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Research paper

Competitive ELISA method for novel estrogen-negative breast cancer biomarker quantitation

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ABSTRACT

Estrogen-negative (ER^-) breast cancer, is recognized as an aggressive subtype, more difficult to treat, with poor survival and prognosis. They are hormonally unresponsive, with no readily effective and specific target therapy. We have previously identified N^w-hydroxy L-Arginine (NOHA) as a blood-based biomarker to distinguish between ER^- and ER+ breast cancer tumors based upon disease burden, progression and molecular phenotype (U.S. Utility Patent 10,073,099). In this study we have demonstrated a competitive ELISA based assay for NOHA measurement using a proprietary monoclonal antibody (mAb) specific for NOHA (U.S. provisional patent 62/754,053). The ELISA assay was evaluated on sensitivity, selectivity, precision, dilution linearity and percent recovery parameters. The assay showed sensitivity at ≥60 pg/ml NOHA antigen with 1 ng/ml NOHA mAb, and maintained NOHA antigen specificity even in the presence of other closely related cationic amino acids (i.e. L-Arginine, D-Arginine, L-Lysine, D-Lysine, L-Ornithine, and L-Citrulline). The reliability of the ELISA protocol was confirmed with the low percent-covariance, for all tested parameters of sensitivity (≤8.2%), selectivity (≤8.6%), precision (≤12.6%), dilution linearity (≤11.2%) and recovery (≤6.7%). Additionally, we can demonstrate NOHA quantification by this ELISA assay to complement the sensitivity achievable with LC-MS (in both assay buffer and with patient plasma samples), thus suggesting it's utility as a simple yet sensitive methodology that might help in ER^- breast cancer prognosis, and disease progression monitoring without the need for expensive analytical equipment (such as LC-MS), large lab space, or specialized technical training.

1. Introduction

Breast cancer remains a major cause of mortality among women (Chavez et al., 2010), accounting for 15–20% of all cancer related deaths in women in the United States (Garcia et al., 2007). Recent large-scale gene expression profiling studies revealed significant differences in gene expression patterns among estrogen–negative (ER^-) and estrogen–positive (ER^+) tumors (Sorlie et al., 2001). Between these two major breast cancer subsets, ER^- is considered to be very aggressive and more difficult to treat than ER^+ tumors. It has a greater ethnic disparity, worse prognosis, and almost twice the risk of mortality; especially, among younger patients of African American, and Jewish origins, than those of Caucasian, Hispanic, or Asian descent (Sorlie et al., 2001; Glynn et al., 2010). Unlike ER^+ tumors, ER^- tumors are hormonally unresponsive, and not treatable by endocrine–targeted therapy such as tamoxifen and aromatase inhibitor protocols; and there is no truly effective and/or specific targeted therapy (Glynn et al., 2010; Cardoso et al., 2016; Hon et al., 2016). Currently, both early and advance stage ER^- breast tumors are treated predominantly by adjuvant chemotherapy. Therefore, there is a need for a fundamental biomarker that can monitor ER^- breast tumor prognosis, disease progression, and treatment outcome management.

Inflammation has been widely recognized as an inducer of cancer initiation, promotion and progression (Hussain et al., 2000; Ridnour et al., 2013; Smith and Kang, 2013). Aberrant changes (i.e. fluctuations) in the inflammation–responsive nitric oxide synthase enzyme (NOS2), than those NOS2 levels seen in healthy individuals, has been observed in multiple solid tumors, including breast cancer (Thomsen et al., 1995; Ambs et al., 1998; Vakkala et al., 2000; Ekmekcioglu et al., 2006; Yu et al., 2006). Upregulation of NOS2, which leads to an increase in nitric oxide (NO) production, has been associated with poor survival in ER^- breast cancer patients only (and not in ER^+ breast tumor patients) (Glynn et al., 2010; Heinecke et al., 2014). Whereas the use of several pro-inflammatory proteins (viz., COX2, NF-kB, IL-6, IL-8, S100 calcium binding protein, and VEGF) have shown inconsistent results as tumor biomarkers (Gluz et al., 2009). A thorough delineation of NOS2–NO

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biology and its association in tumor development and progression, therefore, warrants further investigation.

Interestingly, while all three isoforms of the nitric oxide synthase enzyme (i.e., NOS1, NOS2 and NOS3) can generate N^{w}-hydroxy-L-Arginine (NOHA) as a stable intermediate during NO formation (Stuehr et al., 1991; Pervin et al., 2008); NOHA produced by NOS2 (via a calcium independent pathway) gets effluxed into the circulation, (Garlichs et al., 2000). Once effluxed, NOHA competes with L-arginine (which is at ≥100–fold higher concentration than NOHA) for the same amino acid carrier (system y + carrier); but since the K_m for NOHA transport, as well as for its utilization by NOS isozyme is ≥2-fold higher than L-Arginine, NOHA re-uptake and metabolism remains highly restricted (if not impossible) (Stuehr et al., 1991; Klatt et al., 1993; Hecker et al., 1995). Such strict association of NOHA efflux from NOS2 expressing cells, along with its highly restricted re-uptake and metabolism favors NOHA as an effective breast cancer biomarker (with promising potential with other solid–cancer types).

Our previous studies via LC-MS analysis, have shown NOHA, U.S. Patent., 10,073,099; (Mohan et al., 2016; Mohan et al., 2017; Mohan et al., 2018) as a novel less-invasive biomarker to distinguish ER^- breast cancer from ER^+ breast tumor. These studies were based upon an analysis of disease burden, progression and molecular phenotype. In this study, we validate a competitive ELISA based assay, that uses a proprietary NOHA-specific monoclonal antibody (mAb) (U.S. provisional patent 62/754,053). We have assessed the ELISA assay on its response for NOHA sensitivity, precision (viz., inter-day and intra-day assay reproducibility), dilution linearity, and recovery parameters. We additionally conducted a comparative analysis for the NOHA measurement by the ELISA assay with an LC-MS protocol.

2. Methods

2.1. Reagents and supplies

All general lab chemicals were purchased (at ≥95% purity) from Sigma-Aldrich (St. Louis, MO). General lab supplies were purchased from VWR international (Bridgeport, NJ) and Celltreat Scientific products (Pepperell, MA). Deionized water at 18.2 MΩ was used in all experimental protocol.

2.2. Patient plasma samples

De-identified patient plasma samples, that were previously diagnosed with low-grade, stage-1 breast tumor (as per Nottingham grading system) (Rakha et al., 2008), and categorized based on estrogen-hormone-receptor orientation (viz., ER^- versus ER^+, $n = 50$), were obtained from cooperative human tissue network (Philadelphia, PA), Tissue-for-Research (Atlanta, GA), and Conversant Bio (Huntsville, AL). Healthy patient plasma samples ($n = 50$) as a control, was obtained from cooperative human tissue network (Philadelphia, PA).

2.3. BSA-NOHA strip preparation

NOHA was conjugated to BSA using Imject™ BSA Protein Conjugation Kit from Fisher Scientific (Grand Island, NY). 3 µg/ml of BSA-NOHA conjugate in 0.1 M Sodium Carbonate buffer pH 9.5, at 100 µl/well was added to medium-binding 96 well flat bottom 12xF8 strip plate from USA Scientific (Ocala, FL), and incubated overnight at 4 °C. The plates were then washed with 1 × wash buffer (i.e. 1 × phosphate buffered saline or PBS, 0.025% Tween 20, 0.004% Zwitterion 3–14, pH 6.8–7); and then blocked with 75 µl blocking buffer (1 × PBS, with 1% BSA), and incubated for 2 h at 25 °C. The wells were decanted and 250 µl of Tris Buffer (pH 8.5) with 2.5% sucrose was added to each well and incubated for another 1 h at 25 °C. The wells were then decanted and dried in a vacuum desiccator, before storing in individual re-sealable plastic bag (with desiccant sachet) at 4 °C for ELISA assay use.

2.4. ELISA standard curve development and assay validation

To develop the standard curve for NOHA quantitation, varying concentrations of NOHA antigen (150–0 ng/ml) mixed with 5 ng/ml of NOHA mAb were incubated for 2 h, at 25 °C. For NOHA mAb sensitivity testing, varying concentration of NOHA (20–0 ng/ml) and NOHA mAb (10–1 ng/ml) were mixed at two different ratios of NOHA:NOHA mAb (i.e. 2:1, and 5:1) and incubated for 2 h at 25 °C. For NOHA mAb specificity testing, we mixed 20 ng/ml of NOHA antigen and 50 ng/ml of a closely-related cationic amino acid (viz., L-Arginine, D-Arginine, L-Lysine, D-Lysine, L-Ornithine, and L-citrulline), with 5 ng/ml NOHA mAb, before incubating them for 2 h at 25 °C.

For assay precision detection, each replicate for 50, 20 and 10 ng/ml of NOHA mixed with 5 ng/ml NOHA mAb were incubated for 2 h at 25 °C, before detecting the presence of NOHA by our competitive ELISA at two independent time point, either on the same day (for intra-day precision), or on two consecutive days (for inter-day precision). Additionally, to assess dilution linearity for the assay, each replicate for 50, 20 and 10 ng/ml of NOHA mixed with 5 ng/ml NOHA mAb were diluted 2-fold in 1 × PBS and incubated for 2 h at 25 °C. Furthermore, to measure percent recovery, each replicate at varying NOHA concentrations (50, 20 and 10 ng/ml) mixed with 5 ng/ml of NOHA mAb, were spiked with additional NOHA concentrations (of 50, 30 and 15 ng/ml) before starting their incubation for 2 h at 25 °C. The initial NOHA antigen concentration was chosen as 10–50 ng/ml for these precision and dilution linearity assays to ensure that there was sufficient NOHA antigen available for initial NOHA-mAb interaction, before testing for secondary competitive NOHA mAb binding to BSA-NOHA strips. The 2 h incubation with NOHA antigen and mAb samples (for standard curve development and each of the various assay validation procedures) was performed to allow primary mAb-NOHA complex formation, before performing the competitive ELISA protocol.

2.5. Competitive ELISA

BSA-NOHA ELISA strips were washed atleast 3 times with 200 µl of 1 × wash buffer. 100 µl of mAb-NOHA complex mixture was added to each ELISA strip well, and incubated for 1 h, at 25 °C. After incubation, well contents were discarded, and washed with 200 µl of 1 × wash buffer, for at least 8 times, before adding 100 µl of polyclonal horseradish-peroxidase (HL-HRP) conjugate from Abcam (Cambridge, MA), at 1:20,000 dilution (in 1 × wash buffer). The wells were incubated again for 1 h, at 25 °C, before decanting the well contents and washing them with 200 µl of 1 × wash buffer, at least 8 times. 100 µl of tetramethyl-benzidine (TMB) substrate from Mossbio (Pasadena, MD) was added to each well. The wells were incubated for 10 min in the dark, and the HRP-TMB interaction was stopped with 100 µl 0.1 N HCl stop solution. All wells were read for absorbance at 450 nm using a VersaMax® Spectrophotometer (Molecular Devices, NH). The resultant absorbance values were compared with their theoretical NOHA antigen concentrations. A polynomial second-order trendline and R-squared value were added to the standard curve to assess its confidence in NOHA measurement.

2.6. LC-MS assay

Our LC-MS methodology (Mohan et al., 2017) was selectively adopted from Nemkov et al. (2015). For this study, assay buffer (viz., water with 0.05% trifluoroacetic) used to achieve a final theoretical concentration of 50, and 100 ng/ml NOHA, was quantified using a 150 mm × 2.1 mm Alltima HP HILIC 3 µm column, based on isocratic separation with 20% mobile phase A (i.e., water containing 0.05% trifluoroacetic) and 80% mobile phase B at a flow rate of 0.1 ml/min for 6 min using Agilent Technologies 1200 series HPLC system. The $[M + H]^+$ ions were analyzed in the multiple reaction-monitoring mode of the Agilent Technologies 6460 triple quadrupole mass spectrometer

Table 1
ELISA standard curve for NOHA antigen measurement. NA represents not applicable value.

Theoretical NOHA antigen well concentration (ng/ml) + 5 ng/ml of NOHA mAb	Log_{10} NOHA	Observed/calculated NOHA antigen (ng/ml)
150	5.18	149.7
100	5.00	100.2
50	4.70	49.8
20	4.30	19.6
10	4.00	9.7
0	NA	–

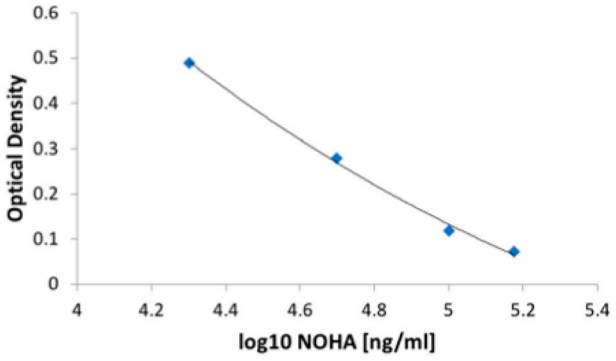

Fig. 1. ELISA standard curve. The NOHA antigen of 150–0 ng/ml with 5 ng/ml monoclonal NOHA antibody (mAB) was used. The mean O.D. values (Y-axis) from duplicate readings was plotted against the log NOHA antigen standard concentrations (X-axis), as an XY scatter plot. A polynomial order-2 trendline (i.e. y = 0.1488 × 2–1.8976× + 5.9014), with R-squared confidence value (of ≥0.997) were added.

equipped with an electrospray ion (ESI) source. The spray voltage was set at 4.5 kV. The flow rates of nebulizer gas (N_2) and curtain gas (N_2) were maintained at 9 Arb and 7 Arb, respectively. Fragmentation occurred at collision gas pressure of 1.5 mTorr.

2.7. Statistical analysis

Results are presented as mean ± standard deviation, along with % covariance, from 4 repetitive samples ($n = 4$), for each tested criteria/condition, unless otherwise stated. The standard curve polynomial second-order tread-line and R-squared values were developed using Microsoft Excel® software. Statistical comparisons among groups were performed using Student's *t*-test (GraphPad Prism, Version 8.0, La Jolla, CA). Statistical significance was concluded with $p < .01$.

3. Results

3.1. ELISA standard curve and assay validation

Our ELISA standard curve scatter plot between absorbance values and log NOHA antigen concentration (of 150–0 ng/ml) received a R-squared confidence value of ≥0.99, for a polynomial order-2 trendline (Table 1; Fig. 1), thus indicating the theoretical NOHA concentration to strictly associate with their experimentally observed NOHA absorbance values, thereby allowing their fitting on the regression line, with negligible deviation. This ELISA standard curve was then used to validate the assay protocol for NOHA quantitation based on sensitivity, selectivity, precision, dilution linearity and recovery parameters.

For assay sensitivity testing, we used two different mixing ratios for NOHA antigen mixed with NOHA mAb (viz., at 2:1 and 5:1). 1 ng/ml of NOHA mAb was the lowest effective concentration to quantify NOHA antigen (Table 2 A, B). Our results also showed 0.06 ng/ml (viz., 60 pg/ml) as the lowest NOHA antigen concentration that could be quantified

Table 2
Sensitivity assessment to identify the lowest effective NOHA mAb concentration in ELISA.

A.

In well concentration after 1:1 mix of reagents		In-well Ratio	Observed/calculated NOHA antigen	% Covariance
Theoretical NOHA antigen (ng/ml)	NOHA mAb (ng/ml)		Mean ± SD (ng/ml)	
20	10	2:1	20.1 ± 0.6	8.2
16	8	2:1	15.9 ± 0.4	5.3
8	4	2:1	8.1 ± 0.3	6.8
4	2	2:1	3.9 ± 0.2	7.3
2	1*	2:1*	2.1 ± 0.1*	8.1*
1.6	0.8	2:1	NQ	NQ
0.8	0.4	2:1	NQ	NQ
0	0	Blank	–	–

B.

In well concentration after 1:1 mix of reagents		In-well Ratio	Observed/calculated NOHA antigen	% Covariance
Theoretical NOHA antigen (ng/ml)	NOHA mAb (ng/ml)		Mean ± SD (ng/ml)	
10	2	5:1	9.8 ± 0.5	8.1
5	1*	5:1*	5.1 ± 0.2*	7.9*
2.5	0.5	5:1	NQ	NQ
0	0	Blank	–	–

Assessment was made at NOHA antigen to NOHA mAb ratios of [A] 2:1, and [B] 5:1, as mean ± standard deviation (SD). *, represents the lowest effective NOHA mAb concentration, to quantify NOHA antigen concentration based on observable NOHA mAb complexation with NOHA antigen, at different mAb-NOHA ratios; $n = 4$, $p < .01$. NQ represents not quantifiable NOHA concentrations.

Table 3
Sensitivity assessment to identify lowest NOHA antigen concentration in ELISA, as mean ± standard deviation (SD).

In well concentration after 1:1 mix of reagents		Observed/calculated NOHA antigen	% Covariance
Theoretical NOHA antigen (ng/ml)	NOHA mAb (ng/ml)	Mean ± SD (ng/ml)	
20	1	19.8 ± 0.4	8.4
10	1	9.7 ± 0.5	8.6
4	1	3.9 ± 0.2	6.9
1	1	0.94 ± 0.04	5.9
0.4	1	0.39 ± 0.02	5.4
0.2	1	0.19 ± 0.02	6.9
0.1	1	0.09 ± 0.01	6.2
0.06*	1	0.057 ± 0.003*	7.9
0.05	1	NQ	NQ
0	1	NQ	NQ
0	0	Blank	–

*, Represents the lowest effective NOHA antigen concentration, that is quantifiable using 1 ng/ml monoclonal NOHA mAb, $n = 4$, $p < .01$. NQ represents not quantifiable NOHA concentrations.

with 1 ng/ml NOHA mAb (Table 3). The selectivity analysis showed ELISA quantified NOHA values to be similar (if not same) to their expected theoretical values, when tested in the presence of other cationic amino acids that would closely resemble NOHA (viz., Arginine, Lysine, Citrulline, Ornithine); and irrespective of other cationic amino acid enantiomeric state (i.e. D– versus L– form; Table 4). A comparison of the ELISA based quantification of NOHA antigen replicates that were assayed the same day (for intra assay precision assessment, Table 5), and on two consecutive days (for inter-assay precision determination, Table 6), showed NOHA levels that were similar to their expected theoretical concentrations.

A similar assessment of NOHA concentrations between theoretical and ELISA based quantitation showed statistically similar NOHA values in samples that were 2-fold diluted (for assessing precision linearity; Table 7), and in samples additionally spiked with known NOHA concentrations (to determine NOHA percent recovery; Table 8).

3.2. ELISA versus LC-MS comparative analysis

NOHA measurement by ELISA methodology and with a standardized LC-MS assay showed similar results between the two assays, that were statistically similar to their desired theoretical NOHA concentrations (Table 9). Furthermore, NOHA measurement in ER^-/ER^+ breast cancer patient plasma samples (diagnosed with low-grade, stage-1 tumor), by ELISA assay and LC-MS method were also found to complement one another, with statistically distinct NOHA reduction for ER^- breast cancer plasma samples. (Fig. 2).

4. Discussion

This study provides the first validation of a competitive ELISA for NOHA quantification with a proprietary NOHA mAb. Previous studies involving LC-MS methodology have shown NOHA reduction among ER^- breast cancer patients (with stage-1. low-grade tumor) to be associated with initial disease prognosis, with potential for tumor progression monitoring based on progressive NOHA reduction assessment (Mohan et al., 2016; Mohan et al., 2017; Mohan et al., 2018). We chose to develop an antibody based immuno-assay (such as ELISA) to measure NOHA that would otherwise be achievable only with an LC-MS methodology. Through the validation of this competitive ELISA, we believe to have developed a simple, yet sensitive methodology for NOHA quantitation, without the need for expensive equipment, lab space or specialized technical training.

To develop the standard curve for NOHA quantitation (with a R-squared confidence value of ≥0.99) we found it essential to maintain the ratio (for NOHA antigen to NOHA mAb) to range from 30:1 to 1:1. We adopted a wide range for NOHA antigen concentration (i.e. 150–0 ng/ml), based on our prior LC-MS standard curve, and NOHA antigen values seen in healthy and ER^-/ER^+ patient plasma (Mohan et al., 2017). Having this wide range in NOHA antigen to NOHA mAb ratio, allowed us to establish the varying degree of NOHA-mAb primary-complex formation, that needs to occur prior to any secondary interaction of any unbound NOHA mAb with the BSA-NOHA in the ELISA strip wells. After establishing the competitive ELISA standard curve for NOHA measurement, we tested the robustness (or ruggedness) of the protocol, prior to conducting any assay validation.

We determined our competitive ELISA assay ruggedness by creating variations to the reagents and supplies that were used in the protocol.

Table 5
Measurement of intra-day precision of ELISA Assay, as mean ± standard deviation (SD).

Theoretical NOHA Antigen concentration (ng/ml) + 5 ng/ml NOHA mAb	Number of replicated (n)	Calculated/ observed NOHA antigen (ng/ml)	% Covariance
		Mean ± SD	
50	4	49.8 ± 1.2	2.5
20	4	19.9 ± 0.7	3.7
10	4	10.1 ± 0.7	7.2

Results represent no statistically significant variation between theoretical and observed/calculated NOHA concentrations, $n = 4$, $p < .01$.

Table 6
Measurement of inter-day precision of ELISA Assay, as mean ± standard deviation (SD).

Theoretical NOHA antigen concentration (ng/ml) + 5 ng/ml NOHA mAb	Number of replicated (*n*)	Calculated/ observed NOHA antigen (ng/ml)	% Covariance
		Mean ± SD	
50	4	50.1 ± 1.8	3.5
20	4	20.4 ± 1.7	8.1
10	4	9.8 ± 1.3	12.6

Results represent no statistically significant variation between theoretical and observed/calculated NOHA concentrations, $n = 4$, $p < .01$.

Table 4
Selectivity measurement of ELISA assay, as mean ± standard deviation (SD).

Theoretical NOHA antigen (ng/ml) + 5 ng/ml NOHA mAb	20	20	20	20	20	20
Spiked Cationic amino acids	L-Arginine	D-Arginine	L-Lysine	D-Lysine	L-Citrulline	L-Ornithine
Spiked Cationic amino acids concentration (ng/ml)	50	50	50	50	50	50
Number of replicates (*n*)	4	4	4	4	4	4
Calculated/observed NOHA antigen Mean ± SD (ng/ml)	20.1 ± 0.9	19.7 ± 0.8	20.1 ± 0.8	19.9 ± 0.8	20.1 ± 0.8	20.1 ± 0.8
% Covariance	8.6	7.2	8.2	7.4	8.3	8.4

Results represents selectivity of NOHA mAb to only NOHA, in the presence of other cationic amino acids, irrespective of their enantiomeric state (i.e. D-form versus L-form), $n = 4$, $p < .01$.

Table 7
Assessment of ELISA dilution linearity for NOHA measurement, as mean ± standard deviation (SD).

Theoretical NOHA antigen concentration (ng/ml) + 5 ng/ml NOHA mAb	Dilution fold	Number of replicates	Expected NOHA (ng/ml)	Calculated/observed NOHA antigen (ng/ml)	% Covariance
				Mean ± SD	
50	2	4	25	24.9 ± 1.1	4.4
20	2	4	10	9.8 ± 0.9	9.7
10	2	4	5	5.3 ± 0.6	11.2

Results represent no statistically significant variation between theoretical and observed/calculated NOHA concentrations, $n = 4$, $p < .01$.

Table 8
NOHA recovery assessment in ELISA assay, as mean ± standard deviation (SD).

Theoretical NOHA antigen concentration (ng/ml) + 5 ng/ml NOHA mAb	Spiked NOHA (ng/ml)	Number of replicates	Expected NOHA (ng/ml)	Calculated/observed NOHA antigen (ng/ml)	% Covariance
				Mean ± SD	
50	50	4	100	98.0.4 ± 5.1	5.2
20	30	4	50	50.6 ± 3.4	6.7
10	15	4	25	24.6 ± 1.3	5.4

Results represent no statistically significant variation between theoretical and observed/calculated NOHA concentrations, $n = 4$, $p < .01$.

These variations involved, using commercially available polyclonal HL-HRP conjugate and TMB substrate from two-different manufacturer-designated batches; using BSA-NOHA ELISA strips that were prepared at two independent instances; and using either of two 25× stock wash buffers to prepare 1×-wash buffer for the assay. The stock buffer solutions and BSA-NOHA strips, used to test the assay robustness, were prepared within 3 months from their use. Since the overall assay outcome remained unaffected by any of these variations in supplies and reagent usage, it supports the ruggedness of the assay protocol. It also provides confidence in using ≤3 month-old BSA-NOHA strips and stock solutions without any stability concerns on the overall assay. We believe this preliminary ruggedness or robustness testing for our in-house assay protocol is beneficial, as it helps to avoid future demand for subsequent validations that may otherwise be needed at instances of such supply and reagent changes.

Table 9
Comparative analysis of NOHA quantification between ELISA and LC-MS methods, as mean ± standard deviation (SD).

Theoretical NOHA antigen concentration (ng/ml) + 5 ng/ml NOHA mAb	Number of replicates	Calculated/observed NOHA antigen (ng/ml)		% Covariance	
		Mean ± SD			
		By ELISA	By LC-MS	By ELISA	By LC-MS
100	4	98.6 ± 5.3#	99.5 ± 5.6	5.4	5.7
50	4	50.4 ± 3.9#	50.2 ± 4.3	7.8	8.5

Results represent no statistical significance between theoretical and observed values for NOHA measurements between ELISA and LC-MS assays.
#, represents comparable NOHA measurements between ELISA and LC-MS assays.

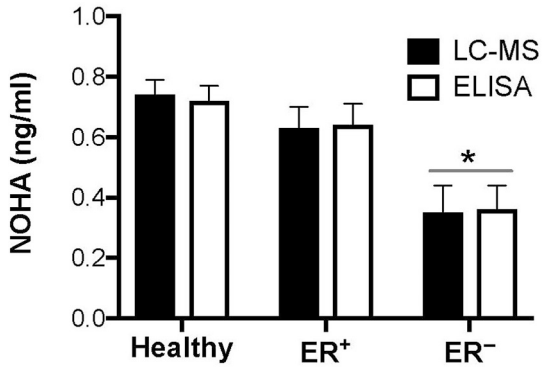

Fig. 2. Plasma NOHA comparison between ELISA and LC-MS. Low-grade, stage 1 plasma samples from ER−/ER+ patients and healthy control subjects, were used for comparative assay study. *, represents statistical significance in NOHA measurement in ER^- breast cancer patient from ER^+ breast cancer and healthy patients, at $n = 50$, $p < .01$.

To validate the NOHA competitive ELISA assay, we conducted fundamental assessment of its sensitivity, selectivity, precision, dilution linearity and recovery parameters. In all of our validation studies, we used 5 ng/ml of NOHA mAb, to ensure optimum NOHA-mAb primary-complex formation, for subsequent NOHA quantitation by the competitive ELISA assay.

The sensitivity analysis involved identification of the minimum NOHA mAb required for NOHA quantification (from background), and also determined the minimum NOHA antigen that can be quantified, with the lowest NOHA mAb concentration. We chose the NOHA antigen to NOHA mAb concentrations of 2:1 and 5:1 to ensure that there was sufficient NOHA antigen available for initial NOHA-mAb interaction, before testing for secondary competitive NOHA mAb binding to BSA-NOHA strips, at all tested NOHA mAb concentrations. The ELISA sensitivity at pg/ml range (viz., 60 pg/ml or 0.06 ng/ml) supports its utility as a tool to monitor NOHA reduction in ER^- breast cancer patients, for prognosis and disease progression. Since NOHA is an intermediate form of a cationic amino acid (i.e. L-Arginine) we wanted to test the selectivity of our NOHA mAb to bind only to NOHA and not to any other closely related cationic amino acids (viz., Arginine, citrulline, Lysine and ornithine). The selectivity assays showed that, the presence of any additional cationic amino acids, irrespective of their enantiomeric state, and at concentrations that are more than twice that of NOHA antigen (which otherwise could favor non-selective binding to NOHA mAb), to have no dampening effect on NOHA quantitation by this ELISA methodology; thus suggesting that the proprietary NOHA mAb used in the assay was highly selective in recognizing only NOHA antigen in a given sample. The high selectivity for NOHA mAb epitope binding to only NOHA antigen was additionally observed in a supplemental study (data not shown here), where we conducted a comparative analyses of ELISA results from BSA-NOHA coated 8-well strips to those obtained using 8-

well strips coated with KLH-NOHA conjugate, or with 10-mer NOHA polypeptide attached to Pierce™ amine-binding, maleic anhydride activated plates (Rockford, IL).

To validate the assay precision, we repeatedly measured (viz., $n = 4$) selective NOHA concentrations at two different instances on the same day (as intra-day testing), or at different time-points over two consecutive days (as inter-day testing). The intent of these precision analyses was to capture the potential variability in assay reproducibility and to assess repetitiveness. However, we did not assess the inter-assay or intra-assay precision analysis based on individual skill level, as ELISA assay in general does not require highly-trained technical skills. The small standard-deviation and small-coefficient of variance percent values under intra-day and inter-day testing conditions, suggests the high-assay reproducibility and repetitiveness precision for the NOHA ELISA methodology; which would be an essential quality to boost confidence with this assay to monitor NOHA for ER^- breast cancer prognosis and disease progression.

Dilution linearity assessment of the methodology was performed to demonstrate that a sample, when diluted within the NOHA ELISA standard curve range, is capable of providing a measurement for NOHA that is similar to their theoretically determined value. Likewise, the detection response of the assay in determining the amount of NOHA that got added/spiked to an original NOHA containing sample matrix was tested as the recovery parameter. In both dilution linearity and recovery parameter analyses, the ELISA based NOHA measurement was similar to their theoretical value with low standard deviation and percent co-variance, thus improving the overall confidence of the assay for NOHA quantitation. Furthermore, when NOHA measurement by the ELISA assay was compared with NOHA levels from a standardized LC-MS assay, the values were similar to one another and also with their expected theoretical concentrations. Of added importance, the NOHA measurement between ELISA and LC-MS assay in ER^-/ER^+ breast cancer patient plasma samples (diagnosed with low-grade, stage-1 tumor) showed similar results with no statistically significant distinction. This validation in patient plasma samples suggests the use of this ELISA assay as a suitable replacement to otherwise labor-intensive LC-MS analysis for NOHA measurement in ER^-/ER^+ patients.

The present NOHA ELISA assay shows potential to be developed in to a low-cost, easy to use, point-of-care diagnostic tool that could be useful with patient screening for aggressive type of cancer (such as the ER^- breast cancer) at rural settings (within USA), and around the world, where access to care and/or diagnostic centers are limited. It shows future promise for monitoring clinical and pathologic response to neoadjuvant therapy in ER^- breast cancer patients, and could be useful to reduce overtreatment of patients with ineffective therapy. The NOHA ELISA assay could also be used to continuously monitor clinical trial outcome during new therapy/drug development (for ER^- breast cancer and/or other solid tumors), and thus, might play a significant role in the development of personalized medicine.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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