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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.

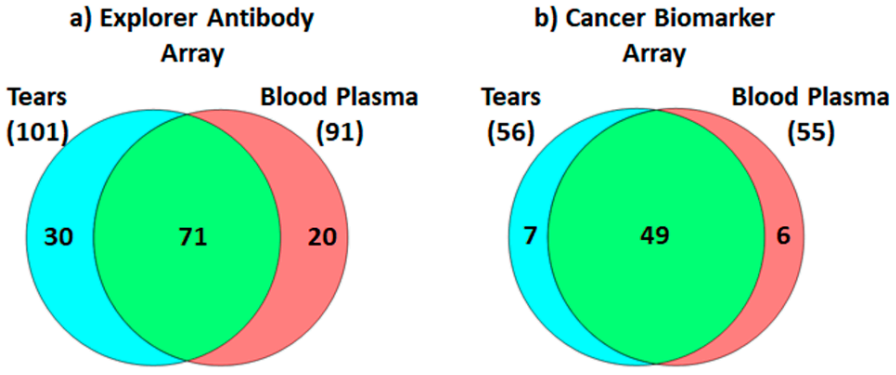

Figure 1. Microarray data analyzed for common protein expression in tears and plasma: (**a**) Explorer biomarker array, and (**b**) Cancer biomarker array.

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.

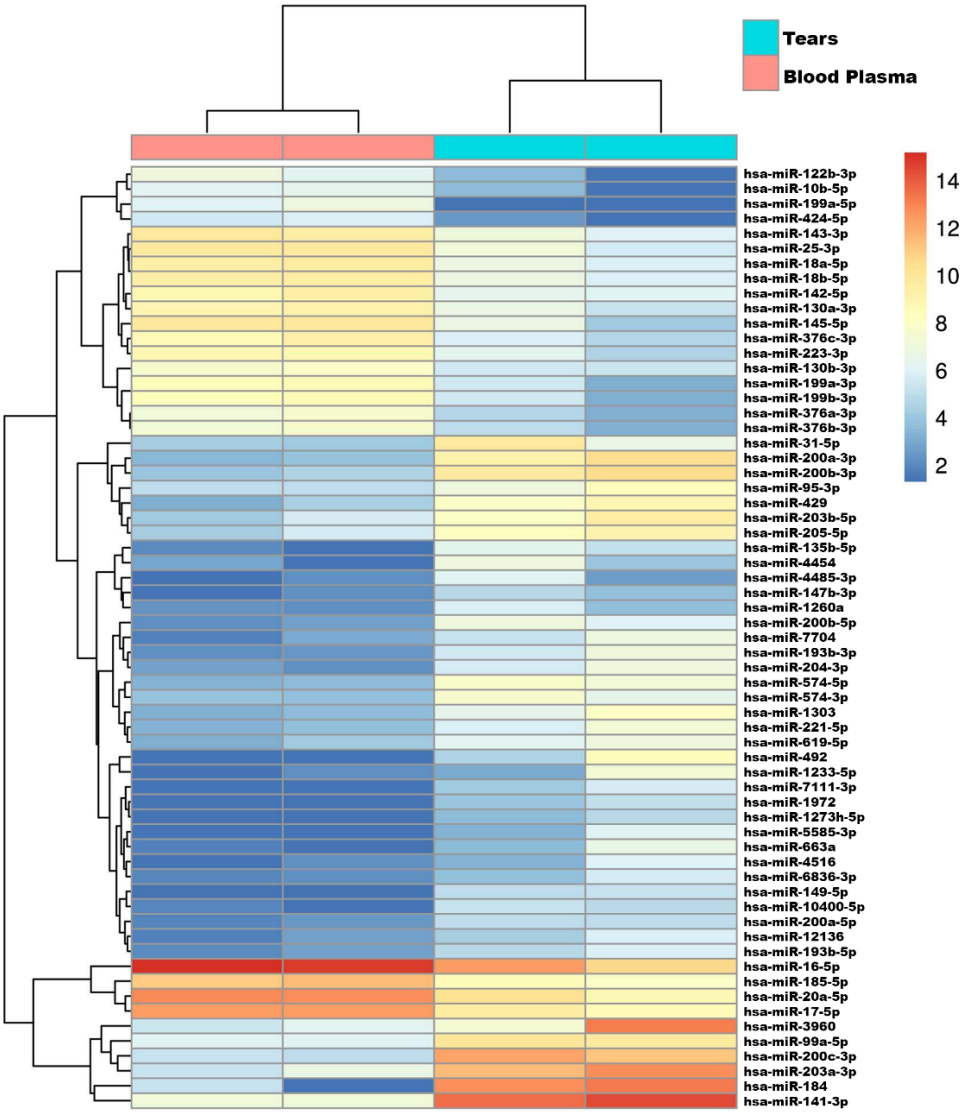

Figure 2. The heatmap represents the cluster analysis of miRNA log fold change in tears and plasma ($p < 0.01$).

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 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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