Auria: Why Tears?

Tears as a diagnostic fluid has been studied for decades by groups around the world. Tears and Blood can be thought of as two sides of the same clinical picture. Blood, while an important diagnostic fluid, contains what's called a large dynamic range. Meaning components as large as red blood cells or lymphocytes, down to very small proteins. When trying to find protein biomarkers to diagnosis for diseases and conditions such as cancer, those large molecules can get in the way of important biomarker identification. Large molecules can be removed, however, the techniques a clinical lab must use to reach low molecular weight proteins can damage or even remove proteins of interest. Our peer reviewed communication titled **"Tears as the Next Diagnostic Biofluid: A Comparative Study between Ocular Fluid and Blood"** provides evidence that many of the same proteins that can be detected in blood can also be detected in tears, however, tears provide the means to get to proteins of interest quicker, easier, and in higher concentrations*.

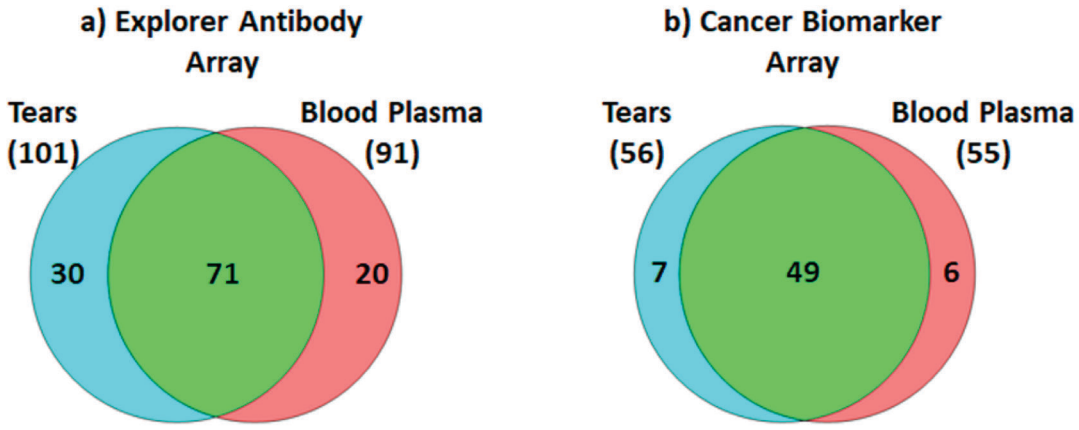


Figure 1: Microarray data analyzed for common protein expression in tears and plasma: (a) Explorer biomarker array; and (b) Cancer biomarker array.

How is a tear sample collected?

An important question in the process is, how is a tear sample collected? Proteins can be easily collected using a Schirmer strip. The strip is folded and placed gently inside the lower eyelid. The microcapillaries on the inner surface of the lower eyelid act as a filtration mechanism, removing any of the large components in blood that can mask low molecular weight proteins, and allowing the small proteins through. These proteins stick to the strip and are removed using the sample buffer provided in the collection kit. The sample buffer now containing the proteins of interest is what we test in the clinical lab.

*Ravishankar, P.; Daily, A. **Tears as the Next Diagnostic Biofluid: A Comparative Study between Ocular Fluid and Blood**. Appl. Sci. 2022, 12, 2884. https://doi.org/10.3390/app12062884

Auria: Biomarker Discovery Study

The goal of this study was two fold 1) determine what proteins could be detected in tears of diagnosed breast cancer patients and 2) determine what proteins could be used as a tool to differentiate between breast cancer and control samples. For Step 1, Proteome wide trypsin digestion was utilized to explore and identify any biomarkers present in detectable levels in tear fluid that may be indicative of breast cancer. Step 2 was carried out by analyzing selected biomarkers on an additional data set by ELISA. Tables below provide a brief overview of sample set demographics.

Table 1: Samples for LC MS/MS

Category	LC MS/MS (n=102)
Normal	51
Breast Cancer	51

Table 3: Samples for ELISA

Category	ELISA (n=171)
Normal	96
Breast Cancer	75

Table 2: Breast Cancer Subtypes for LC MS/MS

Breast Cancer Subtype	
IDC	28
ILC	4
DCIS	13
IDC/DCIS	3
Other	1
NR	2

Table 4: Breast Cancer Subtypes for ELISA

Breast Cancer Subtype	
IDC	44
ILC	5
DCIS	18
IDC/DCIS	4
Other	1
NR	3

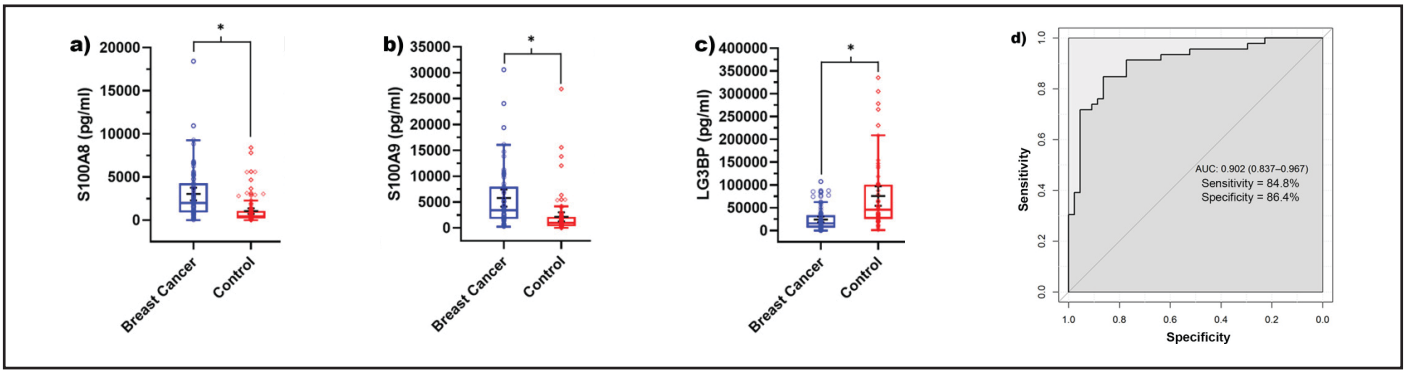


Figure 1: Box plot of S100A8, S100A9, and LG3BP concentrations by ELISA. Receiver operator characteristics curve (ROC) for protein expression of potential breast cancer markers.

In summary, the LC-MS/MS produced a promising data set of 14 biomarkers for potential further analysis. ELISA characterization on the top three biomarkers produced the following diagnostic Parameters: Sensitivity 84% Specificity 86%; providing enough validation to move forward with a larger data set.

Reference: Daily A, Ravishankar P, Harms S, Klimberg VS. **Using tears as a non-invasive source for early detection of breast cancer.** PLoS One. 2022 Apr 26;17(4):e0267676. doi: 10.1371/journal.pone.0267676. PMID: 35471994; PMCID: PMC9041847.

Auria: ELISA Validation Study

The goal of this study was to develop a diagnostic algorithm that could distinguish breast cancer samples as a group with high enough sensitivity and specificity to warrant clinical validation. Three proteins were selected from the biomarker discover study for evaluation by ELISA. Statistical analysis produced three potential diagnostic models which were evaluated at two points with Sensitivity ranging from 52 - 90% and Specificity ranging from 31 - 79% depending on the model and threshold under evaluation.

Given the nuances in various screening modalities and patient journeys within the breast health continuum of care, the samples in this study were further stratified beyond simply "normal" and "breast cancer". Included in the categories of analysis were "call-backs" defined as normal diagnostic mammogram following a screening mammogram, "BiRads Category 3" where the patient's screening and diagnostic imaging is not confirmatory and they are recommended for follow-up imaging and some recommended interval of time (normally 3-6 months) to determine if the area of suspicion has changed at all, and "Benign" where the patient under went a biopsy and the pathology report indicated findings were benign. Break down of sample types are provided in Table 1. Each scenario was evaluated by all models which is why there is a range in specificity and sensitivity.

Table 1: Samples for ELISA Validation Study

Category	Training Set (n=391)	Test Set (n=456)
Normal	223	145
Call-Back	81	83
Breast Cancer	87	21
Benign	–	121
BiRads Cat 3	–	86

Table 2: Breast Cancer Subtype for ELISA Validation Study

Breast Cancer Subtype	Training Set	Test Set
IDC	48	14
ILC	4	3
DCIS	20	2
Multiple	4	0
Other	11	2

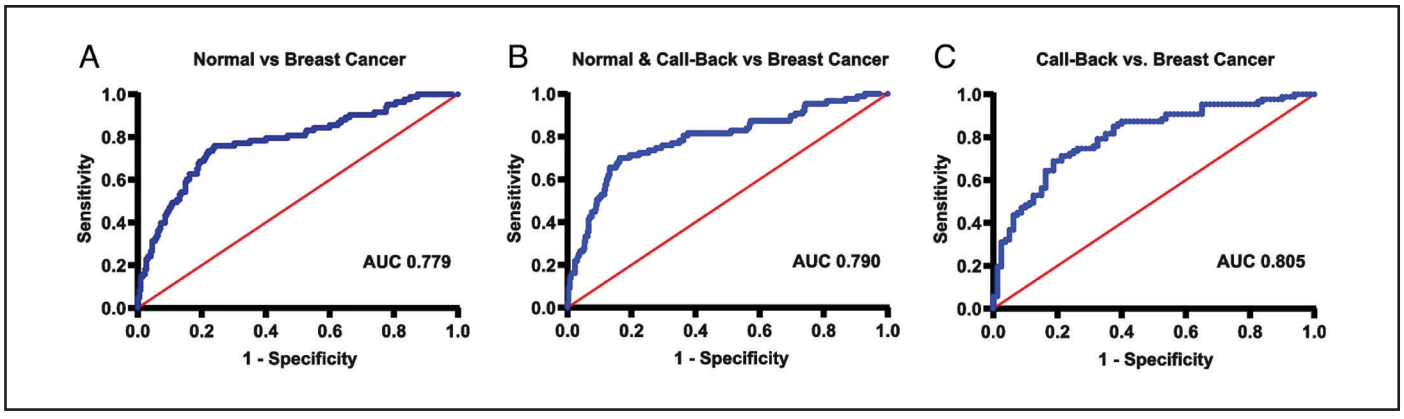


Figure 1: Receiver operator characteristics (ROC) curves comparing (A) control vs breast cancer (B) call-back vs breast cancer (C) control + call-backs vs breast cancer.

In summary a range of diagnostic parameters were observed based on the three models identified Sensitivity 52 - 90% Specificity 31 - 79%. The nature of model has the best potential for cut off optimization, therefore that model was selected for clinical validation moving forward.

Reference: Daily, A., Ravishankar, P., Wang, W. et al. **Development and validation of a short-term breast health measure as a supplement to screening mammography.** Biomark Res 10, 76 (2022). https://doi.org/10.1186/s40364-022-00420-1

Auria: Results Explained

About the Auria Results

Auria quantifies the concentration of specific proteins together with age to determine an Auria result. The result provides biological information regarding potential clinically relevant activity within breast tissue at the time of testing. Early data suggests that scoring clinically significant is associated with an increased likelihood that molecular events are occurring in the host breast tissue. Further characterization of suspect molecular events is recommended by discussing next steps with a healthcare provider.

Auria results were developed from approximately 1000 samples evaluated through the biomarker discovery, ELISA validation, and clinical validation studies. The result classification cutoff was selected using ROC curve and statistical analysis from the ELISA and clinical validation experiments.

Current Diagnostic Parameters

91.5%
Sensitivity

Out of 100 women with breast cancer, Auria would identify 91.5 as clinically significant; 8.5 would be identified as negative.

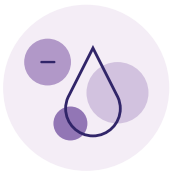
58%
Specificity

Out of 100 women without breast cancer, Auria would identify 58 as negative and 42 as clinically significant.

98% **Negative Predictive Value**

Result Interpretation

Auria® uses innovative proteomic science to give real-time results in aid of early detection.
Auria® does not calculate lifetime breast cancer risk.



Negative

Auria® did not detect elevated protein biomarkers in your tears. You're unlikely to have developing tissue abnormalities associated with breast cancer.*

*False negatives can occur. When you discuss your result with a healthcare provider, ask when you should screen again.

Clinically Significant

Auria® detected elevated levels of protein biomarkers in your tears. Your result may be associated with an abnormality in the breast tissue or breast cancer.*

It's important to know that a clinically significant result is not the same as a cancer diagnosis. If your Auria result is clinically significant additional screening is recommended to rule out cancer.

*False positives can occur. When you discuss your result with a healthcare provider, ask when you should screen again.

Auria: What is CLIA?

What is CLIA and how is it different from FDA?

The Clinical Laboratory Improvement Amendments (CLIA) regulate laboratory testing and require clinical laboratories to be certified by the Center for Medicare and Medicaid Services (CMS) before they can accept human samples for diagnostic testing. Laboratories can obtain multiple types of CLIA certificates, based on the kinds of diagnostic tests they conduct. The FDA, CMS, and CDC are all responsible for elements of CLIA. The main responsibilities of each agency are listed below.

Table 1: CLIA Responsibilities of FDA, CMS, & CDC

FDA	CMS	CDC
• Categorizes test based on complexity either low, medium, or high complexity • Reviews applications companies submit for their product to be CLIA waived • Develops rules/guidelines for CLIA complexity categorization	• Collects user fees • Issues laboratory certificates • Conducts inspections and enforces regulatory compliance • Approves private accreditation organizations for performing inspections, and approves state exemptions • Monitors laboratory performance on Proficiency Testing (PT) and approves PT programs • Publishes CLIA rules and regulations	• Provides analysis, research, and technical assistance • Develops technical standards and laboratory practice guidelines, including standards and guidelines for cytology • Conducts laboratory quality improvement studies • Monitors proficiency testing practices • Develops and distributes professional information and educational resources • Manages the Clinical Laboratory Improvement Advisory Committee (CLIAC)

Reference: https://www.fda.gov/medical-devices/ivd-regulatory-assistance/clinical-laboratory-improvement-amendments-clia

Namida is monitored periodically by CMS through proficiency testing (PT). Since our test is utilizes a “non-regulated” analyte we designed our proficiency testing protocol using Federal (CLIA) Regulations CFR Part 493 Subpart H and 493.1236 which requires us to establish accuracy and reliability through PT at 2 times each year.

Table 2: Explanation of Terms

Explanation of Terms	
LDT	An LDT or Lab Developed test is a designed, developed, and performed within a single laboratory, regulated by the Centers for Medicare and Medicaid Services (CMS), through the Clinical laboratory Improvements Amendments.
Accuracy	The accuracy of a clinical test refers to the “closeness of agreement” between the test results and the true values from an established reference method. For qualitative methods, this means establishing that the method will identify the presence/absence of the analyte.
Precision	The precision of a clinical test refers to the closeness of agreement between a series of test measurements obtained from multiple samplings of the same specimen(s) on the same day/same run, on different days/different runs, and with different employees and different instrumentation (if applicable).
Sensitivity	The sensitivity of a clinical test refers to the ability of the test to correctly identify those patients with the disease; the lowest concentration or amount of the analyte that can be measured or distinguished from a blank.
Specificity	The specificity of a clinical test refers to the ability of the test to correctly identify those patients without the disease; the ability to measure the analyte in the presence of other components expected to be present. The lab must document information regarding interfering substances form product information, references, or its own testing.
Linearity	The linearity of a clinical test refers to the ability of a test to give results that are directly proportional to the concentration of analyte.

Auria: CLIA Validation

The process of the validation of Auria for use as a Lab Developed Test (LDT) offered through the high complexity CLIA laboratory at Namida Lab was designed using the validation guidelines distributed through CLIA and CAP, reviewed and approved by Namida's Medical Director, and reviewed by the Arkansas State Health Department. Once Namida's CLIA license was approved in Arkansas we then procured licenses in 49 states excluding NY.

Data provided, met the CLIA requirements on test performance specifications including precision, analytical accuracy, analytical sensitivity and specificity, reportable range, linearity, clinical accuracy, clinical specificity, and clinical sensitivity. Having met the CLIA regulatory requirements, we provide the required evidence to support the clinical implementation of this test as an LDT for the intended use as a pre-screen for breast cancer.

Table 1: Summary of Analytical Validation

Study Parameter	Sample Description (Name, Number, Replicates)	Results	Comparison to Acceptance Criteria (Pass/Fail)
Accuracy	20 spiked LGF samples	Percent recover between 80 - 120% for all samples	Pass
Intra-Assay Precision	24 replicates of 3 concentrations per analyte	All %CV less than or equal to 15%	Pass
Inter-Assay Precision	Duplicates of 3 concentrations 1x day for 5 days	All %CV less than or equal to 15%	Pass
Sensitivity	20 replicates of blank per protein	S100A8 11% 21.10 pg/ml S100A9 14% CV 24.32 pg/ml	Pass
Linearity	7 unknown concentrations	Percent difference less than 15% for all samples	Pass

Table 2: Summary of Clinical Validation

Study Parameter	Sample Description (Name, Number, Replicates)	Results	Comparison to Acceptance Criteria (Pass/Fail)
Accuracy	26 samples 3 replicates each sample	92%	Pass
Sensitivity	52 LGF samples in duplicate	92%	Pass
Specificity	50 LGF samples in duplicate	54%	Pass