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Biomarker Research

RESEARCH Open Access

Development and validation of a short-term breast health measure as a supplement to screening mammography

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Abstract

Background: There is a growing body of evidence to support tears as a non-traditional biological fluid in clinical laboratory testing. In addition to the simplicity of tear fluid processing, the ability to access key cancer biomarkers in high concentrations quickly and inexpensively is significantly enhanced. Tear fluid is a dynamic environment rich in both proteomic and genomic information, making it an ideal medium for exploring the potential for biological testing modalities.

Methods: All protocols involving human subjects were reviewed and approved by the University of Arkansas IRB committee (13-11-289) prior to sample collection. Study enrollment was open to women ages 18 and over from October 30, 2017-June 19, 2019 at The Breast Center, Fayetteville, AR and Bentonville, AR. Convenience sampling was used and samples were age/sex matched, with enrollment open to individuals at any point of the breast health continuum of care. Tear samples were collected using the Schirmer strip method from 847 women. Concentration of selected tear proteins were evaluated using standard sandwich ELISA techniques and the resulting data, combined with demographic and clinical covariates, was analyzed using logistic regression analysis to build a model for classification of samples.

Results: Logistic regression analysis produced three models, which were then evaluated on cases and controls at two diagnostic thresholds and resulted in sensitivity ranging from 52 to 90% and specificity from 31 to 79%. Sensitivity and specificity variation is dependent on the model being evaluated as well as the selected diagnostic threshold providing avenues for assay optimization.

Conclusions and relevance: The work presented here builds on previous studies focused on biomarker identification in tear samples. Here we show successful early classification of samples using two proteins and minimal clinical covariates.

Keywords: Breast cancer, Cancer biomarkers, Biomarker validation, Tear fluids, ELISA, Receiver operator characteristic curves

Background

Breast cancer is the most diagnosed cancer in women and accounts for up to 31% of all cancer types [1, 2]. It is estimated that 43,250 deaths would be associated with breast cancer in 2022 in the US [3]. The most effective way to reduce mortality associated with breast cancer is to increase early detection and therefore intervention leading to proper treatment [4–6].

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The early 1980's saw a major advancement in breast cancer detection due to the implementation of screening mammography; prior to which, most breast cancers were detected by a palpable mass or breast abnormality found by a physician or patient [7]. With screening mammogram, image-based detection jumped from 4% in 1977 to 41% in 1988 with a mortality reduction of 20% [8, 9]. As the technology advanced from film to digital and now to 3D, the detection rate is steady around 60% [10]. Surprisingly, with all of the advancement and promotion of screening mammography, the rate of so-called interval cancers has remained unchanged with a significant increase in the rate of false-positives requiring follow-up [11].

Mammography correctly detects roughly 87% of patients with breast cancer (76% specificity and 87% sensitivity), and this sensitivity rises with age and in women with fatty breast tissue [12–14]. According to a 2017 study, 63.4% of diagnosed breast cancers were stage 0 or stage 1, and 69.6% of invasive cancers were lymph node negative, based on over 400,000 diagnostic imaging breast examinations performed at 92 different radiology centers [15].

Despite the obvious necessity for screening mammography in the reduction of mortality due to breast cancer, adherence to this modality, based on age, is anywhere from 31 to 70% of women [16]. Typically, compliance with screening increases as women age which also correlate with an increased risk of breast cancer. However, the rate of breast cancer diagnosis in women under 50 is steadily increasing and those diagnoses are typically more aggressive and have higher mortality rates [17]. Unfortunately, the same population has the lowest compliance rates with screening mammography. A recent report by radiologists revealed that the mortality rate of breast cancer in women aged 20–39 has stopped declining since 2010 and increased by 0.5% per year [18]. Screening recommendations in this age range (< 50) vary and this period in women's lives is generally when the demand of career and family responsibilities are highest and available time for personal care is lowest. According to a 2020 study, screening mammography in women aged 40 to 49 years reduced mortality by approximately 25% in the first 10 years when compared to waiting until 50 years or older to begin screening [19].

Another factor effecting the accuracy of mammography is breast density. High mammographic density decreases the diagnostic accuracy of screening mammography by masking tumors and is a risk factor for breast cancer on its own. Breast density is connected with young age, pregnancy, lactation, and hormonal treatment [20–22]. In the BI-RADS lexicon, there are four descriptors for breast density, (1) fatty, (2) scattered areas of fibroglandular density, (3) heterogeneously dense, which can obscure small masses, and (4) extremely dense, which reduces mammographic sensitivity [23].

We use tears as our biofluid source for analyzing the biomarkers for breast cancer. It has been documented that systemic effects exert influence on the ocular environment [24]. Tear fluid is a dynamic environment rich in both proteomic and genomic information [25–27]. Studies show that protein patterns in tears have the potential to generate biomarkers for disease state determination and could also provide new sources for treatment options and monitoring [28–30]. Most promising new discoveries in protein biomarkers focus on low molecular weight proteins which in many cases are undetectable without significant pre-processing of the samples [31]. There is a growing body of evidence to support tears as a non-traditional biological fluid [32–34]. In addition to the simplicity of tear fluid processing, the ability to access key cancer biomarkers in high concentrations quickly and inexpensively is significantly enhanced.

We had previously conducted a biomarker discovery study where we reported the 3 proteins namely S100A8, S100A9, and Galectin-3 binding proteins as potential biomarker candidates [35]. Each of these proteins play a major role in the development of breast cancer. S100 proteins' role in cancer is well documented [36]. Previous research groups have reported that S100A8 and S100A9 were elevated in serum and tissue of breast cancer patients [37–40]. Additionally, the increased expression of these two S100 proteins have been associated with non-functional BRAC1 which play a role in metastasis by binding to RAGE receptors [41]. Galectin-3 binding proteins have been shown to be a potential binding site for proteins involved in metastasis and the protein's elevated levels are associated with shorter survival in patients with breast carcinoma [42, 43]. In this study we validate and build on our previous work [35], using a larger patient population and analysis of clinical covariates. By compiling the scores of the large case-control study, we provide the foundation for a "pre-screen" for women with low to average lifetime risk of breast cancer without a palpable mass or area of breast concern as supplement to screening mammography.

Methods

Study population

All protocols involving human subjects were reviewed and approved by the University of Arkansas IRB committee (13-11-289) prior to sample collection. The sampling technique used was a purposive, non-random sampling strategy to recruit women with the requisite inclusion criteria. Tear fluid samples were collected from study participants recruited at The Breast Center, Fayetteville,

AR and Bentonville, AR. Written informed consent was obtained from all participants prior to sample collection. Patients were given the opportunity to enroll if they were being seen for standard yearly screening, imaging to evaluate an area of breast concern, biopsy, and recently diagnosed with breast cancer being evaluated for pre-surgical MRI evaluation. Imaging results, from the procedure at sample collection, as well as any follow-up imaging was obtained through The Breast Center to assist with sample classification. Details for sample classifications are displayed in Table 1.

Sample preparation and ELISA

Tear fluid samples were collected and evaluated for the expression level of S100A8, S100A9, and Galectin-3-Binding Protein (LG3BP) using ELISA (DuoSets ELISA kits, R&D Systems (Minneapolis, MN, USA) based on previously reported protocols [35].

Statistical analysis

R statistical software was used to apply logistic regression to protein concentrations determined by ELISA for comparison. Forward stepwise logistic regression was used to determine diagnostic parameters for the optimal combinations of proteins of interest and subject demographic characteristics. This was done to reduce the number of predictors in the model to a more parsimonious set. The algorithm derived from the logistic regression model was then used to calculate predicted probability scores for each subject. Receiver operator characteristic (ROC) curves were generated using these predicted probability scores against the breast cancer dependent variables and the area under the curve (AUC) was computed accordingly. An AUC of 0.7 or greater was set as the standard of acceptance for a panel of proteins and characteristics. Sensitivity (true positive rate) and specificity (true negative rate) scores were also calculated to assess the accuracy of the test. The data preparation and analysis were conducted in SPSS version 25 [44]. Crosstabulations using chi-square (Pearson's χ^2) tests were performed to examine the relationships between each diagnosis (sensitivity/specificity/accuracy) and three models in each scenario, and a p-value < 0.05 was considered statistically significant.

Results

Sample population

Logistic regression models were built using a cohort dataset of 391 samples (Cohort 1) collected from a single site from October 2017 through December 2018. As shown in Table 2, age range of the entire study population was 22–84 years of age, with an average of 55.81 ± 12.22 yrs. of age. At the conclusion of enrollment, the study population consisted of 87 confirmed breast cancer cases with a subtype distribution of IDC (55%), DCIS (23%), ILC (5%), multiple diagnosis (5%) (i.e. DCIS/IDC, ILC/IDC), remaining diagnosis (13%) comprising, infiltrating mammary carcinoma; infiltrating mammary duct carcinoma; invasive ductal carcinoma; metastatic mammary carcinoma; papillary carcinoma; and invasive cribriform carcinoma. Controls were divided into two groups, Normal, taken from subjects who were not called-back for additional imaging after a screening mammogram; and Call-back, taken from subjects who were recalled but the recall did not lead to biopsy, and they were cleared to return to standard yearly screening.

Another cohort dataset of 456 samples (Cohort 2) with an average 53.48 ± 12.14 yrs. of age were collected from the same single site from December 2018 through June 2019. The data set consisted of an additional 21 breast cancer samples with a subtype distribution of IDC (67%), ILC (14%), DCIS (10%), with the remaining falling into the "other" category (10%). Additionally, this dataset contained 121 biopsy confirmed benign samples as well as 86 samples assigned BiRADS 3 with recommendation for short-term follow-up. A summary of sample characteristics for the Cohort 1 and 2 can be found in Table 2.

Table 1 Sample classifications and qualifications

Sample Class	BiRADS	Imaging procedure at sample collection	Outcome
Normal	1,2	Screening MGM- no documented area of concern	Classified as normal and a recommendation to continue normal screening
Call-back	1,2	Screening MGM, or diagnostic - may have documented area of concern	Call-back for further imaging that resulted in a "normal" classification and a return to normal screening.
Category 3	3	Screening or diagnostic - may have documented area of concern	Call-back for further imaging that resulted in a recommendation for additional follow-up.
Benign	4a, 4b, 4c	Screening, Diagnostic, Biopsy - may have documented area of concern	Biopsy with final diagnosis as Benign
Breast Cancer	4a, 4b, 4c, 5	Screening, Diagnostic, Biopsy, MRI - may have documented area of concern	Biopsy confirmed Breast Cancer diagnosis

Table 2 Demographics of patient database

Category	Cohort 1 ($n=391$)	Cohort 2 ($n=456$)	Combined ($n=847$)
Age, *y*, median (range)	57 (22–87)	53 (21–87)	55 (21–87)
Normal			
Normal screening mammogram	223	145	368
Call-Back			
Normal Diagnostic Mammogram	81	83	164
Cases			
Biopsy Confirmed Breast Cancer	87	21	108
IDC	48	14	62
ILC	4	3	7
DCIS	20	2	22
Multiple	4	0	4
Other	11	2	13
Grade			
Low	18	2	20
Intermediate	41	11	52
High	26	7	33
Unknown	2	1	3
Tumor Size (cm)			
<1	28	3	31
1–2	27	11	38
>2	29	7	36
Multiple	9	2	11
Primary tumor with positive node	15	2	17
Unknown	3		3
Receptor Status			
ER−/PR-	1	1	2
ER−/PR+	1	0	1
ER+/PR+	16	2	18
ER+/PR-	3	0	3
ER−/PR−/HER2+	3	0	3
ER+/PR+/HER2-	47	13	60
ER+/PR−/HER2-	4	1	5
ER+/PR+/HER2+	8	2	10
Triple Negative	4	2	6
Benign		121	
Category 3		86	

Logistic regression

Stepwise forward logistic regression analysis was used to identify distinguishing covariate combinations. Due to the non-normal distribution of the three protein analytes (S100A8, S100A9, LG3BP), concentrations were transformed to log values for the analysis. In instances where the average protein values were 0 pg/ml, effectively below the lower limit of detection, the value was recoded to 1 pg/ml enabling the log(protein) to be taken generating a 0 instead of missing.

After assessment for collinearity of the 28 covariates, 11 potential covariates remained for evaluation- three proteins (S100A8, S100A9, LG3BP), age, BMI, HRT, family history of cancer, family history of breast cancer, personal history of cancer, personal history of breast cancer, and breast density. Logistic regressions were conducted on the data set in three conditions; Model 1- Normal vs. Breast Cancer (1); Model 2- Call-back vs. Breast Cancer (2); Model 3- Normal & Call-back vs. Breast Cancer (3), and the Forward Logistic Regression Algorithm formulas

are provided below with Y representing the scores for each model,

$$Model\ 1 : \mathrm{Y} = -6.64 + 1.04^{*}(\mathrm{logS100A8}) + 0.811^{*}(\mathrm{logS100A9}) + 0.62^{*}(\text{breast density}) \quad (1)$$

$$Model\ 2 : \mathrm{Y} = -6.988 + 0.614^{*}(\mathrm{logS100A8}) + 1.081^{*}(\mathrm{logS100A9}) + 0.034^{*}(\text{age}) \quad (2)$$

$$Model\ 3 : \mathrm{Y} = -7.244 + 0.955^{*}(\mathrm{logS100A8}) + 1.047^{*}(\mathrm{logS100A9}) \quad (3)$$

Validation of the models on cohort 1

Receiver operator characteristic curves (ROC) were generated for each model on the cohort 1 dataset and used to select various diagnostic thresholds for analysis (Fig. 1). For each model, two diagnostic thresholds were selected; Scenario 1 utilized the Y (score) where the sum of sensitivity and specificity was maximized. For scenario 2, a Y (score) was selected with preference given to 90% sensitivity to evaluate the potential reduction of false negatives.

For the scenario 1 in the cohort 1 dataset, analysis of Normal vs. Breast Cancer samples produced model 1 which incorporated S100A8, S100A9, and breast density as the predictors. The overall AUC was 0.779 with a maximized sensitivity of 76% and a specificity of 76% when the cut-off point was set as $Y > -0.8565$. These results along with the positive coefficient of the three predictors indicate that S100A8, S100A9, and breast density were significantly associated with a positive breast cancer diagnosis. The AUC score of 0.779 further suggests an acceptable factor combination to predict breast cancer.

Analysis of Call-back vs. Breast Cancer samples produced model 2 which incorporated S100A8, S100A9, and age as the predictors. An AUC of 0.805 revealed that this algorithm is a good indicator of the breast cancer diagnosis with a maximized sensitivity of 70% and a specificity of 81% when the cut-off point was set as $Y > 0.2018$.

Analysis of Normal & Call-back vs. Breast Cancer samples produced a model 3 with S100A8 and S100A9 as the predictors. An AUC of 0.790 suggests a fair to good indicator of the breast cancer diagnosis. When the cut-off value Y was set above -0.9487, sensitivity and specificity were maximized to 70 and 84% respectively.

Evaluation of the models on the combined dataset

Diagnostic parameters (sensitivity and specificity) for scenarios 1 and 2 for models 1, 2, & 3 were evaluated for the entire 847 sample data set to find the most effective model out of all three proposed models. In each case, samples were considered positive if the Y-score was greater than the cut-off and below which they were considered negative. Scenario 1 with Model 1 (M1 S1), a Y-score of -0.8565 resulted in a sensitivity of 61%, specificities of 74 and 62% for Normal & Call-back respectively, and an overall accuracy of 69% (Fig. 2A and C). For model 2 scenario 1 (M2 S1), a Y-score of 0.2018 was used, producing a sensitivity of 64%, specificities of 69 and 73% for Normal & Call-back respectively, and an overall accuracy of 69%. Finally, for model 3 scenario 1 (M3 S1) with a Y-score of -0.9487 resulted in a sensitivity of 81% and specificities of 79 and 77% for Normal & Call-back respectively, and an overall accuracy of

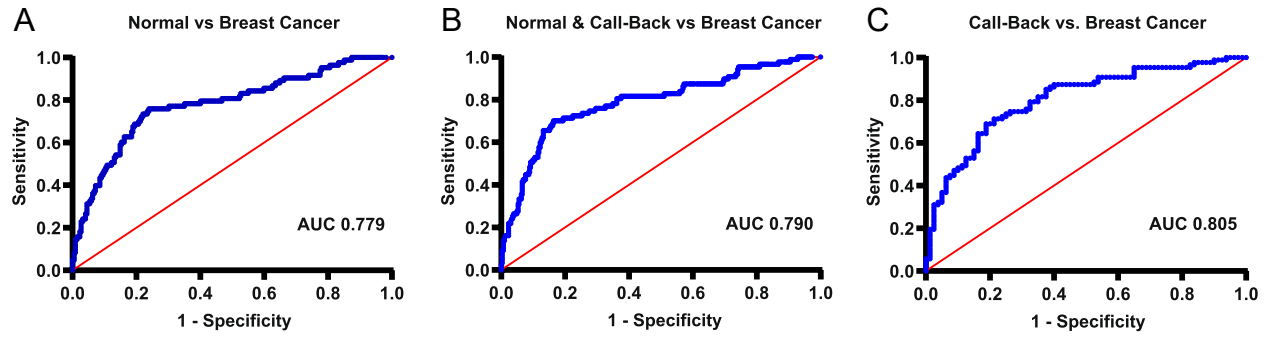

Fig. 1 **A** Receiver Operating Characteristic (ROC) Curves comparing Normal versus Breast Cancer with an AUC of 0.779; **B** Call-back versus Breast Cancer with an AUC of 0.805; **C** Normal & Call-back versus Breast Cancer with an AUC of 0.790

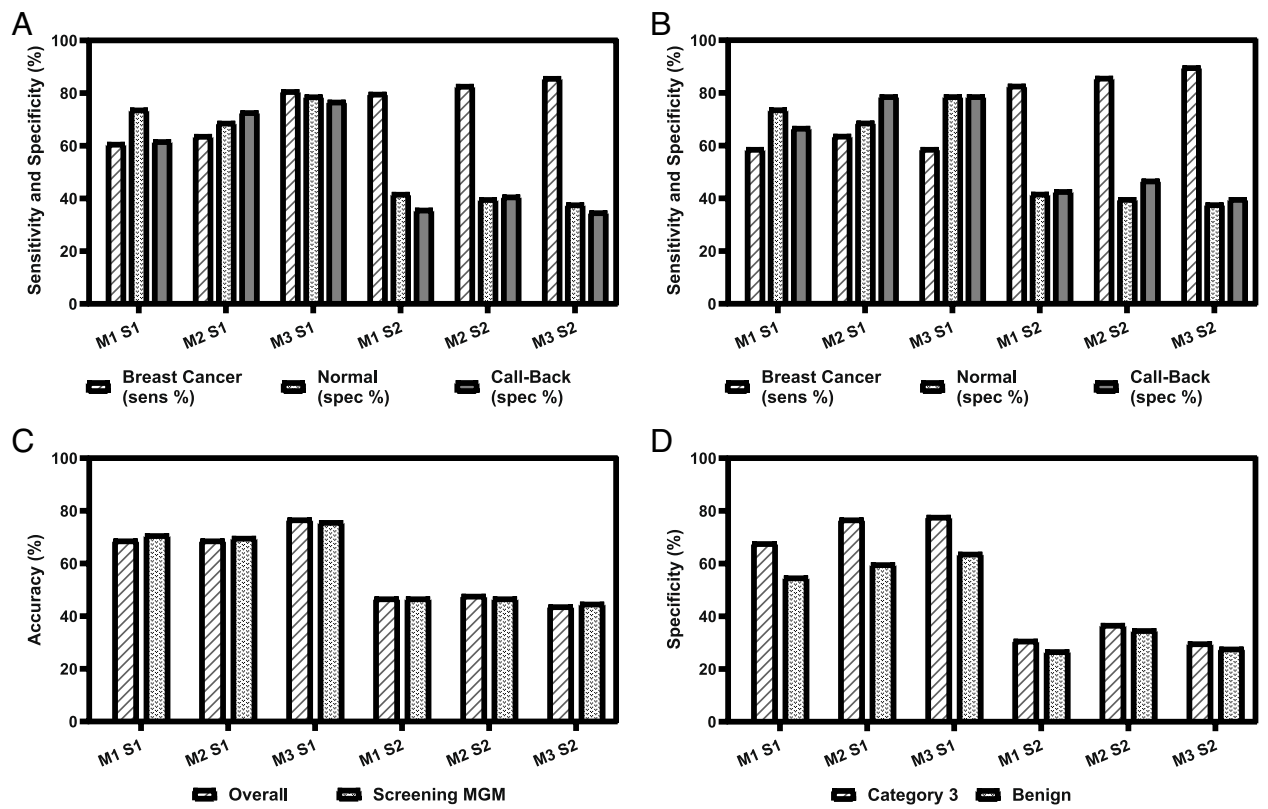

Fig. 2 **A** Comparisons of performance threshold values from the three models (M) produced by logistic regression analysis at two different scenarios (S). **B** Selected samples where the patient entered the continuum of care at the screening mammogram. **C** Accuracy rates for all screening modalities versus accuracy rates for individuals who entered the continuum of care at screening mammogram only. **D** Finally, shown is specificity for biopsy confirmed benign samples as well as samples assigned a BiRADs 3 score

77%. A crosstabulation using a Pearson χ^2 test was conducted and found that there was no statistically significant relationship between diagnosis and M1/M2/M3 for sensitivity and accuracy. However, there was a statistically significant relationship between specificity of normal control and three models in scenario 1 ($p = 0.008$). Model 3 resulted in highest specificity in the prediction of normal population (79%) whereas model 2 had lowest specificity in the prediction of normal population (69%). The significant relationship was also found between the three models and the specificity of call-back controls in scenario 1 ($p = 0.007$). A greater specificity of call-back controls was predicted in the model 3 (77%) as compared to the model 1 (62%) (See Fig. 2A).

A Y-score of -1.8236 was used for Scenario 2 for model 1 (M1 S2), and this resulted in a sensitivity of 80% and specificities of 42 and 36% for Normal & Call-back respectively, and an overall accuracy of 47%. Model 2 scenario 2 (M2 S2) with Y-score of -0.828 produced a sensitivity of 83% and specificities of 40 and 41% for Normal & Call-back respectively, and an overall accuracy of 48%. Model 3 scenario 2 (M3 S2) utilized a Y-score of -2.3226 resulting in a sensitivity of 86% and specificities of 38 and 35% respectively for Normal & Call-back respectively, and an overall accuracy of 44%. A Pearson χ^2 test revealed no statistically significant relationship between diagnosis and M1/M2/M3 for sensitivity, specificity, and accuracy.

All three models and scenarios were then applied to our entire dataset where the participant had entered the breast health continuum of care at screening mammogram to evaluate how the models would perform in a pre-screening application (Fig. 2B and C). For M1 S1 sensitivity was 59% and specificities were 74 and 67% for Normal & Call-back respectively, and an overall accuracy of 71%. M2 S1 produced a sensitivity of 64% and specificities of 69 and 79% for Normal & Call-back respectively, and an overall accuracy of 70%. M3 S1 produced a sensitivity of 59% and specificities of 79 and 79% for Normal & Call-back respectively, and an overall accuracy of 76%. There was no statistically significant relationship between diagnosis and M1/M2/M3 for sensitivity and accuracy. However, there was a statistically significant relationship for specificity in the normal

controls ($p = 0.008$) and a trend was observed in call-back controls ($p = 0.093$). A greater specificity of Normal population was predicted in the model 3 (79%) as compared to the model 2 (69%) (See Fig. 2B).

For the second scenario, M1 S2 resulted in a sensitivity of 83% and specificities of 42 and 43% for Normal & Call-back respectively, and an overall accuracy of 47%. M2 S2 produced a sensitivity of 86% with specificities of 40 and 47% for Normal & Call-back respectively, and an overall accuracy of 47%. M3 S2 produced a sensitivity of 90% and specificities of 36 and 40% for Normal & Call-back respectively, and an overall accuracy of 45%. A Pearson χ^2 test revealed no statistically significant relationship between diagnosis and M1/M2/M3 for sensitivity, specificity, and accuracy.

The final set of samples in the population pool consisted of category 3 and benign to evaluate how the models would perform in a diagnostic application (Fig. 2D). M1 S1 resulted in a specificity of 68 and 55% for category 3 and benign respectively. M2 S1 performed better with a 77% specificity for category 3 and 60% for benign; and M3 S1 produced a specificity of 78% for category 3 and 64% for benign. For the second scenario, M1 S2 resulted in a specificity of 31% for category 3 and 27% for benign. M2 S2 resulted in 37% specificity for category 3 and 35% for benign. Finally, M3 S2 showed a specificity of 30% for category 3 and 28% for benign. There was no statistically significant relationship between category 3, benign, and the three models for specificity in both the scenarios.

Discussion

Here we provide an analysis of the potential capability of tear proteins to be used in the classification of control and breast cancer samples. In this study, we analyze 11 potential clinical covariates, by logistic regression to develop a diagnostic algorithm for sample classification. Methods for the selection of protein biomarkers included in the analysis, S100A8, S100A9, and LG3BP were described previously [35]. Breast cancer samples were compared to two different groups- Normal & Call-back, as the final diagnosis for women in both groups was "normal" for that year, however subjects imaging path differed (Table 1). Individuals in the call-back group experienced an additional imaging step consisting of diagnostic mammogram and in some cases, a diagnostic ultrasound. Women in this group were not recommended to have an additional confirmatory imaging after the diagnostic and were returned to a yearly screening cycle. The call-back group was analyzed separate from normal because reducing false-positive call-backs from imaging is a high priority in breast imaging.

The clinical covariates included in the models were the main differentiating factor as all three utilized S100A8 and S100A9. Model 1 incorporated breast density, model 2 incorporated age, while model 3 only utilized the two protein concentrations. Comparison of the model performance against one another is essential as each model was developed using specific portions of the dataset. Consideration of the covariates dictate where a model could be used in the breast health continuum of care. For example, utilization of model 1 as pre-screening tool would not be feasible as model 1 requires information about tissue density category. Establishment of tissue density is done after imaging and may also change through the course of a woman's lifetime. Additionally, since model 1 had the lowest performance after initial assessment, models 2 and 3 were the focus of further application. The performance of all three models on the entire data set were evaluated using Pearson's χ^2 test and it revealed no statistically significant relationship between diagnosis and M1/M2/M3 for sensitivity, specificity, and accuracy providing evidence that any of the three models could be selected moving forward.

The lower specificity values ranging between 35 and 45% are associated with scenario two for each model. In scenario two, the clinical threshold was selected preferential to sensitivity. Because of the dynamic relationship between sensitivity and specificity, when preference is given to one value the other value often decreases. Scenario 1 in each model tests the y-value where both the sensitivity and specificity are at the highest. For Model 1 the y-value was set at -0.8565 which provided a sensitivity of 75.9% and specificity of 76.1%. When the y-value was preferentially adjusted for higher sensitivity to -1.8236 the predicted sensitivity is now 90% and specificity is 34%. Desired clinical outcomes play a significant role in the selection of the diagnostic threshold. In thinking about the implications of false negatives and false positives, the first preference is to get both numbers as low as possible however if the false negative rate is too high then the threshold is adjusted.

The diversity of diagnosis included in the breast cancer samples allowed for investigation into the performance of the models by cancer subtypes, cancer grade, and tumor size including a small portion of subjects with node metastasis and the relevant sensitivities are reported in the supplemental section. Diagnostic thresholds M2 S2 and M3 S2 were used to evaluate the performance (Supplemental Fig. 1). While tumor size is not an indication of disease severity, it is of interest when considering utility of a biological test prior to screening mammography. Ideally, these models should perform best in a normal breast prior to palpability of a lump or identification of concern. Both models performed best in smaller tumors and lowest performance was in subjects with multiple breast tumors identified

or where metastasis to a node had occurred. We observed a trend when assessing sensitivity of the models by grade where low and intermediate grades of cancer had better sensitivities. The performance of the models for lower grade cancer is not surprising given the roles of S100A8 and S100A9 in recruitment of immune cells essentially prepping the tissue for tumor formation [45–47].

Utilization of screening mammography among insured women in the US hovers around 60% with the lowest participation of 35% in women under 45 years of age [48]. While the detection rate in women under 40 is only 6.5%, these diagnoses are often more aggressive [49]. Screening can only be effective if utilized. It is possible that a simple test offered prior to screening mammography for low to average risk women could increase participation in yearly screening mammography. While there is more development work to be done, given the elegant simplicity of tear sample collection, this could be an interesting medium to explore for a "pre-screening" application.

Limitations of the study include, only one clinical location which limited geographic, racial, ethnic, and economic distribution of subjects. In addition, evaluation of S100A8 and S100A9 has been limited to only breast cancer. Future studies will incorporate additional cancer subtypes. Additionally, most recent developments have focused on employing machine learning tools to develop better diagnostic algorithms and along the same lines, our future work will also focus on developing models with increased specificity by studying more clinical covariates.

Conclusions

In this study, we used tear fluids to determine that cancer biomarkers' protein concentrations and developed a model that is significantly associated with a positive breast cancer diagnosis. We analyzed the protein concentration from 847 individually collected tears samples using logistic regression to develop and validate three models for the identification of positive breast cancer samples with a sensitivity as high as 90%. Our analysis suggests that models developed using tear fluid have clinical validity and could be used in further development of a biological assay to supplement screening mammography for screening adverse individuals or in areas where access to screening mammography is limited.

Abbreviations

BI-RADS: Breast imaging reporting and data system; ROC: Receiver operating characteristics; AUC: Area under the curve; LG3BP: Galectin-3-binding protein; IDC: Intraductal carcinoma; DCIS: Ductal carcinoma in situ.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40364-022-00420-1.

Additional file 1: Fig. 1. Sensitivity of Model 2 and Model 3 according to A. breast cancer subtype, B. grade, and C. tumor size.

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Not applicable.

Authors' contributions

All authors had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Concept and design: AD, SH, VSK. Acquisition, analysis, or interpretation of data: AD, PR, WW, RK. Drafting of the manuscript: AD, VSK. Critical revision of the manuscript for important intellectual content: All authors. Statistical analysis: WW, RK. Obtained funding: AD. Administrative, technical, or material support: AD, PR. Supervision: AD, VSK, SH. The author(s) read and approved the final manuscript.

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Availability of data and materials

All data that support the findings of this study are available from the corresponding authors on reasonable request.

Declarations

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of the University of Arkansas (protocol #13–11-289 and December 2013). Informed consent was obtained from all subjects involved in the study.

Consent for publication

Not applicable.

Competing interests

This investigation has resulted in the achievement of the following patent. Class, I. P. C., and AG01N3072FI USPC. Daily et al. Methods of Detecting Cancer, US 10,451,625 B2, issued October 22, 2019. AD and PR are employees of Namida Lab Inc. AD is an inventor on the patent and serves as the Vice President of Product Development and Innovation. AD owns shares of Namida Lab Inc. PR serves as the Research and Development Scientist at Namida Lab Inc. SH and VSK are on the clinical advisory board of Namida Lab Inc. WW and RK have no competing interests.

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Publisher's Note

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RESEARCH ARTICLE

Using tears as a non-invasive source for early detection of breast cancer

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Abstract

The changing expression levels of ocular proteins in response to systemic disease has been well established in literature. In this study, we examined the ocular proteome to identify protein biomarkers with altered expression levels in women diagnosed with breast cancer. Tear samples were collected from 273 participants using Schirmer strip collection methods. Following protein elution, proteome wide trypsin digestion with Liquid chromatography/tandem mass spectrometry (LC-MS/MS) was used to identify potential protein biomarkers with altered expression levels in breast cancer patients. Selected biomarkers were further validated by enzyme linked immunosorbent assay (ELISA). A total of 102 individual tear samples (51 breast cancer, 51 control) were analyzed by LC-MS/MS which identified 301 proteins. Spectral intensities between the groups were compared and 14 significant proteins (p-value <0.05) were identified as potential biomarkers in breast cancer patients. Three biomarkers, S100A8 (p-value = 0.0069, 7.8-fold increase), S100A9 (p-value = 0.0048, 10.2-fold increase), and Galectin-3 binding protein (p-value = 0.01, 3.0-fold increase) with an increased expression in breast cancer patients were selected for validation using ELISA. Validation by ELISA was conducted using 171 individual tear samples (75 Breast Cancer and 96 Control). Similar to the observed LC-MS/MS results, S100A8 (p-value <0.0001) and S100A9 (p-value <0.0001) showed significantly higher expression in breast cancer patients. However, galectin-3 binding protein had increased expression in the control group. Our results provide further support for using tear proteins to detect non-ocular systemic diseases such as breast cancer. Our work provides crucial details to support the continued evaluation of tear samples in the screening and diagnosis of breast cancer and paves the way for future evaluation of the tear proteome for screening and diagnosis of systemic diseases.

Introduction

With advances in screening techniques, and adjustment of recommended screening guidelines, mortality rates due to breast cancer continue to drop. Despite the estimated drop-in mortality rates, breast cancer still remains the highest cancer diagnosis of women globally [1]. While the United States spends more on cancer screening than any other industrialized

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country, we also have the lowest life expectancy [2]. While family history remains one of the most significant risk factors, the list of factors classifying an individual as "high-risk" continues to grow. Interestingly, family history of breast cancer increases a woman's chance of developing breast cancer by almost two-fold, however less than fifteen percent of all breast cancer diagnosis are attributed to women with family history [3–5].

As research continues to unfold, additional risk factors such as birth control use, hormone replacement therapy, breast tissue density, and obesity continue to increase the number of women who are classified as high-risk [6]. Massive efforts are currently focused on developing a personalized risk-based screening approach that considers individual biological characteristics, circumstances, and lifestyles [7,8]. Results from these studies could allow justification of focusing the most intensive screening on the portion of the population at the highest known risk of cancer formation. Biological tests could play an important role in future cancer screening risk stratification.

With continued advancement in biomarker identification techniques, there is increasing interest in finding markers of disease in non-traditional biological fluids. Breast cancer associated biomarkers have been identified in urine [9], nipple fluid aspirate [10], as well as breast milk [11]. The knowledge gained from identification of disease markers in fluids other than those traditionally associated with cancer diagnosis could improve the ability to not only understand disease instigation and progression, but also narrow the field of who truly needs to be considered high risk.

Biological fluids such as blood and urine have been extensively studied for their clinical value; however, tear fluid is one of the most underrated biofluids that has been gaining interest in recent years [12]. In this study, tears were used as a source for non-traditional biological fluid that could expand upon our current knowledge on crucial breast cancer biomarkers. Tears are transparent, extracellular fluid secreted by the lacrimal glands forming a mechanical and antimicrobial layer protecting the ocular surface [13]. They are comprised mainly of water and electrolytes but also contains a vast range/multitude of/hundreds of proteins/peptides, lipids, glycoproteins, hormones, and small molecule metabolites [12,13]. The importance of tear analysis extends beyond the ocular surface as they are secreted by the lacrimal glands in the eyelids through filtration from blood plasma and can provide valuable/relevant clinical information from unrelated body parts [14,15]. Studies have focused on using tears as a non-invasive source to conduct biomarker discovery studies as a novel and reliable means to predict and diagnose diseases while also serving to monitor disease progression and therapy [16–25]. The simplicity of tear fluid collection and evaluation could potentially provide a convenient, non-invasive method of testing, fitting easily into a personalized risk-based medicine approach [12,20,26,27].

Ease of collection, high protein concentration, and lower complexity of the tear fluid compared to blood make tears an ideal diagnostic fluid [27–29]. Additionally, low molecular weight proteins are easily accessible in tear fluids and can aid in identifying crucial cancer biomarkers [26]. Several preliminary studies utilizing tear fluid have been conducted looking at systemic diseases without ocular diseases, such as cancer (breast, prostate, lung, ovary, and colon) [23,30–32] and neurological diseases (multiple sclerosis, Parkinson's disease) [33–37]. Here data is presented to support using tear proteins to detect breast cancer. In this study, data collected from 273 individual utilizing the Schirmer strip method will be reported.

Materials and methods

Selection criteria and sampling methods

All protocols involving human subjects were reviewed and approved by the University of Arkansas IRB committee (13-11-289) prior to sample collection. The sampling technique used

was a purposive, non-random sampling strategy to recruit women with the requisite inclusion criteria (Table 1). Tear fluid samples were collected from study participants recruited at five breast health and surgery clinics; The Breast Center, Fayetteville, AR, USA; Breast Surgery of Tulsa, Tulsa OK, USA; Knoxville Comprehensive Breast Center, Knoxville, TN, USA; PeaceHealth Southwest, Vancouver, WA, USA; and PeaceHealth St. John Medical Center, Longview, WA, USA. Written informed consent was obtained from all participants prior to sample collection. Participants were recruited from individuals having a yearly screening mammogram, individuals having a biopsy, and individuals recently diagnosed with breast cancer being evaluated for pre-surgical MRI evaluation. Once imaging results were obtained, samples were then classified as: control (normal imaging no biopsy) or diagnosed breast cancer pre-treatment (diagnosed by biopsy).

Tear sample collection

Tear fluid samples were collected using Schirmer strips (Schirmer tear flow test strips, Eye Care and Cure Corp, Tucson, AZ, USA) from the lower conjunctival fornix. Once the Schirmer strip was in place (Fig 1A), the study participant was instructed to close their eyes and keep them closed until the fluid level reached the 25 mm mark or up to five minutes. Following sample collection, the strips were transferred into a 1.5 mL screw top tube containing 1X Phosphate Buffered Saline (1XPBS). Individual samples were centrifuged for 30 seconds using a super-spin mini centrifuge, the buffer was aliquoted and stored at -80˚C until use.

LC-MS/MS; Biomarker discovery

Total protein content was determined for each tear sample using bicinchoninic acid assay (BCA) (Thermo Scientific, Waltham, MA, USA) and samples containing 7μg of protein were prepped for in-solution digests. Solution digests were carried out on all tear fluid samples in 100 mM ammonium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA), following reduction in 10 mM Tris[2-carboxyethyl]phosphine (Pierce, Waltham, MA, USA) and alkylation in 50 mM iodoacetamide (Sigma-Aldrich) by addition of 100 ng porcine trypsin (Promega, Madison, WI, USA) and incubation at 37˚C for 12–16 hours. Peptide products were then acidified in 0.1% formic acid (Fluka, Honeywell Research Chemicals, Morris Plains, NJ, USA).

Table 1. Inclusion/Exclusion criteria.

Breast Cancer		
Exclusion	***Inclusion Pre-Biopsy Patient***	***Inclusion Breast Cancer***
<18 years of age OR >100 years of age	18–100 years of age	18–100 years of age
Concurrent eye infection or trauma	Presenting for the evaluation of an abnormal exam or test (mammogram, ultrasound, MRI, PET, etc.)- they may or may not have a mass present.	Have been diagnosed but have not received treatment.
Acute conjunctivitis	Presenting for the evaluation of a palpable lump or mass	
	Presenting with a mass may be pre- or post-biopsy as long as there is a portion of the mass remaining.	
Control samples		
Exclusion	***Inclusion***	
<18 years of age OR >100 years of age	18–100 years of age	
Concurrent eye infection or trauma	Do not currently have or are being treated for breast cancer.	
Acute conjunctivitis		

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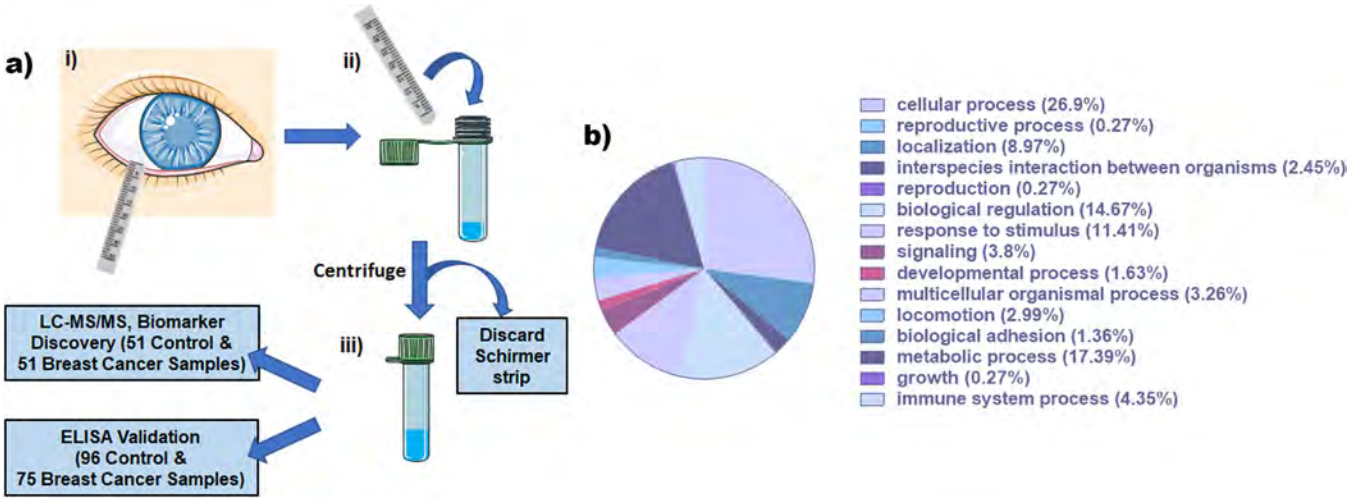

Fig 1. a) Schematic of Tear Collection using Schirmer strip- i) Schirmer strip is placed in the lower conjunctival fornix; ii) wetted strips are placed in screw-top tube prefilled with 225μL of 1XPBS and centrifuged to collect the tears; iii) Schirmer Strips are discarded to collect tears and stored in -80˚C before being analyzed by LC-MS/MS and validated by ELISA. b) Functional classification of 301 mapped proteins in tear samples using PANTHER classification system.

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Tryptic peptides were separated by reverse-phase Jupiter Proteo resin (Phenomenex, Torrance, CA, USA) on a 100 x 0.075 mm column using a nanoAcquity UPLC system (Waters Corporation, Milford, MA, USA). Peptides were eluted using an 80 min gradient from 97:3 to 35:65 buffer A:B ratio [Buffer A = 0.1% formic acid, 0.05% acetonitrile; buffer B = 0.1% formic acid, 75% acetonitrile]. Eluted peptides were ionized by electrospray (1.8 kV) followed by MS/MS analysis using collision-induced dissociation on an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Waltham, MA, USA). MS data was acquired using the FTMS analyzer in profile mode at a resolution of 60,000 over a range of 375 to 1500 m/z. MS/MS data was acquired for the top 15 peaks from each MS scan using the ion trap analyzer in centroid mode and normal mass range with a normalized collision energy of 35.0. Proteins were identified from MS/MS spectra using the Mascot search engine (Matrix Science, Boston, MA, USA) or MaxQuant quantitative proteomics software (Max Planck Institute, Munich, Germany) and the search results were compiled using Scaffold (Proteome Software, Portland, OR, USA). The protein and peptide threshold filters were set at 99% and 95% respectively, with a minimum peptide number of 2.

ELISA biomarker validation

Standard sandwich ELISA procedures using DuoSets ELISA kits purchased from R&D Systems (Minneapolis, MN, USA) were used to evaluate the expression level of S100A8 (SA8), S100A9 (SA9), and Galectin-3-Binding Protein (LG3BP) in tear samples. Assays were conducted according to the manufacturer's guidelines. Based on results from previous optimization tests, tear samples were diluted at 1:10 for SA8 and SA9 analytes and 1:50 for LG3BP. All samples and standards were tested in duplicate. The absorbance was read at 450 nm and 570 nm using a Synergy LX microplate reader (BioTek, Winooski, VT, USA). Absorbance at 570 nm was subtracted from 450 nm for each well. ELISA data was analyzed using Prism version 6.0 (GraphPad, San Diego, CA, USA).

Statistical analysis

For protein discovery, variations in spectral intensities of tryptic fragments mapped to protein IDs in Scaffold software were compared by utilizing One-Way ANOVA in JMP Pro11 software

package. Predicted protein intensities were assessed across groups to elucidate potential biomarkers. An alpha level of 0.05 was used as an indicator of significant expression change between the groups. The functional categories of the identified proteins were determined using an online protein annotation tool, PANTHER (protein annotation through evolutionary relationship, www.pantherdb.org). R statistical software was used to apply logistic regression to protein concentrations determined by ELISA for breast cancer versus control. The probability of breast cancer as a function of protein concentration was obtained and used to create a decision rule. The decision rule was then used to classify each case via the confusion matrix and related metrics such as sensitivity, specificity, receiver operator characteristics (ROC), and area under the curve (AUC).

Results

Sample characteristics

Patient demographics for samples used in LC-MS/MS and ELISA are presented in Table 2. All data sets combined consisted of 273 participants- 102 samples were used for LC-MS/MS analysis (51 breast cancer, 51 control) and 171 for ELISA (75 Breast Cancer and 96 Control). Participants ranged from 23–91 years of age with an average age of 53.75 ± 14.2 years. Most participants were Caucasian (84.56%), followed by African American (3.52%) and Hispanic (1.86%). A modest percentage of participants had a family history of breast cancer (30.31%), and a small portion had a previous history of breast cancer (7.02%). Individuals with a previous history of breast cancer were considered acceptable for the control group if they were no longer undergoing treatment and had not undergone treatment for at least five years and had been returned to standard yearly screening. Of those study subjects for whom breast density was obtained, 57.5% had dense breast tissue. The abbreviation NR was used when clinical or demographic data was not reported by the participating clinical partner.

Study enrollment was not limited to a particular type of breast cancer. This breast cancer sample group included IDC (57.14%), ILC (7.14%), DCIS (24.60%), as well as Metaplastic and Mucinous Carcinoma (1.59%) (Table 3). Grade designation was received for 90 of the 126 samples (Grade I 15.56%, Grade II 54.44%, Grade III 46.83%) (Table 3).

LC-MS/MS

In-solution trypsin digestion followed by LC-MS/MS was conducted and tryptic fragments were mapped for 301 proteins. The functional classifications of these identified proteins (Fig 1B) were primarily involved in cellular (26.9%) and metabolic (17.39%) processes, as well as biological regulation (14.67%). Spectral intensities were imported into JMP Pro11 software for One-way ANOVA and linear regression analysis. An alpha level of 0.05 was used as an indicator of significant expression change between the groups as well as a fold change greater than 2. Variations in spectral intensities of tryptic fragments were evaluated between control vs. breast cancer. Fourteen proteins (Table 4) were identified as potential biomarkers based on their significant p-values ($p < 0.05$) and fold changes; ACTN4, ADH1G, AK1C1, AL1A1, B4E1Z4, CYTN, G3P, K1C9, LDHA, LDHB, LG3BP, S100A8, S100A9, SPRL1. Of the fourteen proteins of interest, three proteins (S100A8, S100A9, and Galectin-3-binding protein) were selected as candidates for initial evaluation by ELISA based on observed fold change, statistical significance, and biological relevance. S100A8 and S100A9 had significantly higher expression levels with p-values of 0.0069 and 0.0048 in breast cancer patients, respectively, with an increased fold-change of 7.8 and 10.2 compared to controls. Similarly, Galectin-3-binding protein (LG3BP) had a 3-fold increase in expression (p-value = 0.01) compared to the control group.

Table 2. Population demographics of tear samples used for LC-MS/MS and ELISA.

		LC-MS/MS			ELISA		
		No. of Patients, %			No. of Patients, %		
		Breast Cancer (N = 51)	Control (N = 51)	Total (N = 102)	Breast Cancer (N = 75)	Control (N = 96)	Total (N = 171)
Age, y							
	<39	2 (3.9)	12 (23.53)	14 (13.73)	2 (2.67)	15 (15.62)	17 (9.94)
	40–49	10 (19.6)	6 (11.76)	16 (15.67)	12 (16)	16 (16.67)	28 (16.38)
	50–59	12 (23.5)	14 (27.45)	26 (25.50)	23 (30.67)	26 (27.08)	49 (28.65)
	60–69	16 (31.4)	6 (11.76)	22 (21.57)	16 (21.33)	13 (13.54)	29 (16.97)
	>70	11 (21.6)	3 (5.89)	14 (13.73)	13 (17.33)	4 (4.17)	17 (9.94)
	NR	-	10 (19.61)	10 (9.80)	9 (12)	22 (22.92)	31 (18.13)
Race							
	African-American	2 (3.92)	2 (3.85)	4 (3.93)	2 (2.67)	4 (4.17)	6 (3.51)
	Asian	1 (1.96)	0 (0.0)	1 (0.98)	1 (1.33)	0 (0.0)	1 (0.58)
	Caucasian	46 (90.2)	43 (82.69)	89 (87.25)	63 (84)	74 (77.08)	137 (80.12)
	Hispanic	1 (1.96)	1 (1.92)	2 (1.96)	1 (1.33)	2 (2.08)	3 (1.75)
	Native Hawaiian or PI	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.67)	0 (0.0)	2 (1.17)
	NR	1 (1.96)	5 (9.62)	6 (5.88)	6 (8)	16 (16.67)	22 (12.87)
History of Breast Cancer							
	Yes	7 (13.72)	5 (9.80)	12 (11.76)	5 (6.67)	0 (0.0)	5 (2.29)
	No	41 (80.39)	26 (50.98)	67 (65.69)	70 (93.33)	96 (100)	166 (97.08)
	NR	3 (5.89)	20 (39.22)	23 (22.55)	-	-	-
Family History of Breast Cancer							
	Yes	22 (43.14)	10 (19.61)	32 (31.37)	29 (38.67)	21 (21.87)	50 (29.24)
	No	29 (56.86)	20 (39.22)	49 (48.04)	42 (56)	48 (50)	90 (52.63)
	NR	0 (0.0)	21 (41.17)	21 (41.17)	4 (5.33)	27 (28.13)	31 (18.13)
Breast Density		(N = 30)	(N = 22)	(N = 52)	(N = 49)	(N = 64)	(N = 113)
	Fatty	1 (3.33)	2 (9.10)	3 (5.77)	1 (2.04)	5 (7.81)	6 (5.31)
	Scattered fibroglandular densities	13 (43.33)	15 (68.18)	28 (53.84)	21 (42.86)	35 (54.69)	56 (49.56)
	Heterogeneously Dense	14 (46.67)	4 (18.18)	18 (34.62)	24 (48.97)	20 (31.25)	44 (38.94)
	Extremely Dense	2 (6.67)	1 (4.54)	3 (5.77)	3 (6.12)	4 (6.25)	7 (6.19)

NR—No clinical or demographic data were reported.

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ELISA

Protein expression levels of S100A8, S100A9, and Galectin-3-binding protein were evaluated by ELISA. Concentrations of S100A8 and S100A9 were found to be elevated in breast cancer (mean concentration of 2997.17 pg/ml for S100A8 and 5729.19 pg/ml for S100A9) compared with the control group (mean concentration of 1003.92 pg/ml for S100A8 and 2107.35 pg/ml S100A9). Student t-test produced a p-value of <0.0001 indicating a statistically significant difference (Fig 2A and 2B). Galectin-3-binding protein was found to be increased in the control

Table 3. Distribution of breast cancer types and grade designations.

LC-MS/MS Sample Pool			ELISA Sample Pool		
		No. of Patients (%)			No. of Patients (%)
Cancer type			Cancer type		
	IDC	28 (54.91)		IDC	44 (58.67)
	ILC	4 (7.84)		ILC	5 (6.67)
	DCIS	13 (25.49)		DCIS	18 (24)
	IDC/DCIS	3 (5.88)		IDC/DCIS	4 (5.33)
	Other	1 (1.96)		Other	1 (1.33)
	NR	2 (3.92)		NR	3 (4)
Grade			Grade		
	I	7 (13.73)		I	7 (9.33)
	II	19 (37.25)		II	30 (40)
	III	13 (25.49)		III	20 (26.67)
	NR	12 (23.53)		NR	18 (24)

NR—No clinical or demographic data were reported.

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group, with a mean of 75263.2 pg/ml, compared to breast cancer group which had a mean of 23747.3 pg/ml and a p-value of <0.0001 (Fig 2C). The receiver operating characteristic (ROC) curve was generated (Fig 2D) using a linear logistic regression analysis, with an area under the curve (AUC) value of 0.902, a sensitivity of 84.8%, a specificity of 86.4% and an accuracy of 85.6%.

Discussion

Here we demonstrate the utility of protein biomarkers isolated from tear samples to differentiate between individuals with a diagnosed breast cancer, a systemic non-ocular disease, and

Table 4. Summary of relevant biomarkers candidates from mass spec analysis.

Protein Name	Gene Name	Function	Cancer vs Control	Fold Change (elevated)
Alpha-actinin-4	ACTN4	Cell adhesion, cell migration, apoptosis regulation.	0.0443	2.3 (CRL)
Alcohol dehydrogenase 1C	ADH1G	Catalytic activity- ethanol, retinol, and other aliphatic alcohol metabolism.	0.0424	3.8 (CRL)
Aldo-keto reductase family 1 member C	AK1C1	Steroid hormone homeostasis, prostaglandin metabolism, metabolic activation of polycyclic aromatic hydrocarbons.	0.0256	3.17 (CRL)
Retinal dehydrogenase 1	AL1A1	Retinol metabolism, ethanol oxidation.	0.0325	1.77 (CRL)
Uncharacterized Protein	B4E1Z4		0.0334	1.7 (BC)
Cystatin-N	CYTN	Regulation of cysteine proteinases, antimicrobial, antiviral.	0.0355	1.68 (CRL)
Glyceraldehyde-3-phosphate dehydrogenase	G3P	Glycolysis, immune response, cytoskeleton organization, apoptosis.	0.0405	1.9 (CRL)
Keratin type 1 cytoskeletal 9	K1C9	Epidermis development, cytoskeletal structure integrity, keratin filament assembly.	0.0428	5.5 (BC)
L-lactate dehydrogenase A chain	LDHA	Oxidoreductase; Involved in the lactate and NAD metabolic process, positive regulation of apoptotic process.	0.0194	2.3 (CRL)
L-lactate dehydrogenase B chain	LDHB		0.0265	3.4 (CRL)
Galectin-3-binding protein	LG3BP	Immune system regulator, cell adhesion.	0.01	3.0 (BC)
S100 A8	S10A8	Inflammation, immune response, inhibitor of casein kinase.	0.0069	7.8 (BC)
S100 A9	S10A9		0.0048	10.2 (BC)
SPARC-like protein 1	SPRL1	Regulates ECM remodeling and cell-matrix interactions and angiogenesis.	0.0371	10.3 (BC)

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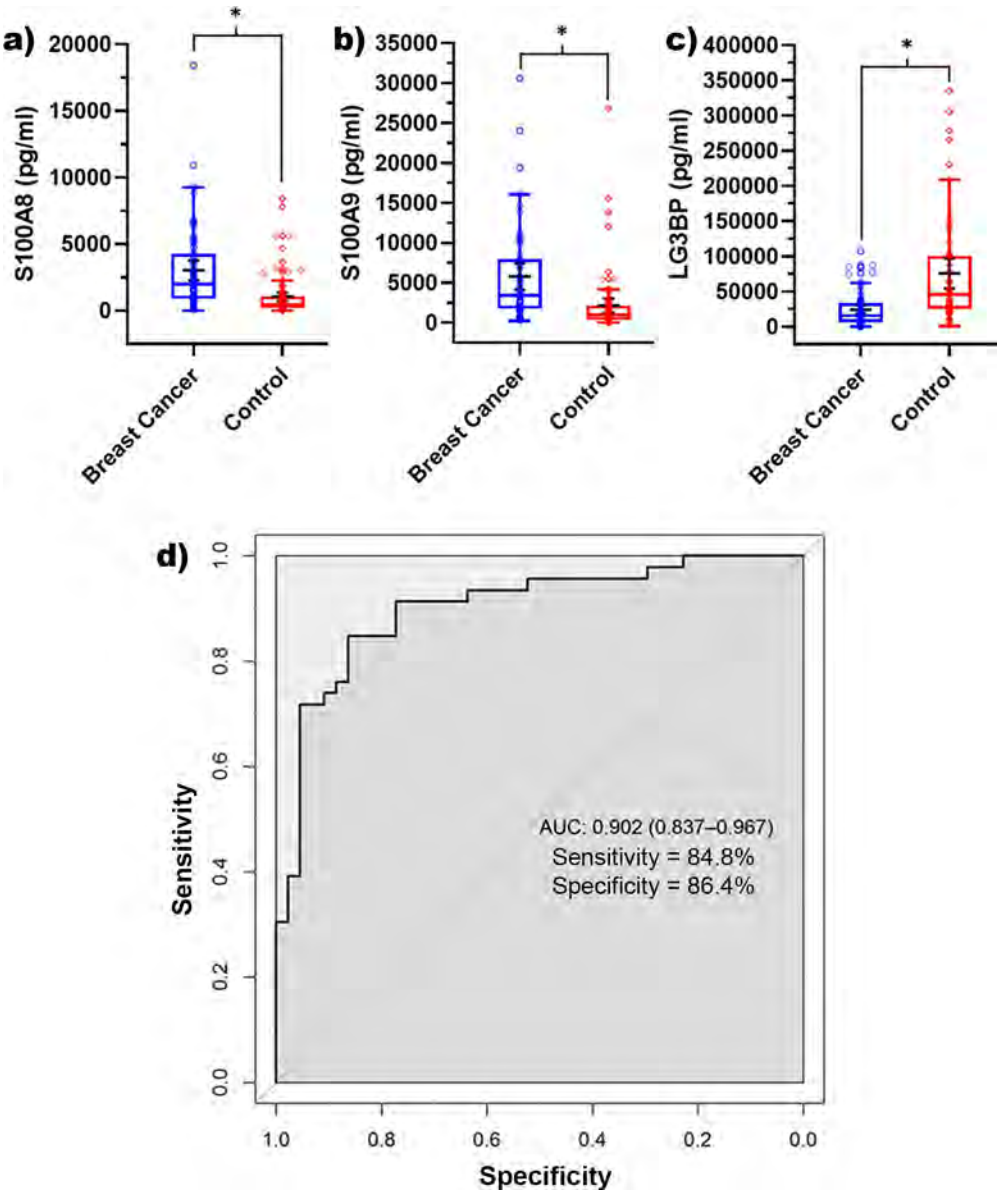

Fig 2. Investigation of biomarkers by ELISA- a) S100A8, b) S100A9, and c) LG3BP expression levels in tear samples between healthy and breast cancer women. (n = 96 control and 75 breast cancer samples, * indicates $p < 0.0001$); d) Receiver Operating Characteristics (ROC) curve for protein expression of potential breast cancer biomarkers. The area under the ROC curve (AUC) represents the accuracy of the combined potential biomarkers for distinguishing between the control and breast cancer sample groups.

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healthy individuals. A Schirmer strip was used as the sample collection source for tears as they can capture a large quantity of intracellular and extracellular proteins on the ocular surface compared to the other commonly used capillary tube method [38]. Total protein content of tear samples collected as determined by BCA, varied from 0.137–1.4 mg/ml. Overall, each sample had a more than adequate concentration of protein to be analyzed by LC-MS/MS. The decision was made to evaluate each sample independently rather than pooling samples to obtain a more accurate representation of the population. As stated earlier in the methods section, 7μg of proteins from each sample were analyzed using LC-MS/MS and tear proteins were

identified using LC-MS/MS and mass intensities of associated peptide fragments were compared between the two groups to identify potential biomarkers. After evaluation, three proteins (S100A8, S100A9, and LG3BP) were selected for validation by performing ELISA based on previously reported association with breast cancer. Conducting validation utilizing a biological assay provided verification of the protein identification from LC-MS/MS.

The S100s are a family of Ca2+ binding proteins, with high sequence and folding similarity, involved in a wide range of biological processes such as proliferation, migration and/or invasion, inflammation and differentiation [39]. These proteins differ in shape and charge which contributes to a wide diversity of protein targets as well as a broad range of functions [40]. Elevated levels of S100A8 and S100A9 detected in tear samples from breast cancer patients supports previously reported results indicating elevated levels in serum and tissue of breast cancer patients [41–44]. A 2018 study reported an increased level of S100A8 expression levels in breast cancer patients with relapse and had significantly lower disease-free survival and overall survival durations [45]. The study further reported S100A8's elevated levels in correlation with estrogen receptor-negative and triple-negative breast cancer clinical subtypes. S100A8 and S100A9 specifically have been shown to have altered expression levels in breast cancer tissues compared with normal tissues, with increased expression levels associated with non-functional BRCA1 (BReast CAncer gene 1) [40,46]. Non-functional BRCA1 leads to increased expression levels of S100A8 and S100A9 which then play a role in metastasis through binding to RAGE (Receptor for Advanced Glycation Endproducts) receptors on the surface of myeloid-derived suppressor cells [47–49]. While supporting literature as well as our data suggests a detectable increased expression in S100A8 and S100A9 in tear samples, the authors acknowledge a previous study on tears indicated reduced expression of S100A8 and S100A9 in pooled tear samples of breast cancer patients compared to normal patients [31]. However, this study does not provide a hypothesis for this contradictory expression profile and the variation in experimental parameters could be responsible for the observed differences (i.e. pooled samples versus individual sample evaluation, use of acetone protein precipitation methods, and evaluation of in-gel digestion versus in-solution trypsin digestion).

Galectin-3 binding protein (LG3BP) is a heavily glycosylated 90 kDa protein that is expressed in bodily secretions produced mostly by epithelial cells in glands, such as breast and tear ducts, as well as cancer cells [50]. LG3BP has been shown to be a binding site for proteins known to be involved in metastasis [51]. In addition, higher serum levels of LG3BP were associated with shorter survival in patients with breast carcinoma [52]. LG3BP was selected as a biomarker due to the elevated level of LG3BP observed by LC-MS/MS. However, ELISA data suggests a reduction in concentration in tears for breast cancer patients. A previous research group performed a comparison of vitamin-D binding protein concentrations in two different races using mass spectrometry, monoclonal and polyclonal ELISA kits [53]. They reported that these expression levels comparing the mass spectrometry results with polyclonal ELISA results had less than 9% variability but showed a higher (~85%) variability with monoclonal ELISA kits. They attributed this effect to the differential isoforms of the proteins detected using the two ELISA methods which varied by genotype. We believe that a similar difference in our ELISA and LC-MS/MS results could be attributed to the monoclonal ELISA kits used to quantify LG3BP.

The Area Under the Curve (AUC) for screening mammography has been reported to be anywhere from 0.67 to 0.84 depending on the modality used (digital vs. film), patient population, and breast density of participants [54,55]. We report an AUC of 0.902 with a sensitivity of 84.8%, a specificity of 86.4% and an accuracy of 85.6% and provides a strong starting point and justification for future research.

Conclusions

The field of tear-based diagnostics is rapidly expanding beyond ocular diseases. Current studies have focused on detecting alterations in the tear proteome in a wide variety of systemic diseases ranging from Alzheimer's to cancer [12,18,21,26,27,56]. Here, we provide an analysis of 273 individually collected tear samples. In this study, we examined the ocular proteome to identify protein biomarkers with altered expression levels in women diagnosed with breast cancer. Biomarker discovery was carried out using LC-MS/MS and selected markers were validated using ELISA. Our work provides data to support the growing body of evidence for continued evaluation of tear samples screening and diagnosis of systemic diseases. While the number of individual tear samples evaluated is large for the field of tear-based proteomics, it is quite small in the field of breast cancer as well as biomarker validation. Significantly larger studies would need to be conducted in order to reach a sound conclusion on the ability of tear proteins to distinguish between control and disease state samples.

Future investigations will focus on the additional biomarkers listed in Table 4 to aid in differentiating breast cancer from control; specifically, SPARC-like protein 1 and lactate dehydrogenase as these markers will provide more insight into ECM remodeling and metabolic processes respectively. Alternative approaches to both collection and sample processing procedures, such as exosome isolation, could allow for evaluation of not only intracellular and extracellular markers but also microRNA [57,58].

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Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of the University of Arkansas (protocol code 13-11-289 and December 2013).

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Author Contributions

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Formal analysis: Anna Daily, Prashanth Ravishankar.

Funding acquisition: Anna Daily.

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Methodology: Anna Daily, Steve Harms, V. Suzanne Klimberg.

Project administration: Anna Daily, Steve Harms, V. Suzanne Klimberg.

Resources: Anna Daily, Prashanth Ravishankar, Steve Harms, V. Suzanne Klimberg.

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Visualization: Anna Daily, Prashanth Ravishankar.

Writing – original draft: Anna Daily, Prashanth Ravishankar.

Writing – review & editing: Anna Daily, Prashanth Ravishankar.

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Communication

Tears as the Next Diagnostic Biofluid: A Comparative Study between Ocular Fluid and Blood

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Abstract: The need to easily isolate small molecular weight proteins and genomic fragments has prompted a search for an alternative biofluid to blood that has traversed sweat, urine, saliva, and even breath. In this study, both the genomic and proteomic profiles of tears and blood are evaluated to determine the similarity and differences between the two biofluids. Both fluids were tested utilizing microarray panels for identifying proteins as well as isolation of microRNA for sequencing. As anticipated, most (118) of the proteins detected in plasma were also detected in the tear samples, with tear samples also showing 34 unique proteins that were not found in the plasma. Over 400 microRNAs were isolated in both samples with 250 microRNA fragments commonly expressed in both tears and blood. This preliminary analysis, along with simplicity of collection and processing, lends credence to further investigate tears as an alternative biofluid to blood.

Keywords: tear fluid; blood plasma; protein microarrays; miRNA; biological fluids

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1. Introduction

With the advancement in proteomic, metabolomic, and genomic technologies, emphasis on finding novel biological markers for systemic diseases have gained a lot of traction in recent years. The main objective of biomarker discovery is identifying protein markers that can improve early diagnosis and track therapeutic intervention efficacy; all while advancing the medical field to create predictive, preventive and personalized medicine. Most of the studies have focused on biomarker discovery using blood serum and/or plasma, as blood is generally regarded as an ideal fluid for the evaluation of systemic diseases. However, a variety of body fluids such as cerebrospinal fluid, urine, saliva, and tears have gained traction as source for novel biomarkers [1].

The challenge in progressing these research studies from the bench to the bedside lies in the dynamic complexity of a blood sample. A standard blood sample contains a multitude of components, such as, large red blood cells, proteins, lipids, small ions, and metabolites [2]. The blood cells need to be removed for effective detection of the analytes and thus requires preprocessing techniques [3–5]. Most new biomarker discoveries involve relatively small or low molecular weight proteins which may be undetectable without a significant amount of sample preprocessing. These standard research laboratory preprocessing techniques do not translate well to commercial labs due to the high throughput efficiency required adding to the cost and time associated with it [4,6,7]. Additionally, the blood collection is an invasive procedure that can test patient compliance. This has led to a quest to find a suitable and alternative biofluid source that expresses similar biomarker profiles with extensive research revolving around biofluids like sweat, urine, saliva, and tears. In this article, tears are tested as a suitable alternative to blood as it provides a clearer picture of the proteome over blood.

Tears are transparent, extracellular fluids secreted by the lacrimal gland and are comprised of three layers- outer lipid layer, middle aqueous layer, and epithelium-covering mucoid layer [8]. They are considered as a hypotonic ultrafiltrate of blood plasma and

contain a mixture of proteins, lipids, mucins, and small molecule metabolites [9], thus, making tears clinically relevant as they can provide information from unrelated body parts. Several proteomics studies reveal the presence of 500–1500 proteins involved in multiple signaling pathways [10–13]. Tears can be collected using several tools such as microcapillary tubes, polyester/polyvinyl wicks, and Schirmer strips [14]. Schirmer strips are more commonly employed for tear collection as they are used in clinical settings for standard ophthalmologic testing of dry eye disease. These strips are relatively simple to use, rapid, and reliable compared to capillary tubes which requires performing a delicate procedure [9].

In this article, tears and blood were collected from the same healthy subjects to compare the protein and microRNA (miRNA) compositions and show that tears can serve as an ideal biofluid source with more accessibility and less complexity than blood.

2. Materials and Methods

2.1. Study Population

Informed consent was obtained from all subjects prior to the study to collect plasma and tear samples. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Advarra IRB committee and assigned protocol #CVDTRS001, dated May 2020. Inclusion criteria required participants to be over the age of eighteen, without active eye infection. For this portion of the study, the population was kept minimal to allow ensure feasibility and allow for proof of concept. Samples were collected from one female and one male, both were aged 33.

2.2. Plasma Sample Collection

From each subject, peripheral venous blood was drawn aseptically by venipuncture into a sterile ethylenediaminetetraacetic acid (EDTA) containing BD Vacutainer® tube. The blood was processed by centrifuging at 18,000× *g* at 4 °C for 15 min and the supernatant was separated carefully to collect the blood plasma. The collected plasma was stored at −80 °C until further use.

2.3. Tear Sample Collection

Tear fluid was collected from the same subject to aid in the direct comparison of proteins obtained from the two different biofluid sources-ocular fluid and blood plasma. To be eligible for tear sample collection, participants were required to be over the age of eighteen without active eye infection.

Based on previously established methods [9,14], tear fluid was collected using Schirmer's strip. Briefly, a Schirmer strip is placed in the subjects' lower eyelid to collect the ocular surface proteins. Once the strip reached 25 mm or 5 min was completed, the Schirmer strip was placed in a tube containing 225 µL of 1× Phosphate Buffered Saline (1XPBS). The tube was processed by centrifuging at 2500× *g* RPM for 5 min. The strips were discarded, and the tear fluid was stored for further analysis.

2.4. Microarray Sample Preparation and Analysis

Tear and blood samples were compared via protein microarray analysis. An Explorer Antibody Array with 656 antibodies and a Cancer BioMarker Antibody Array with 247 unique antibodies of cancer biomarkers were used for this purpose (Full Moon BioSystems, Sunnyvale, CA, USA). The tear fluid and blood plasma samples were prepared, biotinylated, and incubated according to the manufacturer's protocol. Following protein conjugation, the slides were treated with Cy3-Streptavidin (Thermo Fisher, Camarillo, CA, USA), rinsed, and dried. The fluorescent intensities were measured using GenePix 4000B (Molecular Devices LLC, San Jose, CA, USA) and normalized within each array slide to determine the average signal intensity. A protein with a true signal was dictated if the fluorescence intensity was twice the average signal intensity of the empty spots.

The fold change was calculated as the ratio of normalized intensity of tear fluids over normalized intensity of blood plasma. A fold change value of 2 represented double the expression in tears and a value of 0.5 represented half the expression level in tears.

2.5. RNA Extraction for Small RNA-Seq

RNA was extracted from 250 µL plasma or 200 µL tears with Quick-cfRNA Serum & Plasma Kit (Zymo Research, Cat # R1059, Irvine, CA, USA) as per manufacturer's recommendations. Briefly, the samples were centrifuged at 12,000× *g* for 15 min to remove any debris and mixed with Quick-cfRNA digestion buffer with the same volume as the sample. Proteinase K was added to the same and incubated for 2 h at 37 °C. Following which, Quick-cfRNA binding buffer was added to the digested samples. The samples were further treated with RNA prep and recovery buffer. RNA samples were washed and eluted from the columns with 10 µL of RNase free water.

2.6. Library Prep with RealSeq-Dual

Library preparation was performed with RealSeq-Dual as recommended by the manufacturer (RealSeq Biosciences, Santa Cruz, CA, USA) with 10 µL of RNA extracted from the Quick-cfRNA Serum & Plasma assay kit. Half the volume of each library was amplified by 22 cycles of PCR. Libraries from all samples were pooled for sequencing in the same flow cell of a NextSeq single-end 75 nt reads and dual 6 nt indexes. FastQ files were trimmed of adapter sequences by using Cutadapt with the following parameters [15]: cutadapt -u 1 -a TGGAATTCTCGGGTGCCAAGG -m 15. Trimmed reads were aligned to the corresponding reference by using Bowtie [16]. Differential expression was calculated using the DESeq2 package [17].

3. Results and Discussion

3.1. Microarray

An exploratory protein microarray study, which is a high-throughput ELISA based antibody array that gives qualitative/semi-quantitative protein expression profiling, was conducted. The tear fluid and blood plasma samples were prepared and probed with an Explorer Antibody Array and a Cancer Biomarker Array. We chose the explorer array and cancer array to aid in understanding the overall protein expression levels and one that is associated with a systemic disease. As depicted in Figure 1, the Explorer array identified 121 proteins to have a positive signal in which 71 proteins were commonly expressed across both the tears and plasma samples. They also showed 30 proteins to be uniquely expressed in tears and 20 proteins in plasma. In the cancer biomarker array, 62 proteins had a positive signal with 49 proteins commonly identified in both samples with an additional seven proteins expressed specifically in tears and six proteins unique to plasma. Proteins detected in both tears and plasma samples from the microarray panels are listed in Table 1. Appendix A, Table A1 lists the proteins identified unique to the two sample types.

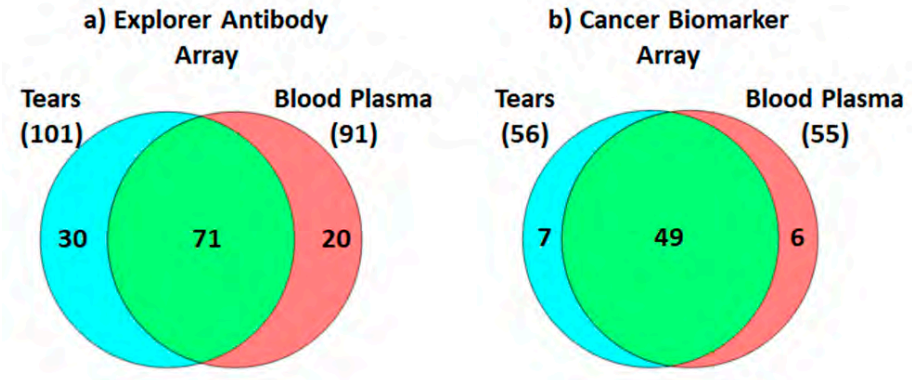

Figure 1. Microarray data analyzed for common protein expression in tears and plasma: (**a**) Explorer biomarker array, and (**b**) Cancer biomarker array.

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

The proteins found unique to tears were further analyzed using the Panther Database and resulted in 28 pathway hits. These results revealed that the expressed tear proteins were involved in FGF, EGF, and JAK/STAT signaling pathways, and included proteins

Table 1. List of common proteins detected via microarray in tears and plasma samples.

Present in Tear and Plasma			
ADP-ribosylation Factor (ARF-6)	CNPase	IL-12	Parathyroid Hormone-related Protein
Alpha-fetoprotein (AFP)	Collagen I	IL-15	Pax-5
Apoptosis Inducing Factor (AIF)	Collagen II	Interleukin-1 receptor associated kinase (IRAK)	PDGFR alpha
Alpha 1 Antichymotrypsin (ACT)	Collagen III	Kappa Light Chain	PDGFR, beta
Alpha Lactalbumin	Collagen IV	Keratin 19	PLC gamma 1
alpha-1-antitrypsin	Cullin-3 (CUL-3)	Keratin 8	Prohibitin
Bcl-2-like protein 1 (bcl-XL)	Cyclin B1	Ku (p70/p80)	Prolactin Receptor
Beta actin	Desmin	Lambda Light Chain	pS2
Beta-2-Microglobulin	Epithelial Specific Antigen	Laminin B1/b1	Rad18
Biotin	Ferritin	Laminin Receptor	Ras
Chemokine receptor type 6 (CXCR6)	Fibronectin	Laminin-s	sIL-2R alpha
Cancer Antigen 15-3 (CA15-3)	Follicle Stimulating Hormone (FSH)	Calprotectin	Silencer of Death Domain (SODD)
Cancer Antigen 19-9 (CA19-9)	Follistatin	Mammaglobin B	Streptavidin
Tyrosine-protein kinase ABL1 (c-Abl)	Gai1	Mek2	TGF beta Receptor III
Cadherin-pan	Glucagon	MGMT	TGF beta1
Caspase 1	Glycogen Synthase Kinase 3b (GSK3b)	MHC II (HLA-DP and DR)	Thymidylate Synthase
Caspase 7 (Mch 3)	Granulocyte Colony Stimulating Factor	MMP-1	Thyroglobulin
CD1	Glutathione S-transferase (GST)	MMP-10	Thyroid Hormone Receptor alpha
CD10	Haptoglobin	MMP-11	TIMP-1
CD1b	Heat Shock Protein 27/hsp27	MMP-2	TR2
CD2	Hepatocyte Growth Factor Receptor (MET)	MMP-2 (72 kDa Collagenase IV)	Transferrin
CD35/CR1	hPL	MMP-7	Tubulin alpha
CD40L Receptor	human Albumin	MSH2	Tubulin gamma
CD42b	IgA	Mucin 2	Uracil-DNA Glycosylase (UNG)
CD45/T200/LCA	IgA	MUTYH	VCAM-1
CD57	IgG	MyoD1	VEGFB
Cdk1/p34cdc2	IgG	Neutrophil Elastase	Vitamin D Receptor (VDR)
Cdk7	IgM	Nitric Oxide Synthase, brain (bNOS)	Xanthine Oxidase
Cdk8	IgM (m-Heavy Chain)	p130	
CEA/CD66e	p19ARF	p130cas	

The proteins found unique to tears were further analyzed using the Panther Database and resulted in 28 pathway hits. These results revealed that the expressed tear proteins

were involved in FGF, EGF, and JAK/STAT signaling pathways, and included proteins associated with metabolic processes and angiogenesis.

Most of the proteins detected in plasma were also detected in the tear samples; however, many low molecular weight proteins showed a higher intensity in tears when compared to blood. This effect could be attributed to larger molecular weight proteins overshadowing smaller ones in plasma or due to the preprocessing leading to damage of the proteins.

3.2. MicroRNA

MicroRNAs are small nucleotide, non-coding RNAs that suppresses target mRNA translation and stability. miRNA sequencing of the sample was used to identify and quantitatively decode the entire population of microRNAs that were common between the tear and plasma samples and compare them. Initial analysis after data normalization revealed that over 400 miRNAs were identified in all the samples with 250 miRNAs commonly expressed in both the tear and plasma samples. Further analysis by filtering to a p-value cutoff of 0.01 showed 64 miRNAs to possess differential expression in the commonly expressed markers. Figure 2 shows the miRNA heatmap created by filtering the log fold change to a p-value cutoff of 0.01.

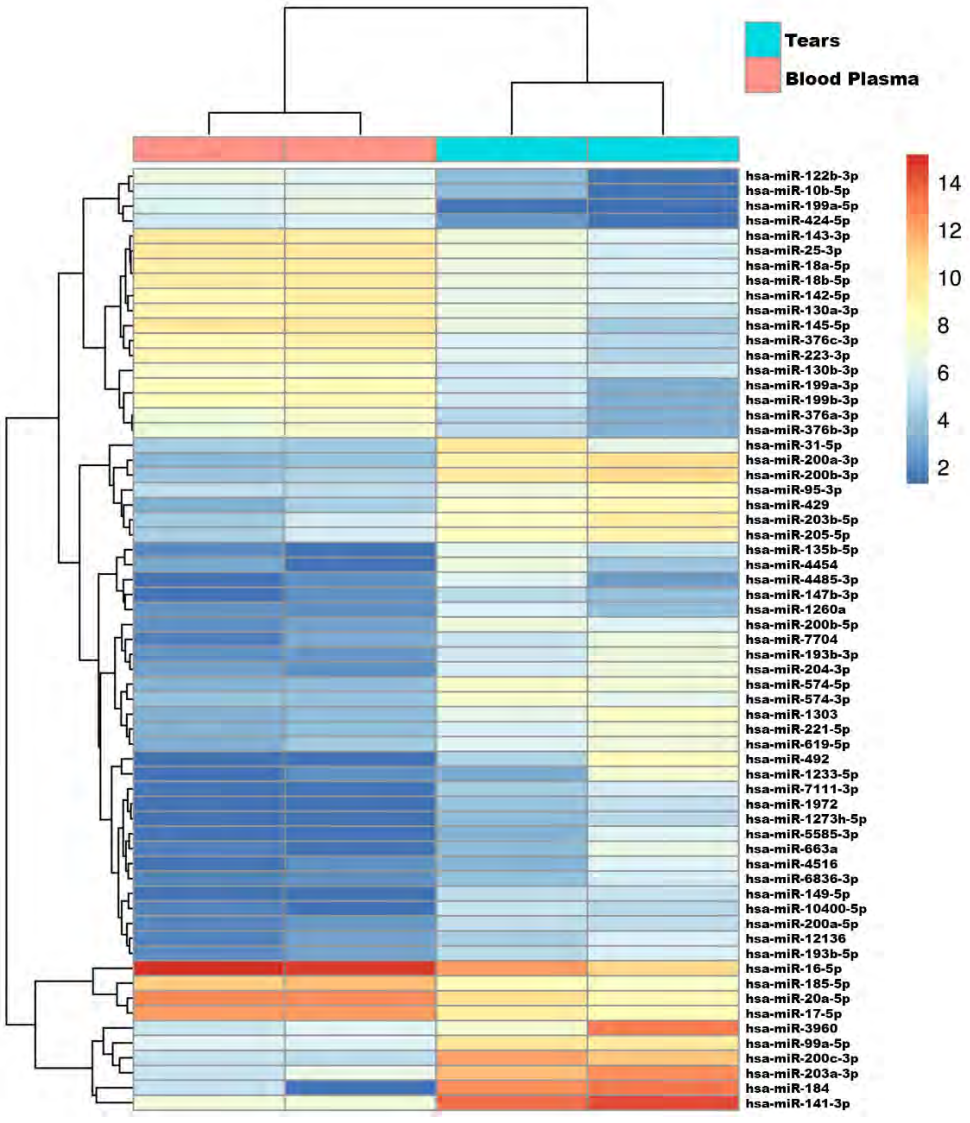

Figure 2. The heatmap represents the cluster analysis of miRNA log fold change in tears and plasma ($p < 0.01$).

MicroRNAs have been demonstrated to serve as useful biomarkers for studying various diseases and conditions. Along with our results which show overlapping miRNA expression in blood plasma and tears, other studies have also shown similar overlapping

MicroRNAs have been demonstrated to serve as useful biomarkers for studying various diseases and conditions. Along with our results which show overlapping miRNA expression in blood plasma and tears, other studies have also shown similar overlapping effect with serum, cerebrospinal fluid, and saliva in traumatic brain injuries [18,19]. Additionally, multiple studies have investigated the proteomic and genomic profile of tears, due to the simplicity of collection and processing, in order to identify their potential in differentiating healthy and disease states. One such study reported the miRNA expression in tears of normal and Sjögren Syndrome patients showed four upregulated and 10 down-regulated miRNA markers [20].

Exosome research has gained attention in recent years by clinicians and researchers as a source of cancer-specific biomarkers [21–23]. However, separation methods of exosomes in blood have proven inefficient as isolation of these small extracellular vesicles is severely hindered by coagulation. Efforts are underway by the International Society of Extracellular Vesicles to create a standardized protocol for isolating EVs from blood sample. Conversely, exosome isolation from tears is relatively easy compared to blood as it does not have any coagulant factors and does not require any special treatment. A recent study published from Kobe University identified miR-21 and miR-200c to be highly expressed in metastatic breast cancer patients' tear exosomes [24].

This overlap of protein and miRNA expression in plasma and tears clearly shows that tears can serve as an alternative biofluid with additional research. The ease of collection and reduced pre-processing times can be easily translated to diagnostic settings. Additionally, it opens another avenue to identify unique protein and miRNA expression that may be associated with diseased conditions that were not previously accessible in blood plasma.

With the advancement of -omic technologies, we are getting closer to creating personalized, preventive medicine and having the ability to make informed decisions in treatment regimes. Research studies have been performed to identify potential biomarkers for pathological conditions beyond ocular diseases especially in Alzheimer's disease and cancer among other systemic diseases [24–28]. Multiple biomarkers associated with a systemic disease is becoming more common as it can achieve overall performance in sensitivity and specificity [29,30]. The biomarker discovery information gained in similar studies will provide an optimal analytical strategy and aid future researchers to build custom microarray panels associated with a certain systemic disease.

Our study serves to communicate that protein and miRNA from blood shows similar composition in tears with less processing techniques and time making it more attractive as a non-invasive source. The results reported were obtained from healthy individuals and a comparative study involving patients with a systemic disease will be conducted as part of our future study. Similar research will aid in addressing the clinical issues and help in creating a clear pipeline for tear biomarker discovery by comparing it is well established methods of discovery, qualification, and validation that has been conducted in blood.

Future comparative studies will involve collection of tears from healthy individuals and cancer patients to create a repertoire of protein and understanding the miRNA compositions. Additionally, we aim to study the effect of miRNAs on proteins using microarray platforms.

4. Conclusions

Our preliminary experiments demonstrate the considerable overlap of proteins and miRNAs in tears and plasma samples. The non-invasive accessibility of tears, ease of collection, minimal to no preprocessing techniques, and reduced processing times make tears as an enticing alternative to explore their potential diagnostic utility over blood. This study paves path to conduct further large-scale research aimed at standardizing tear collection, storage, extraction, as well as their impact on analysis across research and clinical settings that currently exists for blood.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Advarra (protocol #CVDTRS001, dated May 2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data presented in this study are available upon request to the corresponding author.

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Conflicts of Interest: Namida Lab Inc. is a privately funded company and provided funds for this study. Both authors are employees of Namida Lab Inc.; Daily, A. serves as the Vice President of Product Development and Innovation and Ravishankar, P. is a Research and Development Scientist at Namida Lab Inc.

Appendix A

Table A1. List of proteins detected unique to tears and plasma samples.

Present in Tears		Present in Plasma	
14.3.3 gamma	Keratin 8/18	Adiponectin	IGF-BP3
14.3.3, Pan	LewisA	Apolipoprotein E3 (Apo E3)	IL-5
anti-Sm	LewisB	beta-Nerve Growth Factor (beta-NGF)	LH
Calponin	Metastasis-associated protein MTA1 (MAT1)	Bim (BOD)	Mucin 3 (MUC3)
CD25/IL-2 Receptor a	MLH1	CD71/Transferrin Receptor	NOS-i
CD45RO	Mucin 5AC	Collagen II	p27Kip1
CD84	Mucin-13 (MUC13)	CREB	p63 (p53 Family Member)
EMA/CA15-3/MUC-1	Myeloperoxidase	DFF40 (DNA Fragmentation Factor 40)/CAD	Parkin
Heat Shock Protein 70/hsp70	Myostatin	E2F-5	PARP (Poly ADP-Ribose Polymerase)
Heparan Sulfate Proteoglycan	Paxillin	Eotaxin-3	Plasma Cell Marker
HER3/erbB3	PR3 (Proteinase 3)	Factor VIII Related Antigen	Plasminogen
HPV 16	S100A4	Fibronectin	Retinol Binding Protein
IL-1RA	S100A6	HRP	SV40 Large T Antigen
Involucrin	SHP-1		
Keratin 10	TAG 72/CA72-4		
Keratin 16	Thymidine Phosphorylase		
Keratin 5/6/18	Transglutaminase II		

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Multiomic Approaches for Cancer Biomarker Discovery in Liquid Biopsies: Advances and Challenges

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ABSTRACT: Cancer is a complex and heterogeneous disease that poses a significant threat to global health. Early diagnosis and treatment are critical for improving patient outcomes, and the use of liquid biopsies has emerged as a promising approach for cancer detection and monitoring. Traditionally, cancer diagnosis has relied on invasive tissue biopsies, the collection of which can prove challenging for patients and the results of which may not always provide accurate results due to tumor heterogeneity. Liquid biopsies have gained increasing attention as they provide a non-invasive and accessible source of cancer biomarkers, which can be used to diagnose cancer, monitor treatment response, and detect relapse. The integration of -omics technologies, such as proteomics, genomics, and metabolomics, has further enhanced the capabilities of liquid biopsies by introducing precision oncology and enabling the tailoring of treatment for individual patients based on their unique tumor biology. In this review, we will discuss the challenges and advances in the field of cancer liquid biopsies and the integration of -omics technologies for different types of liquid biopsies, including blood, tear, urine, sweat, saliva, and cerebrospinal fluid.

KEYWORDS: Non-invasive biofluids, multiomics, cancer biomarkers

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Introduction

Despite advances in diagnosis and treatment, cancer remains a major public health threat, accounting for nearly 10 million deaths worldwide.[1,2] Timely and accurate detection and diagnosis are critical to improving patient outcomes and survival.[3] The current standard of care involves surgery, radiation, and systemic therapies such as chemotherapy and immunotherapy, or targeted therapy. Appropriate treatment regimens are selected based on the patient's cancer type and tumor staging, which in turn is determined by tumor profiling involving invasive resection/biopsies from the primary tumor.

Currently, the standard method of profiling tumors involves obtaining resected tumor samples through invasive surgeries. While necessary, obtaining both tumor quantity and quality can be a challenge. In addition, obtaining a holistic image of a tumor that has metastasized to multiple locations can be exceedingly difficult. Establishing a longitudinal tumor profile over the course of treatments would be highly useful to monitor the effectiveness of therapies and potential for recurrence, however subjecting patients to multiple invasive biopsies is less than ideal. The answer to these challenges could be found through the utilization of liquid biopsies which have emerged as a promising and more comprehensive approach in providing non-invasive biomarkers for diagnosis, prognosis, and treatment response monitoring of disease.[4-7]

Liquid biopsies (LB) are minimally invasive diagnostic tools that involve the sampling and analysis of biofluids for screening, diagnostic, and prognostic purposes. The main body of research and development has been conducted in blood.[8] However, tears, saliva, urine, cerebrospinal fluid, and other biofluids serve as sources as well. Circulating biomarkers, such as circulating tumor cells (CTCs), cell-free DNA (cfDNA), and exosomes are just a few of the analytes that can be extracted from liquid biopsies for clinical evaluation and hold great promise for revealing the molecular landscape and fate of primary tumors. These biomarkers provide information on the cancer's molecular and genetic makeup, allowing clinicians to monitor disease progression and response to treatment over the entire journey of the cancer patient. Incorporation of the isolation of these analytes from biofluids is a critical step in the liquid biopsy workflow.

CTCs are rare tumor cells shed into the bloodstream, providing insights into tumor metastasis and progression.[9,10] Various techniques have been developed for CTC isolation, including immunomagnetic separation and microfluidic devices. The CellSearch system, based on immunomagnetic capture using EpCAM antibodies, is a widely used method for CTC enrichment, and is the only FDA-approved device for CTC detection and enumeration[11] Additionally, microfluidic devices offer label-free and size-based CTC isolation, enhancing the sensitivity of detection.[12]

ctDNA is fragmented DNA released into circulation by apoptotic and necrotic cells.[13] Isolation methods for ctDNA involve plasma or serum extraction followed by DNA purification. Commercial kits, such as QIAamp Circulating Nucleic Acid Kit, provide efficient extraction, although it is a manual

platform.[14] To analyze ctDNA mutations, platforms such as digital droplet PCR (ddPCR) and high-throughput next-generation sequencing (NGS) are most commonly used.[15]

Exosomes are small membranous vesicles secreted by cells that contain a wide array of biomolecules, such as proteins, nucleic acids, and lipids.[16,17] Ultracentrifugation, size-exclusion chromatography, and immunoaffinity capture are common methods for exosome isolation. ExoQuick and exoEasy are 2 of the most frequently used and commercially available platforms.[18] Further characterization of exosomes involves techniques such as nanoparticle tracking analysis (NTA) and electron microscopy.[19,20]

The integration of -omics technologies, genomics, proteomics, transcriptomics, and metabolomics into liquid biopsies has enabled a more comprehensive analysis of the tumor's molecular and genetic profile, giving way to a more personalized approach to cancer treatment by identifying specific targets for therapy.[21-26] Genomics is the hallmark of biomarker research and aids researchers to study the gene expression and regulation providing a deeper understanding of the molecular mechanisms involved in a disease at a genome level.[27-29] Similarly, proteomics has long served as a powerful technology to identify new protein biomarkers. By measuring the abundance of proteins, proteomics sheds light on a level above gene expression and the integration of these 2 fields provides a comprehensive view of cellular responses from transcription to translation. Recent advances have shown the importance of metabolomics and transcriptomics in their relevance to understanding cancer progression and monitoring. Metabolomics provides a comprehensive understanding of the metabolic state of a biological system which can be used to identify disease markers.[30-32] Transcriptomics, on the other hand, serves as a powerful tool for studying mRNA expression and regulation, allowing researchers to identify the mRNAs that are active in a particular biological process, and how they are regulated.[33-36] Combined, these -omics technologies serve as tools to provide complementary information and help researchers gain a better understanding of the complex biological processes that underlie health and disease. By utilizing these techniques, we can identify crucial biomarkers that can improve the accuracy and specificity of liquid biopsy tests.

This article highlights the advances in the field of cancer liquid biopsies using -omics technologies that were published in the last decade. We specifically focused on blood plasma as it is widely adopted and is a minimally invasive biofluid with more focus on other non-traditional biofluids such as urine, tears, saliva, sweat, and cerebrospinal fluid due to their non-invasive sample collection. We further discuss the challenges that need to be addressed moving forward to create personalized diagnostic tests that can be used in clinical testing for applications such as early cancer detection, progression, and treatment efficacy monitoring.

Liquid Biopsies: Blood

Blood-based liquid biopsies are the most extensively studied approach for cancer detection and monitoring.[37] Peripheral blood has long served as a source of oncological markers, providing insight into primary and metastatic tumor biology given that its circulatory nature allows it to easily remain in contact with primary and metastatic tumors.[38-43] The accuracy of the information derived from liquid biopsies is dependent on many factors, including the quality of pre-analytical workflow involving sample collection, processing, and storage.

The post-collection processing to obtain the desired blood fraction is a critical component of the pre-analytical workflow affecting blood-based liquid biopsies. Serum and plasma are the commonly used blood fractions used for investigation of cancer biomarkers. Plasma is obtained by adding an anticoagulant, followed by centrifugation to remove cellular material from the collected specimen.[44] However, the large proteomic dynamic range as well as potential for hemolysis during the collection process can lead to high false-positive results.[45] Based on the Plasma Proteomic Project by the Human Proteome Organization (HUPO), raw plasma is recommended over serum due to the lower degree of ex vivo degradation.[46] The timing of sample collection can also impact liquid biopsy results, with fluctuating levels of circulating tumor cells and circulating tumor DNA throughout the day and treatment course.[47] Additionally, a recent study mapped the expression variation of plasma proteins to provide insights into potential biomarkers and treatments for various age-related functional disorders. They reported that the functional analysis of the proteome revealed a significant difference in the plasma proteomic profiles between young adults and middle-aged adults or elderly adults.[48] Thus, for the purpose of biomarker discovery, it is important to establish standardized protocols for sample collection, such as using the same time of day and same collection methods across a diverse patient population.

Table 1 summarizes the recent developments in the field of cancer biomarker discovery using blood plasma. With regard to blood-based liquid biopsies, previous studies have leveraged mass spectrometry-based proteomics to identify candidate biomarkers for the detection of pancreatic cancer. They identified several proteins that were differentially expressed in patients with pancreatic cancer compared to healthy controls, including S100A6, S100A8, and S100A9.[49] Another research group employed liquid chromatography-tandem mass spectrometry to investigate novel plasma biomarkers in dissecting tumor stages and post-surgical outcomes by using albumin and immunoglobulin G-depleted plasma samples from individuals with hepatocellular carcinoma and cholangiocarcinoma.[50] The results showed an upregulation of the following proteins in hepatocellular carcinoma and a down regulation in cholangiocarcinoma-afamin, alpha-2-HS-glycoprotein, apolipoprotein B-100, clusterin, hepatocyte growth factor-like protein, and

Table 1. Recent cancer biomarker discoveries in blood.

BIOMARKER TYPE	CANCER TYPE	ANALYTE	DETECTION METHOD	MAIN FINDING	REFERENCES
Protein	Breast cancer	sEVs (plasma)	Reverse phase protein array	Upregulated in BC: FAK, fibronectin (diagnostic)	Vinik *et al.*[56]
	Oral squamous carcinoma	ALIX (serum)	ELISA	Elevated levels of ALIX in patients with OSCC compared to healthy controls	Nakamichi *et al.*[57]
	Esophageal SCC	ANXA1, VIM, hK14, RSPO3 (serum)	Olink Multiplex Oncology II (PEA)	Upregulated in ESCC compared to normal: ANXA1, VIM Downregulated: hK14, RSPO3	Yang *et al.*[58]
DNA	Gastric cancer	cfDNA (plasma)	GeneQuant	Higher cfDNA levels compared to normal controls	Normando *et al.*[59]
	Hepatocellular carcinoma (HCC)	cfDNA	Ultra-deep sequencing (NGS)	*JAK1* mutations	Labgaa *et al.*[60]
miRNA	Breast cancer	ct-miRNA		Increases in ct-miR-148a-3p and ct-miR-37a-5p	Di Cosimo *et al.*[61]
	NSCLC	miRNA (serum)		Decreases in miR-382 levels	Luo *et al.*[62]
Metabolites	Pancreatic ductal adenocarcinoma (PDAC)	554 known metabolites	Orbitrap Elite or Q-Exactive high resolution MS	Metabolites positively associated with PDAC: glycylvaline, aspartylphenylalanine, pyroglutamylglycine, phenylalanylphenylalanine, phenylalanylleucine, and tryptophylglutamate and amino acids aspartate and glutamate. Metabolites inversely associated with PDAC: tyrosylglutamine and α-glutamyltyrosine, fibrinogen cleavage peptide DSGEGDFXAEGGGVR, and glutathione related amino acid cysteine-glutathione disulfide	Stolzenberg-Solomon *et al.*[63]

kininogen-1. Similarly, they also reported that the expression of Ig lambda chain V region 4A was upregulated in cholangiocarcinoma and had the opposite effect in hepatocellular carcinoma. Geary *et al.* used a sequential window acquisition of all theoretical fragment ion mass spectra (SWATH-MS) to identify proteomics signatures in late-stage cancer patients and reported a wellness score.[51] SWATH-MS technology has high precision and accuracy that allows for protein quantification without the need for labels and has been used to identify digitized proteomic signatures in other several cancer types including breast, colorectal, endometrial, and lung cancers.[52-55] Such studies provide insights into the clinical application of these biomarkers in the diagnosis and follow-up of cancers.

Another rapidly growing area within the vast field of genomics is the detection of specific mutations in ctDNA using digital droplet PCR or next-generation sequencing. For example, a study used targeted amplicon sequencing to detect EGFR mutations in ctDNA from non-small cell lung cancer patients. Results suggested that ctDNA analysis was concordant with tissue biopsy analysis in 85% of cases.[64] In addition to proteomics and genomics, metabolomics has been used to identify metabolic alterations that are associated with cancer. A study used untargeted metabolomics to identify metabolic changes in pre-diagnostic serum (up to 24 years prior to diagnosis) from pancreatic ductal adenocarcinoma patients. They identified over 30 metabolites that were significantly altered related to subclinical disease, including glycylvaline, tryptophylgutamate, and aspartate.[63]

The integration of transcriptomic analysis has enabled longitudinal monitoring of response to treatment as well as the prediction of treatment response to neoadjuvant therapies. A study by Di Cosimo *et al.* analyzed expression levels of ct-miRNA in plasma samples from HER2-positive breast cancer patients during treatment with trastuzumab and revealed increases in ct-miR-148a-3p and ct-miR-37a-5p as early as 2 weeks following treatment commencement for patients attaining pathological complete response (pCR).[61] Another study highlighted the diagnostic and prognostic capabilities of miRNA in NSCLC by isolating exosomes from NSCLC patient serum to analyze miR-382 expression. They found a significant drop in miR-382 levels in NSCLC cases compared to healthy controls, as well as improved

sensitivity and specificity when combining the miR-382 expression with carcinoembryonic antigen.[62] On the immunotherapy front, miRNA profiling is also enabling the development of prognostic scores that predict overall survival to immunotherapy in advanced-stage NSCLC. Moreover, Rajakumar *et al.* demonstrated how the 5-microRNA risk score they defined (miRisk) proved superior to tissue-based PDL1 scoring/staining currently used in the clinic.[65]

However, several challenges need to be addressed for the clinical implementation of these biomarkers, including the low abundance of CTCs and tumor-derived exosomes (TDEs) in the blood, the heterogeneity of ctDNA, and the lack of standardized collection and analysis methods.

Liquid Biopsies: Urine

Following blood, urine is one of the most commonly used biofluids for isolating markers for urine and nonurinary tract diseases. Urine-based liquid biopsies have gained significant attention in recent years as a non-invasive method for the diagnosis and monitoring of cancer, given its less invasive sampling nature.

Urine samples can be collected using various methods, including midstream voided urine, catheterization, and urine bags.[66] Midstream (clean-voided) urine is the most common method used for urine collection, which involves collecting the urine in a clean container after discarding the initial stream to avoid contamination.[67] Catheterization is a more invasive method that involves inserting a catheter into the bladder to collect urine.[68] Urine bags are also used for urine collection in infants and young children.[69] Regardless of the collection method used, it is essential to follow standard operating procedures to avoid contamination and ensure the accuracy and reliability of results. Urine samples should be collected in sterile containers and processed as soon as possible to avoid degradation of the biomarkers of interest.

While nucleic acids and metabolites can also be analyzed, proteins are the most common biomarkers analyzed in urine. Given that following ultrafiltration of plasma, less than 1% is excreted as urine, the protein concentration remaining in urine is significantly lower than that of plasma, containing up to 150 mg of protein in approximately 1.5 L of urine output a day.[70,71] The main protein found in urine is albumin, however, low abundant proteins can also be identified, such as prostate-specific antigen (PSA) for prostate cancer and urokinase plasminogen activator receptor (uPAR) for pancreatic cancer.[72,73] Potential biomarkers for ovarian, breast, and pancreatic cancer have been identified through proteomic analysis of urine as well. Stockley *et al.* found MCM5 (mini chromosome maintenance 5) levels to be significantly increased in urine samples of both ovarian and endometrial cancer patients compared to controls, with overall sensitivities of 61.5% and 87.8%, respectively.[74] Similarly, in breast cancer patients, investigators found 59 urinary proteins to be differentially expressed compared to healthy control subjects.[75] Tomiyama *et al.* isolated extracellular vesicles (EVs) in urine samples and following proteomic analysis, they found 6 EV proteins to be significantly upregulated in bladder cancer patients compared to healthy individuals.[76]

Nucleic acids, including DNA and RNA, are also analytes sourced from urine-based liquid biopsies. These markers provide valuable insight into mutations and alterations at the DNA and RNA levels, which can indicate the presence of cancer. For instance, urine-based liquid biopsies have been used to detect mutations in the telomerase reverse transcriptase (TERT) promoter region in bladder cancer patients. Specifically, Hosen *et al.* investigated pre-diagnostic urine samples from asymptomatic patients who subsequently developed bladder cancer and were able to identify TERT promoter mutations at a sensitivity of 46.67% and specificity of 100%.[77]

Amino acids, organic acids, and lipids are of additional interest to investigate in urine. A recent study identified over 20 different urinary volatile metabolites to be altered in bladder cancer patients. Specifically, they reported 11 metabolites to be down-regulated in bladder cancer patients, and 12 metabolites to be up-regulated, including 2-furaldehyde and 4-metholoctane.[78] Table 2 summarizes the recent developments in cancer biomarker discovery using urine as a non-invasive biofluid.

One of the main challenges with urine analysis is the variabilities observed in the inter- and intra-individual samples which is dictated by several factors such as sex, age, and lifestyle.[84-86] Additionally, urine samples have relatively low proteins but high salt concentrations thus necessitating the need for preprocessing samples to lower the salt concentrations via dialysis or precipitation.

Liquid Biopsies: Tears

Tears are an easily and continuously accessible biological fluid that holds great promise for the identification of biomarkers.[87] Tears are clear, extracellular fluids produced by the lacrimal gland and are composed of 3 distinct layers: the inner mucin layer, middle aqueous layer, and the outer lipid layer. They are ultrafiltrate of blood plasma and contain a diverse mixture of molecules, including proteins, microRNAs, lipids, mucins, and small molecule metabolites.[88] They serve as an affordable source and require minimal storage requirements as tear samples can be preserved for years without undergoing any degradation when frozen and stored at low temperatures (−20°C to −80°C).[89] Moreover, basal, open-eye tears have a high concentration of proteins, usually ranging from 6 to 11 mg/mL.[87,90] Additionally, tears are highly sensitive to changes in systemic and ocular conditions, which makes them a valuable diagnostic tool for monitoring and evaluating various health conditions.[87]

Microcapillary tubes, polyester/polyvinyl wicks, and Schirmer strips are some of the methods employed for tear collection.[91] Schirmer strips are more common in clinical settings as they are used in standard ophthalmologic testing for dry eye disease. Compared to microcapillary tubes, Schirmer strips are

Table 2. Recent developments in cancer biomarker discovery using urine as a biofluid source.

BIOMARKER TYPE	CANCER TYPE	ANALYTE	DETECTION METHOD	MAIN FINDING	REFERENCES
Protein	Lung cancer	FTL, MAPK1IP1L, FGB, RAB33B, RAB15	LC-MS/MS	The combination of these 5 urinary markers discriminated LC from control groups and other cancers	Zhang *et al.*[79]
	Ovarian, endometrial cancer	MCM5	ELISA	MCM5 levels higher in both ovarian and endometrial cancer: overall sensitivity 61.5% and 87.8%, respectively.	Stockley *et al.*[74]
Metabolite	Lung cancer	Cotinine	Gas chromatography-mass spectrometry method	Urinary cotinine levels in Lung Cancer cases significantly higher compared to controls. Urinary cotinine can be used for prediction of disease risk.	Thomas *et al.*[80]
ctDNA	Breast cancer	ctDNA	ddPCR	Significantly higher levels of baseline urinary ctDNA in early breast cancer patients compared to healthy controls. Additionally, significant declines in urinary ctDNA levels post treatment were correlated with disease outcome.	Zhang *et al.*[81]
miRNA	Cervical cancer	oncomiRs (miR-21, miR-199a, and miR-155-5p), and tumor suppressors (miR-34a, miR-145, and miR-218)	qRT-PCR	Upregulation of miR-21-5p, miR-199a-5p, and miR-155-5p, and downregulation of miR-145-5p, miR-34a-5p, and miR-218-5p in cervical pre-cancer and cancer patients compared to healthy controls.	Aftab *et al.*[82]
cfDNA	Lung cancer	*EGFR* mutations	ddPCR	Urine testing detected the same mutation as in tissue at 60% sensitivity. Combining plasma results with urine increased sensitivity to 88%.	Satapathy *et al.*[83]

easier to use, quicker, and more reliable.[92] One of the main difficulties in tear analysis is the low volume of tears (~3-10 µL) collected and researchers typically pool the samples to address this issue. Sample pooling offers the advantage of increased volumes for analysis while reducing the inter- and intra-subject variability, but this method does not provide individual variability. With the recent technological advancements of high-speed, high-resolution mass spectrometers, several new studies perform analysis on individually collected tear samples and have reported single samples with 500 to 1500 identified proteins involved in multiple signaling pathways.[93-96] Additionally, a recent study by our group conducted a comparative analysis of tears and blood plasma collected from the same individual and evaluated the samples by protein microarrays and small RNA sequencing. We reported that the Explorer protein microarray identified 71 proteins and RNAseq identified 250 microRNAs that were commonly expressed across both samples.[97]

As these biofluids offer a plethora of biomarkers, they have been studied in breast and colon cancers. Lebrecht *et al.* reported the differences between healthy and breast cancer patients by the tear proteome profiling utilizing mass spectrometry.[98,99] Further studies by the same group noted that the proteins were involved in immune-response pathways, such as C1Q1 and S100A8, and metabolic pathways, such as ALDH3.[100] Our group reported similar results with the upregulation of S100A8 and S100A9 proteins among 9 other proteins that could serve as potential biomarker candidates (sensitivity of 84.8% and specificity of 86.4% with an AUC of 0.902) for differentiating healthy and breast cancer patients.[101] Additionally, a subsequent study was conducted with a large single-tear analysis of over 800 samples on the calgranulin-A and -B proteins to validate these biomarkers along with clinical covariates.[102] We showed the successful early classification of samples using 2 proteins and minimal clinical covariates by proposing 3 models with sensitivity ranging between 52% and 90% and specificity ranging from 31% to 79%. Another recent study by Kaufmann *et al.* reported 18 upregulated and 62 downregulated proteins when they analyzed tears from healthy and colorectal cancer patients.[103]

Besides using proteins as biomarkers for early diagnosis and screening of cancer, microRNAs have also been reported as potential biomarkers. The study evaluated the expression of breast cancer-specific miR-21 and miR-200c and found them

Table 3. Recent cancer biomarker discovery studies involving tears as a non-invasive biofluid source.

BIOMARKER TYPE	CANCER TYPE	ANALYTE	DETECTION METHOD	MAIN FINDING	REFERENCES
Protein	Breast cancer	S100A8, S100A9	LC-MS/MS, ELISA	Upregulated S100A8 and S100A9 in Breast Cancer patients compared to controls	Daily *et al.*[101]
	Colon cancer	PLOD1, COL14A1, SPARCL1, NPMI,	LC-MS/MS	Upregulated in colorectal cancer compared to normal mucosa	Kaufmann *et al.*[103]
miRNAs	Breast cancer	miR-21, miR-200c	qRT-PCR	miR-21, miR-200c highly expressed in metastatic breast cancer patients compared to controls	Inubushi *et al.*[104]

to be significantly higher in tear exosomes collected from metastatic breast cancer patients compared to healthy volunteers.[104] This study also revealed that the tear exosomal markers, CD9 and CD63, were significantly expressed in higher quantities compared to serum exosomes, which could be due to the loss of exosomes during the serum sample preparation. Furthermore, the same group explored the possibility of developing an antibody-conjugated nanocavity-based platform which was fabricated using molecular imprinting approach. These platforms can potentially serve as a sensor for detecting cancers using tear exosomes.[105] Table 3 summarizes the recent advancements in using tears for cancer biomarker discovery.

Tears are advantageous due to their ease of collection, non-invasive nature, and require minimal sample preprocessing while providing crucial genetic and proteomic information. However, similar to the challenges discussed in the previous section, standardized sampling collection and handling methods along with large-scale single tear analysis are required to increase reproducibility, which can address the gaps in the field.

Liquid Biopsies: Saliva

Saliva holds great potential as a non-invasive source of new biomarkers for the diagnosis and prognosis of cancer. It is primarily produced by 3 major salivary glands—the parotid, submandibular, and sublingual glands, along with 300 to 400 minor salivary glands located within the oral cavity.[106] Saliva is a complex fluid that contains proteins, DNA, mRNA, microRNAs, metabolites, and microbiota, making it a promising source of biomarkers and offers several advantages over blood and tissue, including non-invasiveness, easy storage, cost-effectiveness, and dynamic availability for monitoring.[107,108] These biofluids can reflect the physiological state of the body and allow for the monitoring of oral and systemic health and diseases. Additionally, about a third of the proteins found in saliva are also detected in plasma.[109] Salivaomics is a widely used term that refers to a diverse range of technologies utilized to investigate the various types of molecules present in saliva. Several saliva-based diagnostic kits for detection of drugs, COVID, and pregnancy are commercially available and several others evaluating the cardiac risk and malaria are in development.[110]

Proteomic biomarkers have been evaluated in saliva for various cancer types including oral, breast, pancreatic, and lung. Chu *et al.* created an innovative and cost-effective method by integrating salivary immunoglobulin A purification and affinity mass spectrometry to identify biomarker candidates that differentiated healthy individuals from those who had oral cavity squamous cell carcinoma.[111] They reported that 7 salivary autoantibodies had the potential to serve as biomarker candidates and developed a 4-autoantibody diagnostic panel with high sensitivity and specificity consisting of vesicular integral-membrane protein VIP36, prostaglandin reductase 1, Ras-related protein Rab-13, and Cytochrome b-c1 complex subunit 2, mitochondrial. More recent studies have also showed an altered expression level in the salivary metabolites such as lactate, proline, glycine, citrulline, inositol trisphosphate, 2-oxoarginine, and glycerate-2-phosphate in oral cancer patients.[112,113]

A recent meta-analysis conducted by Koopaie *et al.* reported that unstimulated saliva-derived biomarkers for breast cancer, including c-erb-B2 and sialic acid, had sensitivity and specificity comparable to that of serum.[114] Another interesting finding in the study was that the levels of autoantibodies against MUC1, CA 15-3, and adiponectin in saliva demonstrated a stronger correlation with breast cancer than serum levels. With the advancement of microarray panels for genomic analysis, several miRNAs have been studied for their importance in salivary diagnostics. Xie *et al.* reported a model with reasonable sensitivity and specificity, which involved 2 salivary miRNAs—miR-3679-5p and miR-940, that can detect pancreatic cancer.[115] Another group quantified salivary miRNAs using qRT-PCR and reported that hsa-miR-21, hsa-miR-23a, hsa-miR-23b and miR-29c had significant expression in the saliva of pancreatic cancer patients.[116] Table 4 summarizes the fairly new developments in cancer biomarker discovery using saliva as a biofluid source.

Despite the advantages offered by saliva-based biomarkers, most of the published literature involves smaller sample sizes, which poses a limitation. Several studies use healthy controls for comparison with a disease with limited validation of

Table 4. Recent development in cancer biomarker discovery using saliva as a non-invasive biofluid source.

BIOMARKER TYPE	CANCER TYPE	ANALYTE	DETECTION METHOD	MAIN FINDING	REFERENCES
Proteins	Oral squamous cell carcinoma	IL-1β, IL-8 and LGALS3BP	ELISA	Increased levels of IL-1β and IL-8 in OSCC patients compared to controls in Indian population, especially in late stage OSCC. LGALS3BP was found to be significantly elevated in early stage OSCC.	Singh *et al.*[117]
	Oral cavity squamous cell carcinoma	Autoantibodies (AutoAbs)	LC-MS/MS	AutoAbs to CPPED1, GLUD1, LMAN2, PTGR1, RAB13, RAC1, UQCRC2, and p53 presented potential for early detection of OSCC. A panel consisting of 4 autoAbs to LMAN2, PTGR1, RAB13, and UQCRC2 was able to detect 76% of patients with early-stage OSCC.	Chu *et al.*[111]
Metabolites	Oral cavity squamous cell carcinoma	Glycine, proline, citrulline, and ornithine	NMR, LC-MS/MS, LC-Q-TOF	Proline, glutamine, and lactate presented consistently different levels across all 3 analytical platforms between OCC and controls. Glycine, proline, ornithine, and citrulline presented lower levels in stage I and II OCC compared to controls.	Lohavanichbutr *et al.*[112]
	Oral cavity squamous cell carcinoma, oral leukoplakia	A total of 37 metabolites upregulated while 11 compounds downregulated	LC-Q-TOF	Upregulation of 1-methylhistidine, inositol 1,3,4-triphosphate, glycerate-2-phosphate, 4-nitroquinoline-1-oxide, 2-oxoarginine, norcocaine nitroxide, sphinganine-1-phosphate, and pseudouridine was found in leukoplakia and OSCC. Downregulation of l-homocysteic acid, ubiquinone, neuraminic acid, and estradiol valerate was also reported.	Sridharan *et al.*[113]

inflammatory conditions that might be associated with the disease studied. Such complex interactions need to be included in the study design to develop accurate tests that can reduce the false positive rates. Additionally, saliva protein composition databases that support the investigation and comparison of these biofluids are currently available, which can serve to advance the field.[118]

Liquid Biopsies: Sweat

Sweat is a less commonly used biofluid for liquid biopsy compared to those previously discussed for cancer detection. It is secreted by the eccrine and apocrine glands and comprised mainly of water and electrolytes, metabolites, cytokines, cortisol, ammonia, serum components, and other organic compounds.[119,120] Sweat specimens can be collected by non-invasive methodologies that vary from simple and direct collection of sweat off the skin and into testing tubes, to more involved and specifically designed methodologies involving commercially available products, such as Pharmcheck® patches and the Macroduct® Sweat collection system. The Pharmcheck patches are non-occlusive and absorbent bandages composed of a cellulose layer that adheres to the skin and in which larger molecules are deposited. The patch is covered by an external polyurethane layer that protects the patch from external contaminants, allowing it to be worn during normal activities and for longer periods of time.[121] Other non-commercial techniques involve hydrogel micropatches, glass rollers used on the arms, or glass pipettes used on areas such as the forehead, chest, or back.[122,123] A wide variety of sweat collection methods have been used across several studies, depending on the analyte under investigation.

Several studies have shown the promising potential of sweat as a non-invasive liquid biopsy for cancer diagnosis. Calderón-Santiago *et al.* performed metabolic analysis of sweat from patients with lung cancer and found that trisaccharide phosphate best discriminated between lung cancer patients and controls (sensitivity 72.7%, specificity: 76.5%). Moreover, they developed a panel of 5 different metabolites, including suberic acid, tetrahexose, trihexose, nonanedioic acid and monoglyceride (22:2), that achieved a specificity of 80% and sensitivity of 79%.[124] More recently, Monedeiro *et al.* leveraged headspace-gas chromatography coupled to mass spectrometry (HS-GC-MS) to investigate the volatile fraction of sweat collected from healthy controls and individuals with different types of cancer including lung, prostate, gastric, kidney, head and neck, pancreas, colorectal, and lymphoma.[125] Sweat was isolated from PharmChek patches that had previously been applied to patients. Using a machine learning approach, sweat VOC profiles allowed the classification of healthy and diseased patients with 100% accuracy, when considering a panel consisting of 2-ethyl-1hexanol, octanal, and hexanal.[125] On the breast cancer front, Zadák *et al.* report on the diagnostic

Table 5. Recent development in cancer biomarker discovery using sweat as a biofluid source.

BIOMARKER TYPE	CANCER TYPE	ANALYTE	DETECTION METHOD	MAIN FINDING	REFERENCES
Metabolites	Lung cancer	Trihexose, tetrahexose, suberic acid, and nonanedioic acid	Liquid chromatography–tandem mass spectrometry in high-resolution mode (LC–QTOF MS/MS)	A panel formed by trihexose, tetrahexose, suberic acid, MG (22:2), and nonanedioic acid was able to discriminate lung cancer patients vs. controls with specificity of 80% and sensitivity of 79%.	Calderón-Santiago *et al.*[124]
	Lymphoma, lung, prostate, gastric, kidney, head and neck, pancreas, colorectal cancer.	2-ethyl-1-hexanol, hexanal, and octanal	Headspace-gas chromatography coupled to mass spectrometry (HS-GC-MS).	Random Forest analysis and a panel consisting of 2-ethyl-1-hexanol, octanal, and hexanal was used to classify cancer patients vs. healthy controls with 100% accuracy.	Monedeiro *et al.*[125]

Table 6. Recent development in cancer biomarker discovery using cerebrospinal fluid as a biofluid source.

BIOMARKER TYPE	CANCER TYPE	ANALYTE	DETECTION METHOD	MAIN FINDING	REFERENCES
ctDNA	GBM and Brain Metastases	ctDNA	Massive parallel sequencing using MSK-IMPACT platform	CSF ctDNA presented significantly higher sensitivity compared to plasma derived ctDNA for brain tumor genomic alterations.	De Mattos-Arruda *et al.*[134]
miRNA	Glioma	Exosomal miR-21	qRT-PCR	Higher levels of Exosomal-derived miR-21 in glioma patients than controls and no difference was observed in serum-derived exosomal miR-21; miR-21 levels associated with tumor grade and negatively correlated with the overall survival time median values	Shi *et al.*[136]
Metabolites	Glioblastoma (IDH-WT and IDH-mutant)	Metabolite	LC-MS Single reaction monitoring (SRM)	Higher levels of D-2-hydroxyglutarate in CSF of IDH-mutant tumors compared to controls and IDH-wildtype gliomas	Ballester *et al.*[135]

capabilities of metabolite markers isolated from apocrine sweat. Using liquid chromatography with tandem mass spectrometry (LC-MS/MS) and mathematical-statistical methods, they identified 20 metabolites that were able to discriminate between breast cancer patients and healthy controls with a sensitivity of 97% and specificity of 72%.[126] Table 5 summarizes the recent developments using sweat as a source for identifying cancer biomarkers.

Several challenges need to be addressed to make sweat a more reliable and clinically useful liquid biopsy for cancer diagnosis. One of the main challenges is the low concentration of cancer biomarkers in sweat, which makes their detection difficult.[127] Furthermore, sweat is easily influenced by environmental factors such as temperature, humidity, and physical activity, which may affect the accuracy and reproducibility of the results and can vary greatly from skin to skin. However, with the development of more sensitive and specific analytical methods and the standardization of sample collection and processing protocols, sweat may become a promising non-invasive liquid biopsy for cancer diagnosis.

Liquid Biopsies: Cerebrospinal Fluid

Cerebrospinal fluid (CSF) is a clear, colorless fluid that safeguards the central nervous system (CNS) by providing mechanical protection and immunological buffering, as well as enabling nutrient and waste transport within the CNS.[128,129] Secreted primarily by the choroid plexi in the brain's ventricles, CSF circulates within the subarachnoid space surrounding the brain and spinal cord, presenting unique access to CNS-specific biomarkers.[130] CSF is made up of approximately 99% water, with the remaining part consisting of a wide array of biomolecules, including proteins, glucose, ions, nucleic acids, metabolites, vitamins, and neurotransmitters, all of which can be indicative of underlying pathophysiological processes, including cancer.[131] The identification of cancer-related biomarkers in CSF offers the potential for monitoring treatment response and assessment of disease progression in CNS-involved malignancies.[132]

Collection of CSF most commonly involves a lumbar puncture, also referred to as a spinal tap. During this procedure, a puncture needle is carefully inserted into the subarachnoid space of the spinal column, allowing for the withdrawal of

Table 7. Recent development in cancer biomarker discovery using other unconventional biofluid sources.

BIOFLUID SOURCE	BIOMARKER TYPE	CANCER TYPE	ANALYTE	DETECTION METHOD	MAIN FINDING	REFERENCES
Cervical Fluids	Proteins	Endometrial cancer	EC-related proteins	Nano-UHPLC coupled to a tims-TOF pro MS	Significantly higher levels of SERPINH1, VIM, TAGLN, PPIA, CSE1L, and CTNNB1 in EC patients compared to non-EC patients, presenting AUC > 0.8.	Martinez-Garcia *et al.*[137]
Semen	miRNAs	Prostate cancer	Exosomal miRNAs	RT-qPCR	Upregulation of miR-142-5p, miR-128-3p, miR-142-3p, miR-223-3p, miR-212-5p, miR-182-3p, miR-130a-3p, miR-222-3p, miR-187-5p, miR-370-3p and down-regulation of miR-342-3p, miR-374b-5p, miR-217, miR-150-5p among PC samples.	Barceló *et al.*[138]
Breast milk	Proteins	Breast cancer	Lipoproteins, human chorionic gonadotropin (hCG) and Alpha1-antichymotrypsin family proteins	nanoLC-MS/MS	Upregulation in proteins from the human chorionic gonadotropin (hCG) family, lipoproteins family, and Alpha1-antichymotrypsin and Alpha1-antitrypsin family. Downregulation of proteins including mannose receptor, titin iso-form IC, human bile salt, xanthine dehydrogenase/ oxidase, members of the casein family, human heart L-lactate dehydro genase H Chain ternary, and fatty acid synthase.	Aslebagh *et al.*[139]

CSF.[133] The collected fluid is then subjected to a number of analyses to identify disease-specific markers. CSF analysis is particularly relevant in CNS-related malignancies such as glioblastoma, medulloblastoma, and leptomeningeal metastasis brought about by other cancers. Previous studies have reported on the reliability of CSF to act as a source of cell-free ctDNA, presenting levels of ctDNA higher than those reported in plasma.[134] In the case of brain cancer, De Mattos-Arruda *et al.* investigated the genetic landscape of brain tumors through the analysis of cfDNA isolated from CSF.[134] Their results underscored the importance of CSF-derived ctDNA in accurately reflecting genomic alterations present in CNS tumors. Furthermore, the work of Ballester *et al.* leveraged metabolomics to analyze 129 distinct metabolites in CSF samples from patients with a wide variety of CNS tumor types and revealed higher levels of D-2-hydroxyglutarate in the CSF of IDHG-mutant tumors compared to patients of other tumor types or controls (Table 6).[135]

Other Non-invasive Biofluids

A few studies have evaluated the potential of other biological fluids as a source for identifying cancer biomarkers. Martinez-Garcia *et al.* recently reported protein biomarkers in cervical fluids that can serve as a tool for early diagnosis of endometrial cancer.[137] Another group from Spain isolated exosomal vesicles from semen and reported significant changes in the miRNA signatures from these exosomes, distinguishing between prostate cancer patients and healthy controls.[138] Breast milk is another biofluid that has been reported to possess protein biomarkers that correlated with early detection of breast cancer.[139,140] Though these other biofluids have been utilized to identify potential cancer biomarkers, large scale applications are still limited (Table 7).

Challenges and Future Perspectives

Cancer liquid biopsies have emerged as a promising non-invasive diagnostic tool that can provide real-time information on cancer initiation, progression, and response to treatment. Integration of -omics technologies has provided insights into the molecular mechanisms underlying cancer and can guide personalized treatment decisions, furthering the field of precision oncology. Despite the significant advances in cancer liquid biopsies and -omics technologies, many challenges remain and need to be addressed before they can be widely implemented in routine clinical practice. One major challenge is the lack of standardization of liquid biopsy analysis. The pre-analytical, analytical, and post-analytical factors can affect the accuracy and reproducibility of liquid biopsy results. Therefore, standardized protocols and quality control measures need to be established to ensure the reliability and reproducibility of liquid biopsy analysis.

A well-designed -omics study is crucial to obtain meaningful and reliable results that can advance our understanding of biological processes and diseases. Omics technologies generate large amounts of data that require careful planning and execution to ensure that the results are meaningful and biologically relevant. Omics tools require the use of complex statistical tools; with more and more researchers being able to leverage the benefits of machine learning we are witnessing an increase in our ability to identify complex biomarker relationships with clinical factors at a tremendous rate. When designing a biomarker discovery study, researchers should prioritize the importance of maximizing the information gain and addressing the clinical needs through appropriate sample selection along with clear clinical endpoints.

Depending on the biological fluid of interest, the mode for sample collection differs and it is critical to understand the various factors that can introduce bias, such as sample collection protocols, potential contamination, and the importance of incorporating quality checks. An ideal study should involve randomization methods (simple, block, stratified, or adaptive randomizations) of the samples by allocating patients to groups that avoid any potential bias. Importance should be given to the determination of sample sizes to ensure robust results by utilizing statistical power analysis tools. Additionally, proper data sharing practices and ethical considerations need to be implemented, which promote the advancement of the field.

Another challenge is the low sensitivity of liquid biopsy analysis in some cancer types which results in high false negative rates leading to missing out on cancer diagnosis. For instance, liquid biopsy analysis has shown limited sensitivity in detecting early-stage prostate cancer which could be due to the low levels of ctDNA and the high heterogeneity of prostate tumors.[141] Similarly, tests with a low specificity result in high false positives which can lead to overscreening/diagnosis causing more burden on the healthcare system and patients. Therefore, it is crucial that researchers conduct larger cohort studies in order to develop tests with high accuracy.

The clinical implementation of -omics techniques is largely limited by the expensive equipment and technical expertise. We believe that ease of equipment use, standardized sample collection, handling, traceability, reproducibility, and automated analysis of data are required for these advanced and novel technologies to be implemented in daily clinical practice. The time and cost of developing a diagnostic test can be a major obstacle and pose a significant barrier. A collaborative effort between government agencies, academia, and the private sector is highly needed and such large multicenter trials would address these challenges. Lastly, a significant barrier to biomarker-based testing is clinician and insurer acceptance of non-traditional diagnostic tests. This can be addressed by proper education on the potential of biomarkers that provide key health information, evidenced by large clinical datasets, is required to convince physicians, clinicians, and general practitioners for the widespread adaptation of similar technologies in clinics.

As we look to the future of health and healthcare, the holy grail of laboratory medicine is often touted as personalized thresholds for disease. The work being done now to identify which markers hold potential lays the groundwork for this important perspective. To this end, each biofluid holds its own potential for breakthroughs and challenges. The most likely outcome will be that markers from each biofluid can be utilized to build a map personalized for each individual.

Conclusion

This review provides a summary of the recent advances in the field of cancer liquid biopsies that leveraged -omics technologies. We can certainly identify that the biological value of miRNAs, proteins, and other analytes obtained from liquid biopsies offers several advantages; however, they still require further validation. We believe that despite the challenges, continued research and development of cancer liquid biopsies and -omics technologies are anticipated to bring about a transformation in cancer diagnosis and tracking, consequently enhancing the well-being of patients.

Declarations

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Author contributions

Paola Monterroso Diaz: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Writing—original draft; Writing—review & editing. Ashton Leehans: Investigation; Writing—review & editing. Prashanth Ravishankar: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Supervision; Validation; Writing—original draft; Writing—review & editing. Anna Daily: Conceptualization; Methodology; Project administration; Resources; Supervision; Validation; Writing—review & editing.

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All data is provided in the manuscript.

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