Zagazig University Medical Journal
www.zumj.journals.ekb.eg
https://doi.org/10.21608/zumj.2022.161766.2643 Volume 30, Issue 1.4, June 2024, Supplement Issue
Manuscript ID ZUMJ-2209-2643 (R2)
DOI 10.21608/ZUMJ.2022.161766.2643
ORIGINAL ARTICLE

Tissue ELISA and Serum Amperometric Magnetoimmunosensor of ECD-HER2 in Stratifying Subtyping of Egyptian Woman Breast Cancers

Sameh Nakhla[1*], Zynab Elwafe[2], Ahmed Saad [3], Tarek Aref [4], Noha Ragab [5], Inas El-Badry[6], Unai Eletxigerra[7], Susana Campuzano[8], Jesus Martínez-Perdiguero[9], Santos Merino[7], José M. Pingarrón[8], Salah Abdelmoneim[10], Eman El-Abd[1].

[1] Department of Radiation Sciences, Medical Research Institute (MRI), Alexandria University, Egypt.
[2] Department of Radiology, Faculty of Medical Technology, Derna, Libya
[3] Department of Experimental and Clinical Surgery, MRI, Alexandria University, Egypt.
[4] Department of Radiodiagnosis Department, MRI, Alexandria University, Alexandria, Egypt.
[5]Department of Pathology, MRI, Alexandria University, Alexandria, Egypt.
[6]Department of Cancer Management and Research, MRI, Alexandria University, Alexandria, Egypt.
[7]Micro-NanoFabrication Unit, IK4-Tekniker, Eibar, Spain
[8]Departamento de Química Analítica, Facultad de CC. Químicas, Universidad Complutense de Madrid, Madrid, Spain
[9]Universidad del País Vasco/Euskal Herriko Unibertsitatea, Bizkaia, Spain
[10]Department of Clinical Oncology and Nuclear Medicine, faculty of medicine, Alexandria University, Egypt

***Corresponding author**:
Sameh Nakhla

Email:
sameh.nakhlh@alexu.edu.eg

Submit Date 2022-09-11
Revise Date 2022-10-12
Accept Date 2022-10-05

ABSTRACT

Background: Detection of HER2 extracellular domain (ECD) amperometric magnetoimmunosensor (AM) investigated on raw cell lysates, intact breast cancer cells, and commercial and healthy sera except on patients' samples. Our aim was to validate tissue and serum AM of ECD-HER2 in subtyping Egyptian woman breast cancers and to compare it to the current techniques.**Methods:** We collected blood and tissues from 72 women (20 controls, five recurrent breast tumors, and 47 BCs). Tissue (tHER2) and serum HER2 (sHER2); were detected by ELISA and the AM. Results correlated with the clinicopathological parameters, and serum CA15-3 (sCA15-3). **Results:** tHER2 (ELISA) and sCA15-3 significantly differed among the studied groups. tHER2 (ELISA) showed a significant decrease in higher BMI in the HER22+ group, while tHER2 and sHER2 (AM) significantly correlated in the HER23+ group. tHER2 (ELISA) significantly differed among BCs molecular subtypes. Both tHER2 and sHER2 (ELISA) significantly correlated in the HER2 molecular subgroup. sHER2 (AM) showed highly significant sensitivity and specificity in differentiating TN from luminal A at > 9.26 ng/ml and HER2 at 9.6 ng/ ml. However, at > 1.67 ng/ml, tHER2 (ELISA) stratified significantly between BCs molecular and HER2 subgroups. **Conclusions**: Tissue ELISA and serum AM provided accurate quantitative measurements for HER2 in various BC subtypes—at cut-off values lower than those approved by FDA—and would complement the current IHC and ISH techniques.

Keywords: Breast carcinoma; CA 15-3; HER2; immunoassay; immunosensors.

INTRODUCTION

Accurate assessment of Human epidermal growth factor receptor-2 (HER2) status is crucial for both tailored targeted therapeutic and follow-up strategies for breast cancers (BCs) [1]. HER2, a proto-oncogene, encodes a tyrosine kinase (TK) growth factor receptor protein expressed by several normal tissues and has a role in normal cell functions [2]. HER2 receptor has three main domains**:** an extracellular domain (ECD), an intermembrane domain (ICD), and a TK intracellular domain [3]. HER2 ECD is

cleaved by metalloproteases to be activated and released into the blood [4]. HER2 ECD was suggested as a surrogate tumor marker that correlates with over-expression of tissue HER2 (tHER2) and worsens the prognosis of metastatic BC [5]. Enzyme-linked immunosorbent assay (ELISA) was developed and approved by Food and Drug Administration (FDA) to quantify ECD of HER2 in blood and tissue lysates**,** providing low detection limits [3,6]. The HER2 targeted therapy of advanced BC relies on the primary tumor HER2 status**,** and some studies reported HER2 receptor conversion after neoadjuvant chemotherapy (NAC) or during metastasis progression [7]. This conversion will affect the therapeutic decision and treatment response**,** and thus it necessitate**s** re-evaluation in post-NAC specimens and metastatic lesions.

Biosensors, analytical devices containing a bio-recognition layer on the surface of a transducer, can offer a clear advantage in converting a biological event into an electronic signal and distinguishing a specific biomarker in complex matrices [8]. Immunosensors are well established as valid alternatives to classical analyses of cancer biomarkers by offering the advantages of being easy to use, rapid, inexpensive, and capable of multianalyte testing [9]. Several biosensors were developed and used successfully to quantify HER2 with low detection limits [10, 11, 12, 13, 14].

Cancer antigen 15-3 (CA 15-3) is a frequently used marker in BC that detects the soluble moiety of the transmembrane mucin-1 (MUC1) protein- a coreceptor of HER2 and a key player in aberrant O-glycosylation and distant metastasis [15.16,17]. According to various guidelines, CA15-3 may be used in monitoring and follow-up [18]. Nowadays, the electrochemiluminescence (ECL) detection method of CA 15-3 became the most used method because of its high sensitivity and low detection limit [18].The current study compare**s** the efficiency, specificity, and sensitivity of the conventional tHER2 immunohistochemistry (IHC) with ELISA and an AM assay in detecting tHER2 and serum (sHER2)**,** respectively. The correlation between clinicopathological parameters and CA 15-3 level was also investigated.

METHODS

Subjects (72) were randomly selected from patients referred to the Alexandria University (AU) Medical Research Institute (MRI) in Egypt from August 2017 to November 2018, and the sample size was based on previous research [17]. Blood (preoperative) and tissue (during elective surgery) samples were collected from subjects according to the ethical rules approved by the ethics committee of the MRI, AU, Egypt (IORG 0008812) based on the ethical standards of the Declaration of Helsinki (1964) and its subsequent amendments. Written informed consent was obtained from all participants. After history taking, clinical examinations, and routine laboratory, and radiological investigations, subjects were subdivided into the following groups:

1. Control group including 20 women with no evidence of any primary or secondary breast cancer as detected by mammography and breast ultrasound (US) (if required).
2. Recurrent breast tumor group including five women.
3. HER2 negative breast cancer group [Zero (6) & 1^{+} (8)] including 14 women with breast carcinomas.
4. HER2 equivocal breast cancer group (2^{+}) including 21 women with breast carcinomas.
5. HER2 positive breast cancer group (3^{+}) including 12 women with breast carcinomas.

Pathological investigations were performed [17, 18]. Patients were treated and followed up for three years**,** and Nottingham prognostic index (NPI) was calculated [19, 20]. The modified NPI was calculated by the addition of 0.6 values for Her-2 positivity.

Serum and tissue lysates were assayed for HER2 by a ready-to-use ELISA kit (Bioneovan Co, Ltd., China; assay range: 16-1000 Pg/ml) according to the manufacturer's instructions and an AM according to Eletxigerra *et al.* [11]. Briefly, the developed AM assay platform was based on the use of two specific antibodies**:** a capture antibody bound to commercial micromagnetic particles (MBs) and a detection antibody conjugated to horseradish peroxidase (HRP; Sino Biological Inc., China) enzyme that specifically recognizes and enzymatically label**s** the target protein, resulting in the formation of the sandwich immunocomplexes on the MBs. After capturing the resulting magnetic immunoconjugates on the working electrode of screen-printed carbon electrodes (SPCEs; DropSens Spain), the amperometric detection was carried out at -0.20 V vs. the Ag pseudo reference electrode (DropSens Spain) using H_2O_2 (Millipore Milli-Q) as enzyme-substrate and hydroquinone (HQ; Sigma-Aldrich, Spain) as redox mediator.

Under these conditions and after the addition of the substrate, a cathodic current variation, attributed to the enzymatic reduction of the substrate mediated by hydroquinone was obtained which, due to the type of format used, was proportional to the concentration of HER2 in the sample analyzed [21]. For the analysis of tHER2 and sHER2 with the AM in the samples collected from the women and once proven the absence of matrix effect in both challenging matrices, both the tissue extracts and the serum samples were diluted to 40% in 37.5 µL of PBS buffer (Scharlau, Spain) containing 1 M NaCl (Scharlau, Spain), 2% Tween 20 (Scharlau, Spain) and 0.5% BSA (Jackson ImmunoResearch). These were analyzed**,** and the endogenous content of HER2 in them was determined by interpolating the measured amperometric signals into the calibration graph constructed with the AM using HER2 standards.

CA 15-3 was assayed by the electrochemiluminescence immunoassay (ECLIA) [Roche diagnostics GmbH, Germany; assay range = 1-300 U/ml; normal range for healthy non-pregnant women = 28.7-57.8U/ml (cut off value = 34.5 U/ml at 99% confidence interval)] using Cobas ® immunoassay analyzer (Cobas 6000).

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 20 (Armonk, NY: IBM Corp. 2011). Qualitative data were described using numbers and percentages. The Kolmogorov-Smirnov test was used to verify the normality of distribution. Quantitative data were described using range mean and standard deviation (SD). The significance of the obtained results was judged at the 5% level. F-test (analysis of variance: ANOVA) was used for normally distributed quantitative variables, to compare between more than two groups, and Post Hoc test (Tukey) for pairwise comparisons. Mann Whitney test was used for abnormally distributed quantitative variables, to compare two studied groups. Kruskal Wallis test was used for abnormally distributed quantitative variables, to compare between more than two studied groups, and Post Hoc (Dunn's multiple comparisons test) for pairwise comparisons. Spearman coefficient was used to correlate between two distributed abnormally quantitative variables. The receiver operating characteristic curve (ROC) is generated by plotting sensitivity (TPR) on the Y-axis versus 1-specificity (FPR) on the X-axis at different cut-off values. The area under the ROC curve denotes the diagnostic performance of the test. An area more than 50% gives an acceptable performance**,** and an area of about 100% is the best performance for the test. The ROC curve allows also for a comparison of performance between two tests. Survival was analyzed using the Kaplan-Meier survival curve.

RESULTS

The demographic and clinicopathological data are presented in (table 1), Although age significantly differed among the studied groups [p = 0.007 in Her-2 subtypes & p = 0.037 in molecular subtypes), age variation did not show any significant relation to the tHER2 status (p = 0.170) or BC molecular subtypes (p = 0.294); as detected by ELISA. Therefore, the observed significant difference of tHER2 (p = 0.045) among HER2 and BC molecular subtypes (p = 0.017) was age independent.

A significant direct correlation observed between tHER2 and sHER2 levels as detected by ELISA in HER2 enriched subgroup only (r = 0.857, p = 0.014) (Table 2a). Same correlation was detected using AM in $HER2^{3+}$ group only (r = 0.9, p = 0.037) (Table 2b). tHER2 (ELISA) significantly decreased (p = 0.02) with higher Body Mass Index (BMI) in equivocal HER2 (2+) BC subgroup. This group was characterized by being mainly postmenopausal (71.4%), hormonal positive (ER+ & PR+: 76.02%), obese (BMI ≥ 30: 52.4%), and having luminal B (76.2%) BC molecular subtypes. The current results showed for the first time the utility of sHER2 and tHER2 (as detected by Am and ELISA; respectively) in the differentiation of BC molecular subtypes at cut of values > 1.76 (for ELISA; specificity: 85.71-87.5%; sensitivity: 60-100%), > 9.26, and > 9.6 ng/ml (for AM; specificity: 83-100%; sensitivity: 100%) (Figure 1) in both primary and metastatic BC molecular subtypes.

sCA15-3 (p = 0.048) significantly differed among the studied groups with no relevance to the level of HER2. Our study detected no significant correlation between long-term disease-free survival and ER, PR, and HER2 levels. Most of our cases had NPI ranging from excellent to moderate average scores even after the inclusion of HER2 score in the NPI of HER2 positive BC cases (Table 3).

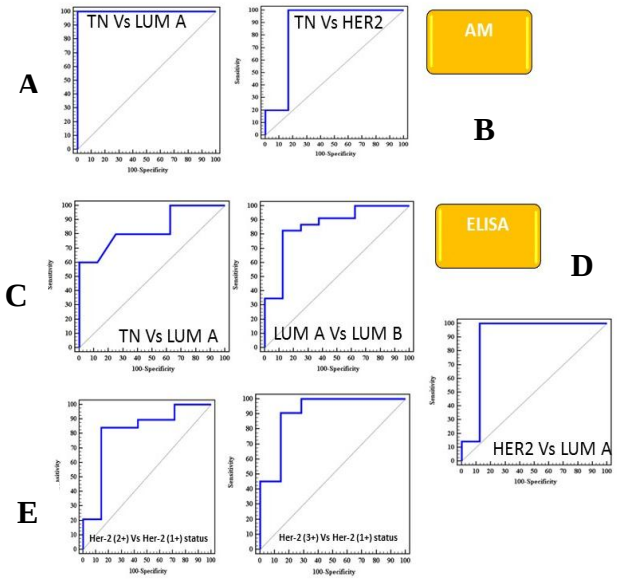

Figure 1. ROC curves to diagnose BC subtypes using sHER2 as detected by AM (A & B) and tHER2 as detected by ELISA (C-G).
A) AUC: 1, *p*: 0.003*, 95% C.I.: 1-1, cut off: >9.26, sensitivity: 100, specificity: 100, PPV: 100, NPV: 100. **B**) AUC: 0.867, *p*: 0.045*, 95% C.I.: 0.615-1.118, cut off: >9.6, sensitivity: 100, specificity: 83.33, PPV: 83.3, NPV: 100; **C**) AUC: 0.850; *p*: 0.040*; 95% C.I.: 0.608-1.092, cut off: >1.67, sensitivity: 60, specificity: 87.5, PPV: 75, NPV: 77.8. **D**) AUC: 0.859, *p*: 0.003*, 95% C.I.: 0.698-1.019, cut off: >1.67, sensitivity: 82.61, specificity: 87.50, PPV: 95, NPV: 60. **E**) AUC: 0.893, *p*: 0.011*, 95% C.I.: 0.691-1.094, cut off: >1.67, sensitivity: 100, specificity: 87.5, PPV: 87.5, NPV: 100. **F**) AUC: 0.812, *p*: 0.016*, 95% C.I.: 0.600-1.024, cut off: >1.67, sensitivity: 84.21, specificity: 85.71, PPV: 94.1, NPV: 66.7. **G**) AUC: 0.909, *p*: 0.004*, 95% C.I.: 0.753-1.065, cut off: >1.67, sensitivity: 90.91, specificity: 85.71, PPV: 90.9, NPV: 85.7. AUC: Area Under a Curve; p value: Probability value; CI: Confidence intervals; NPV: Negative predictive value; PPV: Positive predictive value; #Cut off was chosen according to Youden index; *: Statistically significant at $p \leq 0.05$.

Table 1. The demographic and clinicopathological parameters of the studied groups

Parameters	Control (N = 20)	Recurrent Breast Tumors (N = 5)	Breast Cancer (N = 47)			
			HER2 status (as detected by IHC)			
			Negative		**Equivocal**	**Positive**
			HER2 (0) (N = 6)	HER2 (1^+) (N = 8)	HER2 (2^+) (N = 21)	HER2 (3^+) (N = 12)
	N (%)					
Age (Years) Mean ± SD	48.75 ± 8.67	37.6 ± 15.1	52 ± 13	59 ± 11	55.90 ± 11.71	48.75 ± 10.75
Other diseases						
Prediabetic	1 (5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Diabetes	0 (0)	0 (0)	1 (16.7)	0 (0)	0 (0)	1 (8.3)
Diastolic	0 (0)	0 (0)	0 (0)	0 (0)	1 (4.8)	1 (8.3)
dysfunction	2 (10)	0 (0)	0 (0)	1 (12.5)	4 (19)	1 (8.3)
Hypertension	4 (20)	0 (0)	0 (0)	2 (25)	1 (4.8)	2 (16.7)
Hypertension +	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	0 (0)
diabetes	0 (0)	0 (0)	0 (0)	0 (0)	1 (4.8)	0 (0)
Hypertension +	0 (0)	0 (0)	0 (0)	0 (0)	1 (4.8)	0 (0)
Anaemia						
Hypertension +	1 (5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
CVD	1 (5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Hypertension +	0 (0)	0 (0)	0 (0)	0 (0)	1 (4.8)	0 (0)

Hypercholesteremia	11 (55)	3 (60)	2 (33.3)	5 (62.5)	12 (57)	7 (58.3)
Arthritis	0 (0)	1 (20)	3 (50)	0 (0)	0 (0)	0 (0)
Axillary LN diseases						
Liver diseases						
None						
Unknown						
Menopausal status						
Pre	9 (45)	3 (60)	0 (0)	1 (12.5)	5 (23.8)	6 (50)
Post	10 (50)	0 (0)	4 (66.66)	7 (87.5)	15 (71.4)	3 (25)
Irregular	1 (5)	2 (40)	1 (16.66)	0 (0)	1 (4.8)	3 (25)
Unknown	0 (0)	0 (0)	1 (16.66)	0 (0)	0 (0)	0 (0)
BI-RADS						
0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
1	14 (70)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2	6 (30)	1 (20)	0 (0)	0 (0)	1 (4.8)	0 (0)
3	0 (0)	0 (0)	0 (0)	0 (0)	1 (4.8)	1 (8.3)
4	0 (0)	1 (20)	3 (50)	2 (25)	7 (33.3)	7 (58.3)
5	0 (0)	0 (0)	2 (33.3)	4 (50)	10 (47.6)	3 (25)
6	0 (0)	1 (20)	1 (16.7)	2 (25)	2 (9.5)	1 (8.3)
Unknown	0 (0)	2 (40)	0 (0)	0 (0)	0 (0)	0 (0)
Tumor side						
Left	-	2 (40)	5 (83.3)	6 (75)	13 (61.9)	6 (50)
Right	-	2 (40)	1 (16.7)	1 (12.5)	8 (38.1)	6 (50)
Bilateral	-	1 (20)	0 (0)	1 (12.5)	0 (0)	0 (0)
Tumor histological type						
Benign tumors	-	1 (20)	0 (0)	0 (0)	0 (0)	0 (0)
Desmoid tumor (fibromatosis)	-	2 (40)	0 (0)	0 (0)	0 (0)	0 (0)
Ductal epithelial hyperplasia	-	2 (40)	0 (0)	0 (0)	0 (0)	0 (0)
Lobular granulomatous mastitis	-	0 (0)	0(0)	0 (0)	0 (0)	1 (8.3)
Carcinoma	-	0 (0)	4 (66.7)	7 (87.5)	15 (71.4)	10 (83.3)
In situ carcinoma Ductal	-	0 (0)	1 (16.7)	0 (0)	2 (9.5)	0 (0)
Invasive carcinoma (NOS)	-	0 (0)	0 (0)	0 (0)	1 (4.8)	0 (0)
Mixed ductal & lobular	-	0 (0)	0 (0)	0 (0)	0 (0)	1 (8.3)
Invasive carcinoma (special types)	-	0 (0)	0 (0)	0 (0)	1 (4.8)	0 (0)
Mucinous	-	0 (0)	0 (0)	0 (0)	1 (4.8)	0 (0)
Others	-	0 (0)	0 (0)	0 (0)	1 (4.8)	0 (0)
Paget & IDC with highly grad intraductal component (Comedo)	-	0 (0)	0 (0)	1 (12.5)	0 (0)	0 (0)
IDC & Focal mucoid	-	0 (0)	1 (16.7)	0 (0)	0 (0)	0 (0)

lobular & Paget's Paget's disease & intraductal components Multifocal IDC 10% in situ components (comedo & cribriform) Bifocal Invasive Lobular Carcinoma &in situ lobular components						
Tumor size (cm) < 2 2-4 > 4 Unknown	 - - - -	 0 (0) 1 (20) 2 (40) 2 (40)	 1 (16.7) 2 (33.3) 3 (50) 0 (0)	 0 (0) 7 (87.5) 0 (0) 1 (12.5)	 3 (14.3) 15 (71.4) 3 (14.3) 0 (0)	 1 (8.3) 8 (66.7) 3 (25) 0 (0)
Tumor Grade II III Unknown	 - - -	 0 (0) 0 (0) 0 (0)	 2 (33.3) 3 (50) 1 (16.7)	 6 (75) 2 (25) 0 (0)	 12 (57.1) 5 (23.8) 4 (19)	 9 (75) 3 (25) 0 (0)
Tumor stage DIS I IIA IIIA IB IIB IIIB IV Unknown	 - - - - - - - - -	 0 (0) 0 (0) 0 (0) 0 (0) 0 (0) 0 (0) 0 (0) 0 (0) 0 (0)	 0 (0) 0 (0) 2 (33.3) 1 (16.7) 0 (0) 0 (0) 1 (16.7) 0 (0) 2 (33.3)	 0 (0) 0 (0) 2 (25) 2 (25) 0 (0) 1 (12.5) 1 (12.5) 0 (0) 2 (25)	 1 (4.8) 1 (4.8) 3 (14.3) 5 (23.8) 2 (9.5) 2 (9.5) 2 (9.5) 4 (19) 1 (4.8)	 0 (0) 0 (0) 3 (25) 4 (33.3) 0 (0) 4 (33.3) 0 (0) 1 (8.3) 0 (0)
LNM Negative Positive Unknown	 - -	 4 (80) 1 (20) 0 (0)	 3 (50) 3 (50) 0 (0)	 2 (25) 6 (75) 0 (0)	 9 (42.9) 12 (57.1) 0 (0)	 5 (41.7) 6 (50) 1 (8.3)
Number of the involved LN 0 1 (1-3) 2 (4-9) 3 (>10) Unknown	 - - - - -	 4 (80) 1 (20) 0 (0) 0 (0) 0 (0)	 3 (50) 1 (16.7) 0 (0) 2 (33.3) 0 (0)	 2 (25) 1 (12.5) 3 (37.5) 2 (25) 0 (0)	 9 (42.9) 5 (23.8) 2 (9.5) 5 (23.8) 0 (0)	 5 (41.7) 2 (16.7) 2 (16.7) 2 (16.7) 1 (8.3)
Vascular invasion Positive Negative	 - -	 0 (0) 5 (100)	 6 (100) 0 (0)	 7 (87.5) 1 (12.5)	 18 (85.7) 3 (14.3)	 12 (100) 0 (0)
ER 0 1+ 2+ 3+	 - - - -	 - - - -	 4 (66.7) 0 (0) 0 (0) 2 (33.3)	 1 (12.5) 4 (50) 1 (12.5) 2 (25)	 5 (23.8) 1 (4.8) 6 (28.6) 9 (42.9)	 4 (33.3) 3 (25) 3 (25) 2 (16.7)
PR 0 1+	 - -	 - -	 4 (66.7) 0 (0)	 2 (25) 2 (25)	 5 (23.8) 4 (19)	 4 (33.3) 4 (33.3)

2+	-	-	0 (0)	2 (25)	3 (14.3)	1 (8.3)
3+	-	-	2 (33.3)	2 (25)	9 (42.9)	3 (25)
sHER2 (ng/ml) ELISA (M ± SD)						
Serum	3.61 ± 3.2	2.85 ± 0.37	5.4 ± 5.6	3.18 ± 0.92	3.99 ± 3.64	6.58 ± 6.16
Tissue	-	-	5.23 ± 6.38	1.74 ± 1.61	4.79 ± 4.13	6.80 ± 6.40
AM (M ± SD)						
Serum	-	5.72 ± 0.64	11.16 ± 3.56	7.99 ± 6.76	9.90 ± 6.41	9.15 ± 11.19
Tissue	-	-	28.18 ± 58.7	17.21 ± 45.97	87.21 ± 166.95	125.9 ± 122.34
sCA15-3 (U/ml)						
M ± SD	17.89 ± 8.44	12.18 ± 5.03	21.31 ± 10.51	28.97 ± 9.54	19.55 ± 12.3	16.26 ± 7.46
> 34.5 (cut off value)	1 (5)	0 (0)	0 (0)	2 (25)	2 (9.5)	0 (0)
≤ 34.5	19 (95)	5 (100)	6 (100)	6 (75)	19 (90.5)	12 (100)
BMI (Kg/m^2)						
< 18.5 (underweight)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
18.5-24.99 (healthy)	1 (5)	0 (0)	0 (0)	1 (12.5)	2 (9.5)	1 (8.3)
25-29.99 (overweight)	5 (25)	3 (60)	1 (16.7)	2 (25)	7 (33.3)	4 (33.3)
≥ 30 (obese)	14 (70)	1 (20)	3 (50)	5 (62.5)	11 (52.4)	7 (58.3)
Class I (30-34.99)	6 (30)	1 (20)	3 (50)	3 (37.5)	4 (19)	4 (33.3)
Class II (35-39.99)	5 (25)	0 (0)	0 (0)	2 (25)	5 (23.8)	1 (8.3)
Class III ≥ 40	3 (15)	0 (0)	0 (0)	0 (0)	2 (9.5)	2 (16.6)
Unknown	0 (0)	1 (20)	2 (33.3)	0 (0)	1 (4.8)	0 (0)
Outcomes						
Alive	-	4 (80)	4 (66.7)	8 (100)	19 (90.5)	12 (100)
Died	-	0 (0)	1 (16.7)	0 (0)	2 (9.5)	0 (0)
Unknown	-	1 (20)	1 (16.7)	0 (0)	0 (0)	0 (0)
Free						
Local recurrence	-	3 (60)	4 (66.7)	4 (50)	12 (57.1)	9 (75)
Metastasis	-	1 (20)	0 (0)	0 (0)	2 (9.5)	2 (16.7)
Local recurrence plus metastasis	-	0 (0)	0 (0)	0 (0)	5 (23.8)	0 (0)
Unknown	-	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	-	1 (20)	2 (33.3)	4 (50)	2 (9.5)	1 (8.3) 0 (0)
NPI						
Excellent (2.02-2.4)	-	-	0 (0)	0 (0)	3 (14.3)	2 (16.7)
Good (2.4-3.4)	-	-	1 (16.7)	1 (12.5)	3 (14.3)	3 (25)
Moderate 1 (3.41-4.4)	-	-	3 (50)	1 (12.5)	3 (14.3)	2 (16.7)
Moderate 2 (4.41-5.4)	-	-	0 (0)	2 (25)	2 (9.5)	1 (8.3)
Poor (5.41-6.4)	-	-	1 (16.7)	3 (37.5)	4 (19)	1 (8.3)
Very poor (6.41-6.8)	-	-	0 (0)	0 (0)	2 (9.5)	1 (8.3)
Unknown	-	-	1 (16.7)	1 (12.5)	4 (19)	2 (16.7)
Socioeconomic status						
High	3 (15)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	7 (35)	0 (0)	1 (16.7)	0 (0)	2 (9.5)	4 (33.3)

Middle Low Unknown	10 (50) 0 (0)	4 (80) 1 (20)	2 (33.3) 3 (50)	8 (100) 0 (0)	19 (94.5) 0 (0)	8 (66.7) 0 (0)
Breast feeding Yes No Unknown	 16 (80) 4 (20) 0 (0)	 2# (40) 2 (40) 1 (20)	 3 (50) 0 (0) 3 (50)	 3 (37.5) 5 (62.5) 0 (0)	 17## (81) 4 (19) 0 (0)	 10 (83.3) 2 (16.7) 0 (0)
Number of pregnancies lasting ≥ 6 months 0 1 2 3 ≥ 4 Unknown	 1 (5) 1 (5) 7 (35) 3 (15) 8 (40) 0 (0)	 1 (20) 0 (0) 1 (20) 0 (0) 2 (40) 1 (20)	 0 (0) 0 (0) 0 (0) 1 (16.7) 2 (33.3) 3 (50)	 1 (12.5) 1 (12.5) 4 (50) 0 (0) 2 (25) 0 (0)	 1 (4.8) 0 (0) 6 (28.6) 2 (9.5) 12 (57.1) 0 (0)	 2 (16.7) 1 (8.3) 2 (16.7) 1 (8.3) 6 (50) 0 (0)
Smoking Nonsmokers Passive smokers Active smokers Unknown	 9 (45) 11 (55) 0 (0) 0 (0)	 2 (40) 2 (40) 0 (0) 1 (20)	 2 (33.3) 1 (16.7) 0 (0) 3 (50)	 4 (50) 4 (50) 0 (0) 0 (0)	 7 (33.3) 13 (62) 1 (4.8) 0 (0)	 6 (50) 6 (50) 0 (0) 0 (0)
Family history Yes No Unknown	 4 (20) 16 (80) 0 (0)	 2 (40) 2 (40) 1 (20)	 1 (16.7) 2 (33.3) 3 (50)	 2 (25) 6 (75) 0 (0)	 6 (28.6) 15 (71.4) 0 (0)	 2 (16.7) 10 (83.3) 0 (0)
Oral contraceptives Yes No Unknown	 5 (25) 15 (75) 0 (0)	 1 (20) 3 (60) 1 (20)	 0 (0) 3 (50) 3 (50)	 2 (25) 6 (75) 0 (0)	 4 (19) 17 (81) 0 (0)	 4 (33.3) 8 (66.7) 0 (0)
Hormone replacement therapy Yes No Unknown	 0 (0) 20 (100) 0 (0)	 0 (0) 4 (80) 1 (20)	 0 (0) 3 (50) 3 (50)	 1 (12.5) 7 (87.5) 0 (0)	 2 (9.5) 19 (90.5) 0 (0)	 1 (8.3) 11 (91.7) 0 (0)

LN: lymph node; CVD: cardiovascular diseases; NOS: no special type.

Equivocal status (as detected by IHC) was confirmed by Fluorescence in situ hybridization (FISH).

Table 2a. *Correlation between sHER2 and tHER2 using ELISA and AM assays in molecular subtypes groups*

Parameters	Molecular subtypes							
	TN		LUM A		LUM B		Her-2	
	r_s	p	r_s	p	r_s	p	r_s	P
ELISA (sHER2 vs. tHER2)	0.800	0.104	-0.476	0.233	0.309	0.151	0.857	0.014*
AM (sHER2 vs. tHER2)	0.500	0.391	0.609	0.109	0.224	0.533	1.000	–
ELISA (tHER2 vs. age)	-0.100	0.873	0.595	0.120	0.183	0.403	-0.464	0.294
ELISA (tHER2 vs. CA 15-3)	-0.500	0.391	-0.333	0.420	0.032	0.886	0.286	0.535

rs: Spearman coefficient; *: Statistically significant at $p \leq 0.05$.

Table 2b. *Correlation between sHER2 and tHER2 using ELISA and AM assays in HER2 subtypes groups*

	HER2 status (as detected by IHC)					
	Negative		Her-2 (2+)		Her-2 (3+)	
	r_s	p	r_s	p	r_s	P
ELISA (sHER2 vs. tHER2)	0.330	0.271	0.319	0.183	0.091	0.790
AM (sHER2 vs. tHER2)	0.245	0.420	0.433	0.244	0.900	0.037*
ELISA (tHER2 vs. age)	-0.052	0.865	-0.024	0.923	0.445	0.170
ELISA (tHER2 vs. CA 15-3)	-0.176	0.566	0.118	0.632	0.218	0.519

rs: Spearman coefficient; *: Statistically significant at $p \leq 0.05$.

TABLE 3. Prognosis of HER-2 (2^+) equivocal and HER-2 (3^+) groups according to NPI and the revised NPI

PARAMETERS	Her-2 (2^+) (as detected by IHC) (N = 21)						Her-2 (3^+) (as detected by IHC) (N = 12)					
	NPI N (%)	DFS (%)	OS (%)	Revised NPI N (%)	DFS%	OS%	NPI N (%)	DFS (%)	OS (%)	Revised NPI N (%)	DFS%	OS%
PROGNOSIS	N (%)											
EXCELLENT (2.02 – < 2.4)	3 (14.3)	66.7	100	0 (0)	-	-	2 (16.7)	100	100	0 (0)	-	-
GOOD (2.4 – < 3.4)	3 (14.3)	66.7	66.7	4 (19)	37	66.7	3 (25)	0	100	4 (33.3)	100	100
MODERATE 1 (3.41 – < 4.4)	3 (14.3)	33.3	100	4 (19)	75	100	2 (16.7)	100	100	2 (16.7)	0	100
MODERATE 2 (4.41 – < 5.4)	3 (14.3)	33.3	100	3 (14.3)	33.3	100	1 (8.3)	100	100	2 (16.7)	100	100
POOR (5.41 – < 6.4)	3 (14.3)	33.3	66.7	3 (14.3)	33.3	66.7	1 (8.3)	100	100	0 (0)	-	-
VERY POOR (6.41 – < 6.8)	2 (9.5)	100	100	3 (14.3)	66.7	100	1 (8.3)	0	100	2 (16.7)	50	100
UNKNOWN	4 (19)	75	100	4 (19)	75	100		2 (16.7)	50	100	2 (16.7)	100

DISCUSSION

The current study is the first study validated the utility of sHER2 and tHER2 (as detected by Am and ELISA; respectively) in the differentiation of BC molecular subtypes in both primary and metastatic BC at cut-off values lower

than the approved value (15 ng/ml) for the current assays. It showed that the relation between tissue and serum HER2 in certain BC subgroups depends on the specific characteristics of those groups. The absence of an association between age variation among BC subtypes was detected in the current and previous studies [22,23]. Contrary to our results, significant age variation (p = 0.05) was reported in a study conducted on 49 Egyptian women with IDC where higher expression of HER2—as detected by IHC & RT-PCR—was detected in younger and older ages (100% in < 35 years and 91% in > 50 years**,** respectively), while ages from 35-50 years showed intermediate expression (67%) [24]. this conflicting data might implicate variability in the studied populations, the clinicopathological characteristics, the used assay, and/or the cut-off values.

Although HER2 status is an important diagnostic and prognostic marker in BC, the validity of HER2 extracellular domain (ECD) as a surrogate maker is not approved due to controversial results. However, our current results showed a significant direct correlation between tHER2 and sHER2 levels in some BC subgroups (HER2 enriched subgroup and in HER2 $^{3+}$ groups). Similarly, in a study on 545 Chinese primary BC**s** without prior treatment, sHER2 ECD (measured by immunoassay) related to tHER2 [measured by IHC or fluorescence in situ hybridization (FISH)] [25]. However, 36.9% of patients with tHER-2 over-expression had <15 ng/ml of sHER2 reflecting low shedding activity. Cell culture models revealed that the HER2 shedding activity is alpha-secretase and a disintegrin and metalloproteinase domain-containing protein10 (ADAM10) dependent and the progression-free survival (PFS) of BC patients with tHER-2 IHC $^{3+}$ and sHER-2 ECD > 15 ng/ml was lower than those with serum HER-2 ECD <15 ng/ml [25]. Recently, high association and concordance between sHER2 and tHER2 levels rather than HER2 ICD in HER2 $^{3+}$ subgroup (as detected by IHC) was also reported [5]. Krüger *et al*. attributed the absence of such correlation with another HER2 status to the "heterogeneity of ECD loss at the single-cell level, and in different areas of individual tumors" [26].

The reverse association between tHER2 and body mass index (BMI) in the equivocal HER2 $^{2+}$ BC subgroup stems from the main characteristics of this subgroup: obesity, postmenopausal status, hormonal positive status, and luminal B tumors. Studies documented a positive association between overweight or obesity and the risk of luminal BC molecular subtype amongst postmenopausal women with positive hormonal receptors status [27, 29]. It was suggested that abdominal fat plays an important role in developing specific BC molecular subtypes and insulin resistance amongst postmenopausal women especially those with hormone receptor-positive subtypes [30]. It is evident now that each subtype has a characteristic source of energy and metabolic profile [29, 31, 32]. Luminal subtypes upregulate *de novo* fatty acid synthesis and β-oxidation, TN depends on the exogenous fatty acids, and HER2 enriched subtype relies on the *de novo* fatty acid synthesis, storage, and oxidation [29,31,33].Moreover, recent findings showed inter- and intra-variations in the lipidomes of the various molecular BC subtypes [33]. HER2 can also crosstalk estrogen in the breast adipose tissue and acts as a key driver of breast cancer growth, development, stemness, and drug resistance [32, 34].

Serum HER2 ECD varies according to the used assay, cut-off values, presence of serum interfering factors, kinetics nature of serum HER2 ECD, ethnic population, BC progression, tumor stage, size, and origin of the tumor [35]. In a study on 322 advanced breast cancer, serum HER2 ECD (detected by ELISA) differed significantly between BC molecular subtypes (p < 0.001), tHER2, number of metastatic sites, visceral metastasis, sCA15-3, and carcinoembryonic antigen (CEA) [36]. A cut-off value of 7.4 ng/ml (sensitivity: 72.9%, specificity: 85.3%) was the best diagnostic cut-off value for early-stage BC [37]. Recent meta-analysis for 40 studies revealed low sensitivity and a reasonable specificity, accuracy, and area under the curve (AUC) of serum HER2 levels (detected by ELISA and/or CLIA; reference methods: IHC or IHC/FISH) "as a verification test for initial negative screening test results, especially in low-income regions due to its cost-effectiveness and ease of implementation" [38]. This further supports the value of our current findings of the utility of sHER2 and tHER2 using Am and ELISA**,** respectively**,** in the differentiation of BC molecular subtypes at lower cut-off values in both primary and metastatic BC molecular subtypes.

Despite the significant difference of CA15-3 among the studied groups, no relevance to the level of HER2 was detected in the current study. Similarly, a previous study detected no significant relation between sCA15-3 and any of the tumor prognostic factors, age, or patients 'outcomes [17]. Conversely, Perrie *et al*. showed the validity of serum HER2, CA15.3, and CEA levels for predicting differential therapeutic response and

"for monitoring HER2-targeted therapy in patients with HER2-positive metastatic breast cancer [39]. The average decrease of the three biomarkers with a threshold of > 10% appeared to be the best parameter to distinguish patients who will develop a progressive disease from those who will have complete, partial, or stable responses. Although Dong *et al.* reported a significant correlation between long-term disease-free survival and ER, PR, and HER2 levels, the relation was absent in our study [40]. This would be attributed to the fact that most of our cases had NPI ranging from excellent to average scores, and a lower number of ER-negative and TNBC [41, 42].

CONCLUSIONS

Tissue ELISA and serum AM provided accurate, quantitative, and cost-wise measurements for HER2 in various BC subtypes—at cut-off values lower than those approved by FDA—and would complement the current IHC and ISH techniques for dynamic profiling of HER2 in the diagnosis and/or prognosis of breast cancer. Further multi-Centre studies are strongly recommended. Testing the applicability and validating both ELISA and AM HER2 detection techniques in screening programs for early detection of breast cancer and tailored targeted therapy monitoring is mandatory as well.

Conflict(s) of interest: None
Financial Disclosures: None

REFERENCES

1. **Ahn S, Woo JW, Lee K, Park SY**. HER2 status in breast cancer: changes in guidelines and complicating factors for interpretation. J Pathol Transl Med, 2020; 54: 34-44.
2. **Krishnamurti U, Silverman JF.** HER2 in breast cancer: a review and update. Adv Anat Pathol, 2014; 21: 100-7.
3. **Furrer D, Paquet C, Jacob S, Diorio C.** The human epidermal growth factor receptor 2 (HER2) as a prognostic and predictive biomarker: Molecular insights into HER2 activation and diagnostic implications. Cancer PX, 2018, DOI: 10.5772/intechopen.78271.
4. **Tsé C, Gauchez A, Jacot W, Lamy PJ**. HER2 shedding and serum HER2 extracellular domain: Biology and clinical utility in breast cancer. Cancer Treat Rev, 2012; 38: 133-142.
5. **Fabricio AS, Michilin S, Zancan M, Agnolon V, Peloso L, Dittadi R, et al**. Shed HER2 surrogacy evaluation in primary breast cancer patients: a study assessing tumor tissue HER2 expression at both extracellular and intracellular levels. Scand J Clin Lab, 2019; 79:260-7.
6. **Furrer D, Sanschagrin F, Jacob S, Diorio C.** Advantages and disadvantages of technologies for HER2 testing in breast cancer specimens. Am J Clin Pathol, 2015; 1; 144:686-703.
7. **Lamy PJ, Guachez AS, Tse C, Williams SI.** The HER2 shedding and its consequences in breast cancer. HER2 and Cancer. Hauppauge, NY: Nova Science Publishers, Inc. 2011.
8. **Singh S., Kumar V., Dhanjal D.S., Datta S, Prasad R, Singh J.** Biological biosensors for monitoring and diagnosis. In: Singh J., Vyas A., Wang S., Prasad R. (eds) Microbial biotechnology: Basic research and applications. Microb Biotechnol, Springer Sci Rev, 2020.
9. **Aydin M, Aydin EB, Sezgintürk MK.** Advances in immunosensor technology. Adv Clin Chem, 2021;102:1-62.
10. **Arkan E, Saber R, Karimi Z, Shamsipur M.** A novel antibody-antigen based impedimetric immunosensor for low level detection of HER2 in serum samples of breast cancer patients via modification of a gold nanoparticles decorated multiwall carbon nanotube-ionic liquid electrode. Anal Chim Acta, 2015; 874: 66-74.
11. **Eletxigerra U, Martinez-Perdiguero J, Merino S, Barderas R, Torrente-Rodríguez RM, Villalonga R, et al.** Amperometric magnetoimmunosensor for ErbB2 breast cancer biomarker determination in human serum, cell lysates and intact breast cancer cells. Biosens Bioelectron, 2015; 70:34-41.
12. **Eletxigerra U, Martinez-Perdiguero J, Barderas R, Pingarrón JM, Campuzano S, Merino S.** Surface plasmon resonance immunosensor for ErbB2 breast cancer biomarker determination in human serum and raw cancer cell lysates. Anal Chim Acta, 2016; 905: 156-62.
13. **Pereira AC, Sales MG, Rodrigues LR.** Biosensors for rapid detection of breast cancer biomarkers. In Advanced biosensors for health care applications. Elsevier sci, 2019;71-103.
14. **Şahin S, Caglayan MO, Üstündağ Z.** Recent advances in aptamer-based sensors for breast cancer diagnosis: special cases for nanomaterial-based VEGF, HER2, and MUC1 aptasensors. Mikrochim Acta, 2020;187:549.
15. **Kölbl AC, Andergassen U, Jeschke U.** The Role of glycosylation in breast cancer

metastasis and cancer control. Front Oncol, 2015; 5:219.
16. **Syrkina MS, Vassetzky YS, Rubtsov MA.** MUC1 Story: Great expectations, disappointments, and the renaissance. Curr Med Chem, 2019;26:554-563.
17. **El-Abd E, El-Sheikh M, Zaky S, Fayed W, El-Zoghby S.** Plasma TuM2-PK correlates with tumor size, CRP and CA 15-3 in metastatic breast carcinomas; short versus long term follow-up study of the Egyptian breast cancer patients. Cancer Biomark, 2017; 20:123-33.
18. **Jeong S, Park MJ, Song W, Kim HS.** Current immunoassay methods and their applications to clinically used biomarkers of breast cancer. Clin, 2020; 78:43-57.
19. **Blamey RW, Ellis IO, Pinder SE, Lee AH, Macmillan RD, Morgan DA, et al.** Survival of invasive breast cancer according to the Nottingham Prognostic Index in cases diagnosed in 1990–1999. Eur J Cancer, 2007; 43:1548-55.
20. **Fong Y, Evans J, Brook D, Kenkre J, Jarvis P, Gower-Thomas K.** The Nottingham Prognostic Index: five-and ten-year data for all-cause survival within a screened population. Ann R Coll surg Engl, 2015; 97:137.
21. **Camacho C, Matías JC, Chico B, Cao R, Gómez L, Simpson BK, et al.** Amperometric biosensor for hydrogen peroxide, using supramolecular immobilized horseradish peroxidase on the β- cyclodextrin- coated gold electrode. NYNY, 2007;19: 2538-42.
22. **El-Abd E, Matta CA, Sheta M, El-Kerm Y, Sakr S.** Histopathological characteristics of breast cancer and evaluation of ER alpha and Her-2neu using immunohistochemical and RT-PCR techniques. Alexandria J Med, 2014;50:275-82.
23. **El-Abd E, Matta C, Sheta M., El-Kerm Y, Samy M.** Relation between hypoxic markers P65, P50, CAIX, and tumor stages in invasive ductal carcinoma subtypes. ACRT, 2015; 1–19.
24. **Ismail MF, Aly MS, Khaled HM, Mohamed HM.** Detection of HER-2/neu, c-myc amplification and p53 inactivation by FISH in Egyptian patients with breast cancer. GMS, 2009;7. doi: 10.3205/000062.
25. **Zheng H, Zhong A, Xie S, Wang Y, Sun J, Zhang J, et al.** Elevated serum HER- 2 predicts poor prognosis in breast cancer and is correlated to ADAM10 expression. Cancer Med, 2019;8:679-85.
26. **Krüger JM, Thomas M, Korn R, Dietmann G, Rutz C, Brockhoff G, et al.** Detection of truncated HER2 forms in formalin-fixed, paraffin-embedded breast cancer tissue captures heterogeneity and is not affected by HER2-targeted therapy. Am J Pathol 2013;183:336-43.
27. **Phipps AI, Malone KE, Porter PL, Daling JR, Li CI**. Body size and risk of luminal, HER2-overexpressing, and triple-negative breast cancer in postmenopausal women. CEBP, 2008;17:2078-86.
28. **Crispo A, Montella M, Buono G, Grimaldi M, D'Aiuto M, Capasso I, et al.** Body weight and risk of molecular breast cancer subtypes among postmenopausal Mediterranean women. Curr Res Transl Med, 2016; 64:15-20.
29. **Kang HS, Lee SC, Park YS, Jeon YE, Lee JH, Jung SY, et al.** Protein and lipid MALDI profiles classify breast cancers according to the intrinsic subtype. BMC cancer, 2011;11:465.
30. **Chen Z, Li Z, Li H, Jiang Y.** Metabolomics: a promising diagnostic and therapeutic implement for breast cancer. OncoTargets Ther, 2019; 12:6797.
31. **Bhardwaj P, Au CC, Benito-Martin A, Ladumor H, Oshchepkova S, Moges R, et al.** Estrogens and breast cancer: Mechanisms involved in obesity-related development, growth and progression. J. Steroid Biochem Mol Biol, 2019; 189: 161-70.
32. **Cappelletti V, Iorio E, Miodini P, Silvestri M, Dugo M, Daidone MG, et al.** Metabolic footprints and molecular subtypes in breast cancer. Dis Markers, 2017; 24. https://doi.org/10.1155/2017/7687851
33. **MK, Costa M, Bödvarsdottir SK, Ögmundsdottir HM, Thorsteinsdottir M.** Lipidomic study of cell lines reveals differences between breast cancer subtypes. PloS one, 2020; 15: Eiriksson FF, Nøhr e0231289.
34. **Gandhi N, Das GM.** Metabolic reprogramming in breast cancer and its therapeutic implications. Cell J, 2019;8:89.
35. **Lam L, McAndrew N, Yee M, Fu T, Tchou JC, Zhang H.** Challenges in the clinical utility of the serum test for HER2 ECD. Biochim Biophys Acta Rev Cancer, 2012;1826:199-208.
36. **Zhou J, Liu Y, Wang T, Zhang H, Du M, Zhang S, et al.** Serum HER2 ECD level and its clinical significance in advanced breast cancer patients with different molecular

subtypes. Zhonghua yi xue za zhi, 2014; 94:1384-7.

37. **Ma L, Yang H, Li J, Wang F, Han X, Shi Y.** Study on serum HER2 extracellular domain expression in early stage breast cancer patients. Ann Oncol , 2012; 23:111.
38. **Shamshirian A, Aref AR, Yip GW, Ebrahimi Warkiani M, Heydari K, Razavi Bazaz S, et al.** Diagnostic value of serum HER2 levels in breast cancer: a systematic review and meta-analysis. BMC cancer, 2020;20:1-10.
39. **Perrier A, Boelle PY, Chrétien Y, Gligorov J, Lotz JP, Brault D, et al.** An updated evaluation of serum sHER2, CA15. 3, and CEA levels as biomarkers for the response of patients with metastatic breast cancer to trastuzumab-based therapies. PLoS One, 2020;15: e0227356.
40. **Dong G, Wang D, Liang X, Gao H, Wang L, Yu X, et al.** Factors related to survival rates for breast cancer patients. Int J Clin Exp, 2014;7:3719.
41. **Juneja S, Agarwal R, Agarwal D, Rana P, Singh K, Kaur S.** Correlation of expression of estrogen receptor, progesterone receptor and human epidermal growth factor receptor-2 with histopathological grade in cases of carcinoma breast. Int j contemp med res, 2019; 6: I6-I11.
42. **Al jarroudi O, Zaimi A, Brahmi SA.** Nottingham Prognostic Index is an applicable prognostic tool in non-metastatic triple-negative breast cancer. Asian Pac J Cancer Prev, 2019; 20, 59-63.

To Cite:

Nakhla, S., Elwafe, Z., Saad, A. S., Aref, T., Ragab, N., elbadry, I., Eletxigerra, U. E., Campuzano, S., Martínez-Perdiguero, J., Merino, S. M., M. Pingarrón, J., Abdelmoneim, S., El-Abd, E. Tissue ELISA and Serum Amperometric Magnetoimmunosensor of ECD-HER2 In Stratifying Subtyping of Egyptian Woman Breast Cancers. *Zagazig University Medical Journal,* 2024; (141-153): -. doi: 10.21608/zumj.2022.161766.2643