mainly because of the lack of standardization [15, 32-34]. This index is one of the main determinants of most genomic predictors designed to separate tumours into prognostic subgroups [35, 36]. Despite these limitations, it is possible that careful evaluation, combined with aspects of the Nottingham prognostic index [16], can lead to the characterization of cases into a good or poor response to adjuvant therapy [16, 36]. This hypothesis is supported by the almost perfect agreement between methods, as evidenced by the high Cohen's kappa coefficient.

TNBC are histologically and clinically aggressive tumours that often lead to early recurrence [21, 27]. The frequency of this subtype varies between 10–25% [20, 27], in agreement with the results of the present study (14.1%) (Table 2). TNBC tumours have a worse prognosis than other subtypes [4, 10, 20]. Tumours associated with *BRCA1* gene mutation are usually $ER^-/HER2^-$ or TNBC [1, 4, 37, 38]. In this study, $ER^-/HER2^-$ was identified in 15.3% of cases (Table 2).

An important result of this research was histological and nuclear grade 3 with poor prognosis subtypes (Tables 4 and 5). Univariate analysis of histological grade 3 tumours showed high risks of developing aggressive tumours than control cases (Table 4). Nuclear grade 3 tumours showed the same

patterns (Table 5). These results show the relationship of cases with morphological characteristics of aggressiveness (nuclear and histological). Previous studies reported prognostic and predictive findings that associated luminal B, TNBC, and HER2$^+$ tumours with a high risk of recurrence and poor outcome [10, 39].

In this study, we investigated the agreement between two classification methods that use immunohistochemistry for determining subtypes. The first was based on the 13th St Gallen Conference and used four classic biomarkers. The second was based on two biomarkers (ER/HER2). The results showed an agreement of 99.48 to 100% between methods. These classification systems assessed invasive breast cancers with different prognoses and correlated them with recognized predictive and prognostic factors. However, although an absolute agreement was observed between data, there are reports that classical immunohistochemical markers do not fully recapitulate intrinsic subtypes and are not precise substitutes for true intrinsic molecular subtype status [6, 25].

A limitation of the present study was that HER2 evaluation was based exclusively on immunohistochemistry. Gene amplification by *in situ* hybridization was not performed for equivocal cases (Table 2), generating a bias that can lead to analysis errors. The lack of family history also induced selection bias, as cases with family risk factors were not stratified. Lastly, immunohistochemistry results were not correlated with molecular tests to assess subpopulation or tumour heterogeneity.

5. Conclusion

In conclusion, among women with invasive breast cancer who attended a Brazilian public hospital between 1994 and 2018, the prevalent age group, cancer type, and histological and nuclear grades were 50–69 years, NST, and grade 2, respectively. Subtypes were distributed into luminal B/HER2$^-$ (32%), luminal A (23.4%), TNBC (14.1%), luminal B/HER2$^+$ (9.9%), and HER2$^+$ (6.7%). A high agreement was observed between subtype classification using two and four biomarkers, indicating that invasive breast cancer may be grouped within a sequence of subtypes defined by at least two biomarkers. When combined with overlapping morphological characteristics, these biomarkers can help determine subtypes and, therefore, contribute to predicting clinical outcomes and therapeutic responses. Worldwide efforts to improve the understanding of tumour heterogeneity at the histological and genomic levels are fundamental for designing clinical application tools. Our analysis shows that, in routine practice, it is possible to apply economically accessible tools, such as immunohistochemistry, for invasive breast cancer diagnosis, leading to positive socio-economic impacts. Subtypes of invasive breast cancer can be categorized for treatment purposes based on HER2 and ER statuses in agreement with WHO, thereby representing a low-cost option for institutions with few available resources.

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7. Potential Conflict of Interest Statement

The authors have no conflicts of interest to declare that are relevant to the content of this article.

8. References

[1] WHO Classification of Tumors Editorial Board. Breast Cancer. Lyon: International Agency for Research on Cancer; 2019.

[2] Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet. 2005 May 14-20;365(9472):1687-717. doi: 10.1016/S0140-6736(05)66544-0. PMID: 15894097.

[3] Trudeau M, Charbonneau F, Gelmon K, Laing K, Latreille J, Mackey J, "et al". Selection of adjuvant chemotherapy for treatment of node-positive breast cancer. Lancet Oncol. 2005 Nov;6(11):886-98. doi: 10.1016/S1470-2045(05)70424-1. PMID: 16257797.

[4] Ahn SG, Kim SJ, Kim C, Jeong J. Molecular Classification of Triple-Negative Breast Cancer. J Breast Cancer. 2016 Sep;19(3):223-230. doi: 10.4048/jbc.2016.19.3.223. Epub 2016 Sep 23. PMID: 27721871; PMCID: PMC5053306.

[5] Simon R, Mirlacher M, Sauter G. Tissue microarrays in cancer diagnosis. Expert Rev Mol Diagn. 2003 Jul;3(4):421-30. doi: 10.1586/14737159.3.4.421. PMID: 12877382.

[6] Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000 Jan 7;100(1):57-70. doi: 10.1016/s0092-8674(00)81683-9. PMID: 10647931.

[7] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011 Mar 4;144(5):646-74. doi: 10.1016/j.cell.2011.02.013. PMID: 21376230.

[8] Sørlie T. Molecular portraits of breast cancer: tumour subtypes as distinct disease entities. Eur J Cancer. 2004 Dec;40(18):2667-75. doi: 10.1016/j.ejca.2004.08.021. PMID: 15571950.

[9] Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, "et al". Molecular portraits of human breast tumours. Nature. 2000 Aug 17;406(6797):747-52. doi: 10.1038/35021093. PMID: 10963602.

[10] Prat A, Perou CM. Deconstructing the molecular portraits of breast cancer. Mol Oncol. 2011 Feb;5(1):5-23. doi: 10.1016/j.molonc.2010.11.003. Epub 2010 Nov 24. PMID: 21147047; PMCID: PMC5528267.

[11] Vuong D, Simpson PT, Green B, Cummings MC, Lakhani SR. Molecular classification of breast cancer. Virchows Arch. 2014 Jul;465(1):1-14. doi: 10.1007/s00428-014-1593-7. Epub 2014 May 31. PMID: 24878755.

[12] Bertucci F, Birnbaum D, Goncalves A. Proteomics of breast cancer: principles and potential clinical applications. Mol Cell Proteomics. 2006 Oct;5(10):1772-86. doi: 10.1074/mcp.R600011-MCP200. Epub 2006 May 29. PMID: 16733261.

[13] Shak S. Overview of the trastuzumab (Herceptin) anti-HER2 monoclonal antibody clinical program in HER2-overexpressing metastatic breast cancer. Herceptin Multinational Investigator Study Group. Semin Oncol. 1999 Aug;26(4 Suppl 12):71-7. PMID: 10482196.

[14] Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thürlimann B, "et al". Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International

Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. Ann Oncol. 2013 Sep;24(9):2206-23. doi: 10.1093/annonc/mdt303. Epub 2013 Aug 4. PMID: 23917950; PMCID: PMC3755334.

[15] Amin MB, Greene FL, Edge SB, Compton CC, Gershenwald JE, Brookland RK, "et al". The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. CA Cancer J Clin. 2017 Mar;67(2):93-99. doi: 10.3322/caac.21388. Epub 2017 Jan 17. PMID: 28094848..

[16] Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology. 1991 Nov;19(5):403-10. doi: 10.1111/j.1365-2559.1991.tb00229.x. PMID: 1757079..

[17] Wolff AC, Hammond MEH, Allison KH, Harvey BE, Mangu PB, Bartlett JMS, "et al". Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update. Arch Pathol Lab Med. 2018 Nov;142(11):1364-1382. doi: 10.5858/arpa.2018-0902-SA. Epub 2018 May 30. PMID: 29846104..

[18] Soliman NA, Yussif SM. Ki-67 as a prognostic marker according to breast cancer molecular subtype. Cancer Biol Med. 2016 Dec;13(4):496-504. doi: 10.20892/j.issn.2095-3941.2016.0066. PMID: 28154782; PMCID: PMC5250608..

[19] Cheang MC, Chia SK, Voduc D, Gao D, Leung S, Snider J, "et al". Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. J Natl Cancer Inst. 2009 May 20;101(10):736-50. doi: 10.1093/jnci/djp082. Epub 2009 May 12. PMID: 19436038; PMCID: PMC2684553..

[20] Vieira DSC, Dufloth RM, Schmitt FCL, Zeferino LC. Carcinoma de mama: novos conceitos na classificação. Revista Brasileira de Ginecologia e Obstetrícia [online]. 2008, v. 30, n. 1 [Acessado 1 Setembro 2021] , pp. 42-47. Disponível em: <https://doi.org/10.1590/S0100-72032008000100008>. Epub 07 Abr 2008. ISSN 1806-9339. https://doi.org/10.1590/S0100-72032008000100008.

[21] Ding YC, Steele L, Warden C, Wilczynski S, Mortimer J, Yuan Y, "et al". Molecular subtypes of triple-negative breast cancer in women of different race and ethnicity. Oncotarget. 2019 Jan 4;10(2):198-208. doi: 10.18632/oncotarget.26559. PMID: 30719214; PMCID: PMC6349443.

[22] Eiermann W, Rezai M, Kümmel S, Kühn T, Warm M, Friedrichs K, "et al". The 21-gene recurrence score assay impacts adjuvant therapy recommendations for ER-positive, node-negative and node-positive early breast cancer resulting in a risk-adapted change in chemotherapy use. Ann Oncol. 2013 Mar;24(3):618-24. doi: 10.1093/annonc/mds512. Epub 2012 Nov 7. PMID: 23136233; PMCID: PMC3574549.

[23] Ahn HJ, Jung SJ, Kim TH, Oh MK, Yoon HK. Differences in Clinical Outcomes between Luminal A and B Type Breast Cancers according to the St. Gallen Consensus 2013. J Breast Cancer. 2015 Jun;18(2):149-59. doi: 10.4048/jbc.2015.18.2.149. Epub 2015 Jun 26. PMID: 26155291; PMCID: PMC4490264.

[24] Almeida, A. M. P. T. de, Marquini, H. R., Leite, R. M., & Nai, G. A. (2012). PREVALÊNCIA DE CÂNCER DE MAMA E ASSOCIAÇÃO COM SEUS FATORES PROGNÓSTICOS E PREDITIVOS

DIAGNOSTICADOS NUM HOSPITAL UNIVERSITÁRIO. *Colloquium Vitae. ISSN: 1984-6436*, *4*(1), 27–37. https://revistas.unoeste.br/index.php/cv/article/view/733.

[25] Williams C, Brunskill S, Altman D, Briggs A, Campbell H, Clarke M, "et al". Cost-effectiveness of using prognostic information to select women with breast cancer for adjuvant systemic therapy. Health Technol Assess. 2006 Sep;10(34):iii-iv, ix-xi, 1-204. doi: 10.3310/hta10340. PMID: 16959170.

[26] Payne SJ, Bowen RL, Jones JL, Wells CA. Predictive markers in breast cancer--the present. Histopathology. 2008 Jan;52(1):82-90. doi: 10.1111/j.1365-2559.2007.02897.x. PMID: 18171419.

[27] Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. N Engl J Med. 2010 Nov 11;363(20):1938-48. doi: 10.1056/NEJMra1001389. PMID: 21067385.

[28] Jiang H, Bai X, Zhang C, Zhang X. Evaluation of HER2 gene amplification in breast cancer using nuclei microarray in situ hybridization. Int J Mol Sci. 2012;13(5):5519-27. doi: 10.3390/ijms13055519. Epub 2012 May 8. PMID: 22754312; PMCID: PMC3382781.

[29] Ng CK, Martelotto LG, Gauthier A, Wen HC, Piscuoglio S, Lim RS, "et al". Intra-tumor genetic heterogeneity and alternative driver genetic alterations in breast cancers with heterogeneous HER2 gene amplification. Genome Biol. 2015 May 22;16(1):107. doi: 10.1186/s13059-015-0657-6. PMID: 25994018; PMCID: PMC4440518.

[30] Braun L, Mietzsch F, Seibold P, Schneeweiss A, Schirmacher P, Chang-Claude J, "et al". Intrinsic breast cancer subtypes defined by estrogen receptor signalling-prognostic relevance of progesterone receptor loss. Mod Pathol. 2013 Sep;26(9):1161-71. doi: 10.1038/modpathol.2013.60. Epub 2013 Apr 5. PMID: 23558572.

[31] Nielsen TO, Parker JS, Leung S, Voduc D, Ebbert M, Vickery T, "et al". A comparison of PAM50 intrinsic subtyping with immunohistochemistry and clinical prognostic factors in tamoxifen-treated estrogen receptor-positive breast cancer. Clin Cancer Res. 2010 Nov 1;16(21):5222-32. doi: 10.1158/1078-0432.CCR-10-1282. Epub 2010 Sep 13. PMID: 20837693; PMCID: PMC2970720..

[32] Muftah AA, Aleskandarany MA, Al-Kaabi MM, Sonbul SN, Diez-Rodriguez M, Nolan CC, "et al". Ki67 expression in invasive breast cancer: the use of tissue microarrays compared with whole tissue sections. Breast Cancer Res Treat. 2017 Jul;164(2):341-348. doi: 10.1007/s10549-017-4270-0. Epub 2017 May 6. PMID: 28478613; PMCID: PMC5487701.

[33] Inwald EC, Klinkhammer-Schalke M, Hofstädter F, Zeman F, Koller M, Gerstenhauer M, "et al". Ki-67 is a prognostic parameter in breast cancer patients: results of a large population-based cohort of a cancer registry. Breast Cancer Res Treat. 2013 Jun;139(2):539-52. doi: 10.1007/s10549-013-2560-8. Epub 2013 May 16. PMID: 23674192; PMCID: PMC3669503.

[34] Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, "et al". A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N Engl J Med. 2004 Dec 30;351(27):2817-26. doi: 10.1056/NEJMoa041588. Epub 2004 Dec 10. PMID: 15591335.

[35] Burstein HJ, Temin S, Anderson H, Buchholz TA, Davidson NE, Gelmon KE, "et al". Adjuvant endocrine therapy for women with hormone receptor-positive breast cancer: american society of clinical oncology clinical practice guideline focused update. J Clin Oncol. 2014 Jul 20;32(21):2255-69. doi: 10.1200/JCO.2013.54.2258. Epub 2014 May 27. PMID: 24868023; PMCID: PMC4876310.

[36] Rakha EA, Reis-Filho JS, Baehner F, Dabbs DJ, Decker T, Eusebi V, "et al". Breast cancer prognostic classification in the molecular era: the role of histological grade. Breast Cancer Res. 2010;12(4):207. doi: 10.1186/bcr2607. Epub 2010 Jul 30. PMID: 20804570; PMCID: PMC2949637.

[37] Lakhani SR, Jacquemier J, Sloane JP, Gusterson BA, Anderson TJ, van de Vijver MJ, "et al". Multifactorial analysis of differences between sporadic breast cancers and cancers involving BRCA1 and BRCA2 mutations. J Natl Cancer Inst. 1998 Aug 5;90(15):1138-45. doi: 10.1093/jnci/90.15.1138. PMID: 9701363.

[38] Sheffield BS, Kos Z, Asleh-Aburaya K, Wang XQ, Leung S, Gao D, "et al". Molecular subtype profiling of invasive breast cancers weakly positive for estrogen receptor. Breast Cancer Res Treat. 2016 Feb;155(3):483-90. doi: 10.1007/s10549-016-3689-z. Epub 2016 Feb 4. PMID: 26846986.

[39] Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, "et al". Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol. 2009 Mar 10;27(8):1160-7. doi: 10.1200/JCO.2008.18.1370. Epub 2009 Feb 9. PMID: 19204204; PMCID: PMC2667820.

Appendix

Appendix 1. Supplementary Table 1. Distribution of biomarkers based on St. Gallen Conference14 (group 1) and subtype categorization based on status ER and HER21 (group 2).

IHC Subtypes group 1	ER	PR	HER2	Ki67
Luminal A	+	+/-	-	Low
Luminal B/HER2-	+	+/-	-	High
Luminal B/HER2+	+	+/-	+(3+)	Any
HER2+	-	-	+(3+)	Any
TNBC	-	-	-	Any
IHC Subtypes group 2	**ER**		**HER2**	
ER +/HER2-	+		-	
ER +/HER2+	+		+(3+)	
ER -/HER2+	-		+(3+)	
ER -/HER2-	-		-	

IHC: immunohistochemical; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; Ki67: Proliferation Marker; TNBC: triple negative breast cancer; +: positive; -: negative; +/-: positive or negative; + (3+): positive if at least 10% of tumor cell exhibited a cell membrane staining score of 3+.