BREAST CANCER

original reports

# NOHA: A Promising Biomarker for Determining Estrogen Receptor Status Among Patients With Breast Cancer in Resource-Constrained Settings

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abstract

**PURPOSE** Challenges to breast cancer control in low-and middle-income countries exist because of constrained access to care, including pathology services. Immunohistochemistry (IHC)–based estrogen receptor (ER) analysis is limited-nonexistent because of few and inadequately staffed and equipped pathology laboratories. We have identified N$^{w}$-hydroxy-L-Arginine (NOHA) as a blood-based biomarker to distinguish ER status in US patients with breast cancer. Here, we examine NOHA's clinical utility as an ER IHC alternative in Tanzanian patients.

**MATERIALS AND METHODS** Following informed consent, 70 newly diagnosed, known or suspected patients with breast cancer were enrolled at Kilimanjaro Christian Medical Center; basic, deidentified clinical and sociodemographic data were collected. For each, a needle prick amount of blood was collected on a Noviplex plasma card and stored at –80°C. Plasma cards and unstained tumor pathology slides were shipped regularly to US laboratories for NOHA, histologic and IHC analysis. NOHA and IHC assay operators were blinded to each other's result and patient clinical status. Paired NOHA and IHC results were compared.

**RESULTS** Slides from 43 participants were available for pathological analysis in the United States. Of those with confirmed malignancy (n = 39), 44%, 51%, 5% were ER-positive, ER-negative, and ER inconclusive, respectively. NOHA levels were available among 33 of 43 of those with pathological data and showed distinct threshold levels correlating 100% to tumor ER IHC and disease categorization where a level below 4 nM, from 4 to 8 nM, and above 8 nM signified ER-negative, ER-positive, and no cancer, respectively.

**CONCLUSION** The results are consistent with findings from US patients and suggest NOHA's clinical utility as an accessible IHC replacement in determining ER status among low-and middle-income country patients with breast cancer, promising to extend access to cost-efficient, available hormonal agents and improve outcomes.

JCO Global Oncol 8:e2200192. © 2022 by American Society of Clinical Oncology



## BACKGROUND

Breast cancer is the leading cause of cancer morbidity and mortality in women globally with nearly 2.3 million cases and 700k deaths yearly.[1] Mortality rates are substantially higher among women in low-and middle-income countries (LMICs),[1,2] largely due to advanced stages at diagnosis, reflecting diagnostic delays because of limited care access. Mortality-to-incidence rates in sub-Saharan Africa (SSA) reach 0.55 versus 0.16 in North America.[1,2] Breast cancer incidence rates are projected to increase in SSA because of shifting risk factors as countries transition economically.[3] In East Africa, annual breast cancer cases are expected to climb from 45.7k in 2020 to 125k in 2040.[4] Advancements in breast cancer control require longitudinal assessment of a nation's disease burden, reflecting accurate diagnoses and collection of prognostic information, including that from a dependable pathology report.

Despite a significant burden of late-stage breast cancer in SSA, individuals can benefit from surgery, chemotherapy, or endocrine therapy, depending on tumor biology, advancing both quality of life and survival.[1] Included among the 2019 WHO essential medicines list are two oral, easy to administer, and accessible breast cancer hormonal agents: tamoxifen and anastrozole.[5] The offer of surgery or systemic therapy, including hormonal therapy, requires pathological confirmation of disease[1] with optimal treatment dependent on stage and other markers, the most critical of

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Accepted on November 3, 2022 and published at ascopubs.org/journal/go on December 21, 2022: DOI https://doi.org/10.1200/GO.22.00192

## CONTEXT

### Key Objective

The overall goal of this work was to pilot test an innovative, US-validated and patented blood-based assay, N$^{w}$-hydroxy-L-Arginine, to determine estrogen receptor (ER) status among a cohort of patients with newly diagnosed breast cancer from a single Tanzanian cancer center.

### Knowledge Generated

Offers insight into a promising, sensitive, blood-based technology that differentiates ER-negative versus ER-positive breast cancers and uses inexpensive, easy-to-maintain equipment and reagents, suitable for point-of-care use by laboratory personnel in low-and middle-income countries.

### Relevance

N$^{w}$-hydroxy-L-Arginine holds promise as an attractive and scalable replacement for costly immunohistochemistry-based ER testing, with potential clinical applications extending beyond cancer diagnostics to surveillance, determination of prognosis, and disease monitoring in resource-constrained settings.

which is estrogen receptor (ER) alpha expression.[6] Although prognostic, progesterone receptor (PR) status is not significantly predictive of response to hormonal agents,[7] and costly human epidermal growth factor receptor 2 (HER2)–directed therapies are not widely available in LMICs. Thus, PR analysis is of limited value when added to ER, and HER2 testing is not warranted when related therapy is cost-prohibitive[1,8] in resource-constrained settings such as Tanzania. Fundamentally, ER determination classifies a breast cancer as ER-positive (ER+) versus ER-negative (ER–) and improves the potential of disease control through identification of candidates for cost-efficient and easy-to-access and administer endocrine therapies, promising to improve survival and quality of life.[1] However, there are marked shortages in professional and technical pathology services in LMICs, with many of the lowest pathologist-to-population ratios and associated technical services, such as hormone receptor immunohistochemical (IHC) testing in Africa, including Tanzania.[1,9]

We have identified N$^{w}$-hydroxy-L-Arginine (NOHA) as a blood-based marker that is 100% sensitive and specific, respectively, (95% CI, 94.5% to 100% each) in determining ER status in five ethnically and racially diverse US groups (US Utility Patent 10073099).[10-12] We also find NOHA to be effective in differentiating ER– tumors by grade and molecular phenotype.[10] NOHA can be measured at a fraction of the cost of traditional IHC and can be run on fresh or dried plasma extracted from a needle prick amount of blood.[10,13] In addition, NOHA remains stable in dried plasma for 14 days, at ≤ 42°C, allowing for ease of sample shipment.[13] NOHA promises to be a cost-effective and accessible tool for disease analysis in LMICs.

The overall goal of this work was to pilot test and validate the use of this new, US-validated, blood-based assay to determine ER status among a cohort of patients with newly diagnosed breast cancer from a single Tanzanian cancer center.

## MATERIALS AND METHODS

The Kilimanjaro Christian Medical Centre (KCMC) and the National Institute of Medical Research institutional review boards approved this work (ie, KCMC #2445 and NIMR IHQ/R.8a/Vol. IX/3249). Written informed consent was required from all participants and was collected at enrollment by the KCMC study coordinator. There were no incentives offered for enrollment.

### Settings

This study was done in partnership with KCMC staff in Moshi, serving the Northern Zone of Tanzania. All NOHA analysis was performed in the Mohan laboratory at the University of New England (UNE; Portland, ME). Study-based histologic and hormone receptor, ER, and PR IHC testing was performed at Maine Medical Center (MMC; Portland, ME).

### Participants

A total of 70 individuals were recruited for this study. Participant enrollment was based on a suspected or proven breast cancer diagnosis, before surgical or medical management. Basic clinical and sociodemographic data from participants were collected by KCMC clinical staff and stored in a secure study database, without identifiers.

### Sample Collection, Handling, and Processing

Following informed consent and before any treatment, a finger prick amount of blood (approximately 25 μL) was collected from all participants onto a Shimatzu Noviplex plasma prep card (West Lafayette, IN). After a 3-minute incubation period, the top layer of the Noviplex card was peeled off; the resultant plasma containing disc was air dried for 15 minutes, individually vacuum sealed, and stored at –80°C before shipment directly from KCMC via DHL international overnight service every 3 months. The plasma card shipment protocol was changed after the loss of 34 samples because of the commercial shipper's package mishandling, resulting in sample denaturation.

 

Subsequently, all plasma cards were secured in individually sealed vacuum bags, wrapped in aluminum foil to protect the sample from atmospheric moisture and photon radiation. Secured samples were hand carried by research or clinical volunteers returning to the United States for ultimate stateside shipment to the Mohan laboratory at 2-3 month intervals. This resulted in a total of 36 Noviplex plasma prep card samples available for stateside NOHA analysis.

Surgical specimens from all participants were submitted for routine pathology at KCMC, where five unstained slides were prepared and shipped from KCMC via DHL international overnight service at 3-month intervals for study-based histologic and IHC analysis at MMC. When available, clinical hormone receptor IHC testing was also performed on site at KCMC with results recorded in the secure study database.

Study-based hormone receptor IHC testing was performed on shipped unstained tissues slides from study participants, as described by Allison et al,[14] by a single pathologist (coauthor, M.J.) at MMC, following CAP guidelines wherein a tumor was classified as ER+ if $\geq$ 1% of tumor cells demonstrated nuclear staining. NOHA and IHC assay operators were blinded to each other's results and to patient clinical status. During the study period, among those cases undergoing clinical IHC analysis at KCMC, a tumor was classified as ER+ if at least 10% of cells revealed nuclear staining.

### Sample Preparation for NOHA Enzyme-Linked Immunosorbent Assay Analysis

The dried plasma disk from each enrolled participant was soaked in 100 μL of deionized water for 15 minutes at room temperature (25°C), without stirring. The soaked discs were then placed in 200 μL of extraction buffer (ie, 9:1, acetonitrile-water, v/v), vortexed for 1 minutes, sonicated for 5 minutes, incubated in extraction buffer for 10 minutes, disc discarded, and centrifuged at 14,000 *g* for 5 minutes. The resulting supernatant was dried in a lyophilizer and reconstituted in 50 μL of water and stored at –80°C, until NOHA measurement by enzyme-linked immunosorbent assay (ELISA) assay.

### NOHA ELISA Assay

We previously validated a competitive ELISA assay for NOHA quantification with a proprietary monoclonal antibody, as a simple yet sensitive alternative method to analytical NOHA detection by LC-MS.[11] The competitive NOHA ELISA assay was used here. All reagents and chemicals were purchased from either Invitrogen (Carlsbad, CA) or Sigma-Aldrich (St Louis, MO), and all supplies were obtained from VWR (Bridgeport, NJ). In brief, BSA-NOHA binding strips were washed at least 3 times with 200 μL of 1× phosphate-buffered saline (PBS). 20 μL of reconstituted dried plasma sample from each research participant (as duplicates) was mixed with 5 μL of NOHA-monoclonal antibody at 5 ng/mL and 75 μL 1× PBS. The resultant mixture was added to each well of the bovine serum albumin–NOHA binding strip and incubated for 1 hour at 25°C. After incubation, well contents were discarded and washed 8 times with 200 μL of 1× PBS, before adding 100 μL of polyclonal horseradish peroxidase conjugate from Abcam (Cambridge, MA), at 1:20,000 dilution (in 1× PBS wash buffer). The wells were incubated for 1 hour, at 25°C, before decanting and washing them 8 times with 200 μL of 1× PBS. 50 μL of tetramethylbenzidine substrate from Mossbio (Pasadena, MD) was added to each well and incubated for 10 minutes in the dark, before stopping the horseradish peroxidase-tetramethyl benzidine interaction with 50 μL of 0.1 N HCl. The binding strip wells were read for absorbance at 450 nm using a VersaMax Spectrophotometer (Molecular Devices, NH). Absorbance values were plotted on a polynomial second order trendline, and *R*-squared values were added to the standard curve to assess its confidence in NOHA measurement. Average NOHA value from duplicate assessment of each patient sample was used for IHC comparison.

### NOHA versus IHC Clinical Utility Assessment

As further assessment of the clinical utility of NOHA as a promising replacement for IHC, we examined sample requirements, assay cost, turnaround time, and facility and personnel needs, comparing these with reference standard ER IHC testing in Tanzania.

### Statistical Analysis

Our pilot study assumed that 50% of patients would have ER+ disease on the basis of preliminary published evidence.[15-17] Our sample size of a total of 70 patients (35 ER+ and 35 ER–) was predicted to provide 80% power to detect a one standard deviation difference in means between the two groups with an alpha level of .05. Statistical comparisons of participant NOHA levels versus US-determined ER IHC results were performed using Student's *t* test (GraphPad Prism, Version 8.0, La Jolla, CA). Statistical significance was determined at $P < .05$.

## RESULTS

### Participant Pathology, Staging, and Sociodemographic Data

Because of technical and staffing constraints, complete pathology, clinical staging, and sociodemographic data were available for 53 of 70 enrolled patients (Fig 1). Of these, 33 had associated NOHA and IHC results (Fig 2, Table 1). Sociodemographic data among these 33 participants revealed that all were women, with almost three-fourths being between the self-reported age of 20-60 years and a quarter being above a self-reported age of 60 years. A majority were from the Chagga tribe, reflecting geographic setting of the study in Northern Tanzania. The sociodemographic data collected from the 33 participants with a

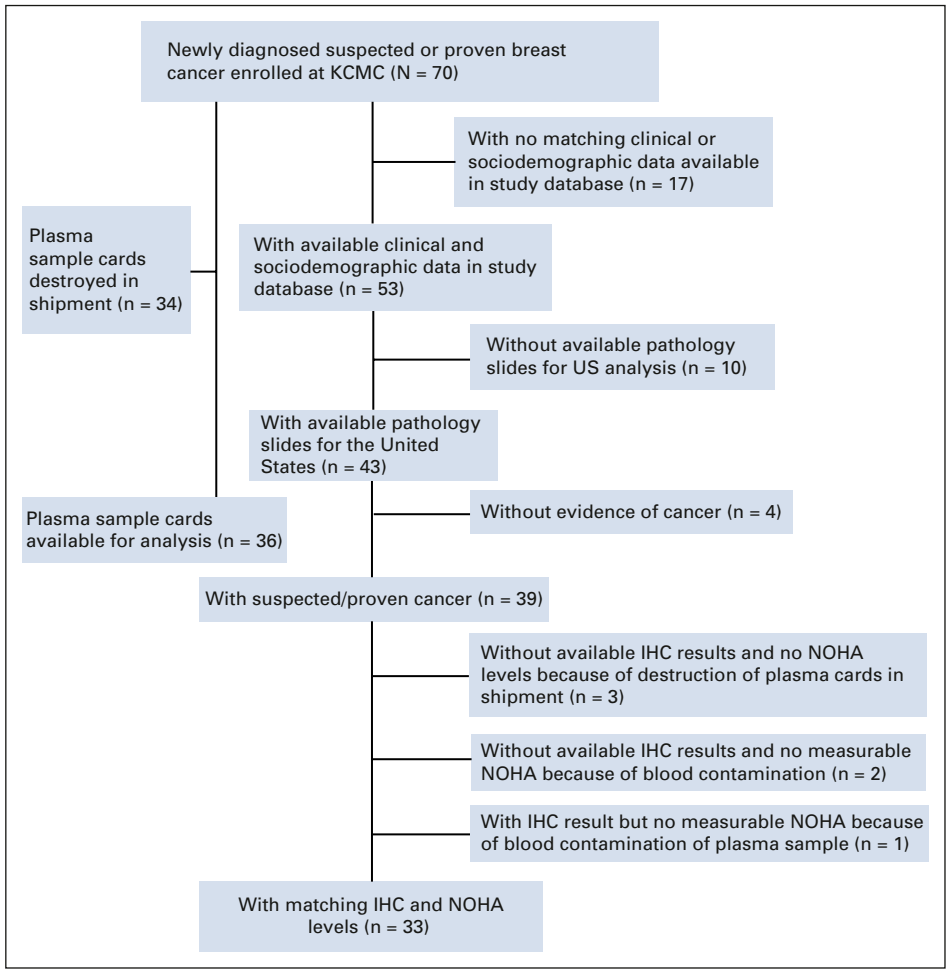


**FIG 1.** Flow diagram. KCMC patient enrollment and available samples for IHC and NOHA testing. IHC, immunohistochemistry; KCMC, Kilimanjaro Christian Medical Center; NOHA, $N^{w}$-hydroxy-L-Arginine.

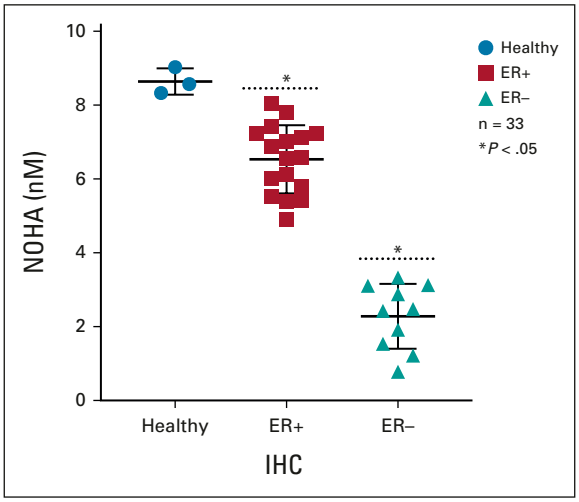


**FIG 2.** NOHA average versus IHC comparison. NOHA < 4 nM in ER– participants, 4-8 nM NOHA in ER+ participants, and > 8 nM NOHA in healthy or no tumor participants. *Represents significance in NOHA on the basis of participant ER status from healthy condition. Solid horizontal line shows average mean per group and the vertical line shows the average deviation in NOHA level on the basis of disease stage. IHC, immunohistochemistry.

complete data set correlated reasonably well with the overall population.

Clinical staging at enrollment revealed that most participants had T4 and/or nodal involvement, with 18% presenting with evidence of metastatic disease. Slides from 43 participants were available for histologic and ER and PR IHC testing in the United States. Of these, four (9%) were not shown to have cancer. Of the remaining 39 patients with IHC results, 33 had matching NOHA values. Most of the 33 had invasive ductal carcinoma (64%). IHC results in these 33 patients revealed that 45% of tumors were ER+ while 55% were ER–, with 10 of the 15 ER+ cases shown to be PR+ (67%). Three of the 33 tumors were ER– and PR+ (9%). A total of 20 participants had hormone receptor IHC testing performed on-site at KCMC. Of these, two had inconclusive IHC results at MMC. Excluding these two cases, IHC concordance was 78% (14 of 18) and 89% (16 of 18) for ER and PR, respectively, comparing on-site with US-run results, recognizing that the two sites varied in their classification of ER+ disease, that is, positivity cutoff values of at least 1% at MMC versus at least 10% at KCMC.

 

**TABLE 1.** Pathology, Staging, and Sociodemographic Characteristics of Enrolled of Total Study Participants (ie, N=70) and of Those With Matching $N^{w}$-Hydroxy-L-Arginine and Immunohistochemistry Levels (ie, n = 33)

| | | % of Study Population | |
|---|---|---|---|
| **Clinical Type** | **Clinical Subtype** | **n = 33** | **N = 70** |
| Pathology | | | |
| ER status | Positive | 45.4 | 42.9 |
| | Negative | 54.6 | 32.8 |
| | Inconclusive | – | 2.9 |
| | Not determined | – | 21.4 |
| PR status | Positive | 51.5 | 32.8 |
| | Negative | 48.5 | 42.9 |
| | Inconclusive | – | 2.9 |
| | Not determined | – | 21.4 |
| Invasive carcinoma subtype | Ductal | 63.6 | 67.1 |
| | Lobular | 6.1 | 5.7 |
| | Others | 3.0 | 1.4 |
| | Not invasive | 6.1 | 8.6 |
| | Not determined | 21.2 | 17.1 |
| Staging | | | |
| Tumor size | T1 | 0 | 2.9 |
| | T2 | 15.2 | 21.4 |
| | T3 | 15.2 | 17.1 |
| | T4 | 69.6 | 58.6 |
| Lymph nodes | N0 | 45.4 | 42.8 |
| | N1 | 24.2 | 20.0 |
| | N2 | 18.2 | 20.0 |
| | N3 | 6.1 | 8.6 |
| | Not detectable | 6.1 | 8.6 |
| Metastases | M0 | 54.5 | 55.7 |
| | M1 | 18.2 | 21.4 |
| | Not documented | 27.3 | 22.9 |
| Sociodemographics | | | |
| Age, years | 20-40 | 18.2 | 24.3 |
| | 41-60 | 54.5 | 54.3 |
| | 61-80 | 21.2 | 17.1 |
| | > 80 | 6.1 | 4.3 |
| Sex | Female | 100 | 100 |
| Tribe | Chagga | 63.6 | 57.1 |
| | Pare | 6.1 | 7.1 |
| | Iraki | 3.0 | 7.1 |
| | Masai | 6.1 | 4.3 |
| | Others | 21.2 | 24.3 |
| Hormone replacement therapy | Never | 93.9 | 92.9 |
| | Not documented | 6.1 | 7.1 |
| Comorbidities | Hypertension | 33.3 | 27.1 |
| | Diabetes | 9.1 | 5.7 |
| | Others | 15.2 | 11.4 |
| | Not documented | 42.4 | 55.7 |

### NOHA to IHC Comparison

Coupled with the 34 cards destroyed during commercial shipment at study outset, unstained slides were not available from three participants for US-based IHC testing, and three of the 36 dried plasma discs from the Noviplex prep cards were blood-contaminated and could not be analyzed for NOHA. This resulted in 33 of 70 participants with matching NOHA and IHC data for comparative ER assessment.

Duplicate NOHA values deviated ≤ 0.2 nM from their average in this study population. As shown in Fig 2, study comparison of participant average NOHA level with IHC ER results revealed a distinct NOHA threshold that correlated 100% to the tumor's IHC categorization as ER–, ER+, and no tumor (Fig 2). A NOHA level < 4.0 nM served as a reliable indicator of ER– breast cancer status. Participants with a NOHA level of 4.0-8.0 nM corresponded to ER+ tumor status, and three cases with a plasma NOHA level above 8.0 nM showed no tumor on study-based analysis of the corresponding pathology specimens.

Additional NOHA-IHC comparisons relevant to the biomarker's clinical utility in the low-resource setting are summarized in Table 2.

## DISCUSSION

In contrast to costly cytotoxic agents and HER2-directed therapies that carry significant risk and require close monitoring by an oncologist and IV administration in a dedicated infusion center, cost-efficient oral hormonal agents are more widely available and easier to administer in low-resource and community settings, including nontraditional settings.[1] Despite the promise of broader availability of these effective therapies, their use is greatly hampered by little to no access to costly and resource-demanding IHC-based ER testing in SSA.[1] Although approximately 80% of breast tumors are ER+ in US women, the proportion of ER+ breast tumors in SSA ranges from 20% to 70%.[15] This variation likely reflects differential risk factor distributions (including reproductive and lifestyle) born from socioeconomic development efforts, histopathologic methods, and genetic heterogeneity across the continent.[15,16] Consistent with the results reported here, a single-institution study in urban Tanzania, using tightly controlled IHC testing methodology, revealed that approximately 50% of the cases presenting for care were ER– with these results subsequently confirmed in a national urban referral hospital population.[16,17] These data reinforce the need for reliable ER determination, supporting effective treatment decision making in Tanzania.

Accurate ER testing requires high-quality histology and IHC facilities as part of a pathological review. A quality control issue in SSA is inappropriate handling of biopsy or excision specimens obtained in the community setting, compounded by sparse to no access to tissue processing facilities resulting in diagnostic delays, high IHC equipment and reagent costs, frequent equipment failure requiring hard-to-access

**TABLE 2.** Index (NOHA) Versus Reference Standard (IHC) ER Testing: Facility Requirements, Costs, and Turnaround Time Comparisons

| | NOHA | IHC |
|---|---|---|
| Specimen | Blood | Tumor tissue |
| Facility needs | Basic laboratory equipped with easy-to-maintain hand-held and desktop equipment | Tissue handling and processing facility |
| Personnel | Laboratory technician | Laboratory technician |
| | | Pathologist |
| Total cost of assay (in USD) | 11.36[a] | Approximately 43[b] |
| Turnaround time | ≤ 2.5 hours | Days to weeks |

Abbreviations: IHC, immunohistochemistry; NOHA, N$^{w}$-hydroxy-L-Arginine; USD, US dollars.

[a]US assay costs (expected to be less in Tanzania because of lower personnel costs).

[b]Cost estimate per personal communication from coauthor Alex Mremi, at Kilimanjaro Christian Medical Centre.

technical support, and need for a skilled pathologist to accurately interpret and distribute results.[1] Access to guidelines-based basic resources and care is unavailable among many with breast cancer in SSA because of these diagnostic barriers.[18] Reflected in the data shown here, lack of access to pathology services in LMIC settings[5] results in both late stage at disease presentation and limited treatment options among those ultimately presenting for care, with a critical component of treatment decision making, both curative and palliative intent, dependent on reliable ER status assessment.

Here, we tested the generalizability of this US-validated,[10,11] blood-based assay to determine breast cancer ER status among a cohort of patients with breast cancer recruited from a single rural Tanzanian cancer center. The results presented provide preliminary evidence that the NOHA biomarker is valid within the Tanzanian context. We confirm that NOHA can be reliably measured via our existing ELISA assay[11] using dried blood samples from TZ.

Consistent with our previous US-based work, among the population studied here, distinct NOHA threshold levels correlate 100% to both tumor ER IHC and disease categorization where a level below 4 nM, from 4 to 8 nM, and above 8 nM signified ER–, ER+ and no cancer, respectively.[10,13] These pilot data suggest that NOHA is equivalent to IHC in breast cancer ER status determination and that NOHA analysis may offer utility in the identification of breast cancer in LMICs where tissue-based diagnosis is limited.

Essential to the low-resource setting, NOHA stability, using plasma cards, was retained during up to 3 months of –80°C storage at KCMC, followed by ambient temperature, that is, at 25-30°C, hand carriage by air, followed by ground shipment to the US-based Mohan laboratory for analysis. This correlates with our prior laboratory-based storage testing outcomes, where NOHA maintained its stability at least 14 days in dried plasma at ambient or higher temperatures of ≤ 42°C.[10,13] These data are critical to real-world LMIC settings where sample storage and shipment conditions are often affected by unreliable cold storage, significant temperature extremes, and unreliable interinstitutional handling. Our own initial international sample shipping experience revealed this where the commercial shipper mishandled 34 plasma sample cards, rendering them unusable. Although this was a major barrier to the work described herein, relative to the scalability of NOHA in Tanzania and elsewhere, it reflected our pilot study design where all NOHA testing was performed in the United States. As the ELISA testing technology relies on easy to acquire and maintain equipment and reagents, it promises to be adaptable for use by point-of-care laboratory personnel receiving local training at the regional or community level, overcoming the sample shipment barriers experienced herein.

As shown in Table 2, the cost to perform the NOHA ELISA in the Mohan (US) laboratory is $11.36/sample, including supplies, sample processing, data analysis, and technician time. It is expected that these costs will be less in Tanzania because of lower personnel expenses. Technical and analytic development efforts are underway for a portable NOHA assay that could further impact point-of-care access to this biomarker. With a turnaround time of ≤ 2.5 h/sample, NOHA would be an attractive and scalable replacement for costly IHC-based ER testing, with promising clinical applications extending beyond cancer diagnostics to surveillance, determination of prognosis, and disease monitoring.[19-21]

This work is highly innovative as, to our knowledge, NOHA is the first blood-based technology that differentiates ER– versus ER+ breast cancers, offering rapid results through use of inexpensive, easy-to-maintain equipment and reagents, suitable for use by laboratory personnel at LMIC point of care. Furthermore, data reported here hold promise relative to NOHA's utility in supporting the diagnosis of breast cancer in clinical settings with little to no access to pathology services. If validated among broader breast cancer populations throughout Tanzania and SSA, this assay could be scaled globally. Taking bold and creative steps to address burgeoning breast cancer morbidity and mortality rates globally holds promise in addressing cancer care disparities.

Limitations to this study include the small sample size and enrollment of patients from a single Tanzanian institution. On the basis of the results presented here, broader assessment of NOHA's clinical utility in the low-resource setting is warranted.

 

## AFFILIATIONS

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## EQUAL CONTRIBUTION

S. Miesfeldt and S. Mohan contributed equally to this work.



## SUPPORT

Supported in part by a Sandy Christman Foundation grant (No. 26056) and through Seed Funds from the University of New England Westbrook College of Health Professions School of Pharmacy, and Office of Research and Scholarship, all of which was awarded to Dr Mohan's laboratory.


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## AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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Open Payments is a public database containing information reported by companies about payments made to US-licensed physicians (Open Payments).

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**Stock and Other Ownership Interests:** Intellia Therapeutics, Fate Therapeutics, Nkarta, CRISPR Therapeutics, Arrowhead Pharmaceuticals

**Susan Miesfeldt**
**Research Funding:** Pfizer (Inst)

No other potential conflicts of interest were reported.


## ACKNOWLEDGMENT

We thank Divya Guthikonda, CRC, Lora Girata, MPH, and Ivette Emery, PhD at Maine Medical Center Research Institute; Ummil R. Koosa and Dr Silvia Kirenga at KCMC; and David Croft at MMC for their contributions. We are grateful to all patients and KCMC staff and volunteers involved in this work.


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