Tear Proteasome Analysis & its role in Early Screening & Staging of Disease

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Introduction

The advent of personalised medicine has made it inevitable to identify unique and novel attributes in patients for improved diagnosis and to monitor efficacy of ongoing treatment regimens. Instead of conventional, invasive sampling methods, researchers have now shifted focus to more achievable and convenient methods of non-invasive sampling such as ear-wax, saliva, aqueous and vitreous humor, tear film and urine among others. This has had a huge impact on improved sensitivity of patient profiling with advent of detection systems that allow quantification of even minute sample volumes. (1) (2) (3).

The Tear-Film: Components & Functions

Tears are secreted by the lacrimal glands, the goblet cells of the conjunctiva, the Meibomian glands located on the edge of the eyelid, along with permeating fluid from corneal and conjunctival tissue (4).

The tear-film consists of three main layers: a) the outermost lipid layer, secreted majorly by the Meibomian glands of the upper and lower eye-lids, with contributions coming from the Zeis and Moll glands and is composed of sterol esters, wax esters, triglycerides, free cholesterol, free fatty acids and polar lipids, b) the intermediate aqueous layer, packed with every possible macro and micro-nutrients, that covers a majority of the volume of the eye, secreted by the lacrimal glands and c) an innermost mucus layer (2) (4).

A range of nutrients and micro-molecules make tears a unique source of body-fluids, containing a range of mucins, glycoproteins, un-glycosylated proteins, peptides, lipids, electrolytes, immunoglobulins, enzymes, non-enzyme proteins, growth-factors and cell debris (4). It covers the outer surface of the eye, provides nutrition, hydration and lubrication to the cornea and underlying structures, helps in maintaining an optically smooth surface to help light enter the retina, acts as a superficial chemical barrier to protect it from pathogens as a part of the innate immune system, and prevents dehydration of the mucosal surface (5) (6).

Why Tear-film Proteomics?

Proteomics – the study of protein molecules responsible for some of the most crucial body functions, provides valuable insights to unveil the underlying causes behind pathogenesis, diagnosis and treatment of ocular (and related systemic) conditions. Ever since the announcement of the Human Genome Project, the correlation between genomic compositions of the human microbiome and human health has been of prime importance. Constant efforts being made to analyse the

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uniqueness of each individual and biomarker testing has emerged as a winner in such a scenario, owing to their precision, sensitivity and reproducibility (3).

There are several choices for non-conventional body fluids that are rich in protein content, making them suitable for quantitative proteomic analysis.

Earwax or 'cerumen' has not yet been studied widely in terms of quantitative proteomic biomarkers, despite having a high protein concentration. Saliva is another less conventional source being analysed for monitoring progression of immunodeficient conditions like HIV and graft vs. host disease and diabetes. A lack of clinical trials limits its application in these conditions. The collection of aqueous and vitreous humor requires invasive techniques (similar to blood) and cannot be ethically collected in healthy adults, if not suffering from ocular complications. Analytical challenges have also limited the utility of urine, broncho-alveolar lavage fluid and nipple-aspirates in proving sensitive and specific biomarker assays for identification of systemic conditions (3).

The protein concentration of tears is relatively high for analytic purposes, its composition varying in concentration depending upon the physiological condition of the individual (4). Close to 2000 tear proteins, 90 small molecule metabolites, few hundred individual lipid species across 7 lipid classes and different types of glycans have been reported in human tears, making it less complex than blood and plasma (with close to 8000 proteins) and, simultaneously, a rich source of biomarkers (6).

Though one may argue that the volume of samples collected from saliva or blood is much higher than tears (maximum volume being 5-10µl at a time), tears are the only body-fluid that are in the closest proximity of the (ocular) disease site which can be collected non-invasively, using Schrimer's Strips or fire-polished microcapillary tubes. This form of easy accessibility and convenience of sample-collection from patients makes it a favourite for both physicians and patients, alike (4) (6).

The importance of proteomic analysis was reflected upon during the launch of the Human Eye Proteome Project (also known as the EyeOme) where 4842 non-redundant proteins identified in the human eye. Adding to this, twenty-three recent papers on the human eye proteome provided an updated resource of 9782 non-redundant proteins in the human eye. Characterisation of such proteins in diseased and healthy individuals becomes necessary to identify the otherwise poorly understood pathogenesis of some of the major, debilitating conditions (7). Hence, tear-film proteomics grabs the attention of avid researchers in present times.

Mass-spectroscopic techniques like electrospray ionisation (ESI), isobaric tags for relative and absolute quantitation (iTRAQ), surface-enhanced laser desorption/ionization (SELDI) and matrix-assisted laser desorption/ionization

(MALDI) coupled with time of flight (TOF) technology are best recommended for analysing tear-fluid samples (3) (8).

Ophthalmologic Conditions & Tear Film Proteasome

Dry Eye Disease (DED), also known as **keratoconjunctivitis sicca (KCS)** is a condition of the lacrimal gland with mixed etiology. It has been linked to metabolic disorders and use of systemic medications such as antihistamines, β-blockers, antidiuretics, and antidepressants. This correlation between metabolic and systemic disorders led DED to be the first ocular condition to be studied using proteomics (1) (6).

Different forms of DED, ranging from mild-moderate DED, evaporative cases (including meibomian gland dysfunction (MGD)) to more severe hyposecretory forms of DED, e.g. Sjögren's syndrome (SS) and ocular graft versus host disease (GVHD) have been analysed using tear-proteomic studies. Possible alteration in the concentrations of inflammatory molecules (cytokines/chemokines) as well as growth factors, mucins, neuromediators and lipids have been linked to different forms of DED (1).

Studies using 2D electrophoresis and differential gel electrophoresis (DIGE) showed lysozyme proline-rich proteins 3 and 4 (LPRR3 / 4) to be significantly down-regulated in several types of DED, making them some of the crucial markers, related to the severity of the condition. A few other proteins that were shown to be down-regulated in studies included prolactin-inducible protein (PIP), lactotransferrin (LT), nasopharyngeal carcinoma-associated proline-rich protein 4 (PRP4), lysozyme (LYZ), cystatin-S (CST4) and α-1 antitrypsin (1) (8). It should however be kept in mind that the concentration of some protein groups like secretoglobin 2A2, lipocalin-1 (LCN-1) and serum albumin remains controversial in a few studies and hence, their stance in determining the severity of DED remains debatable (8).

Finer tests with iTRAQ quantitative proteomics, coupled with 2D nano-LC-nano-ESI-MS/MALDI-TOF showed increased expression of inflammatory proteins, calgranulin A /S100 A8 & calgranulin B / S100 A9, S100 A4 and S100 A11 (calgizzarin) in DED patient tears, making them another potent set of inflammatory biomarkers. Over expression of Anexin A1 (ANXA1), α-enolase, α-1 acid glycoprotein 1was also noticed in DED patients (1) (8).

Inflammatory cytokines/chemokines (such as IL-1, IL-6, TNF-α, metalloproteinase (MMP)-9, IL-17A, IL-1RA, IL-8/CXCL8, IL-22, INF-γ, MIG/CXCL9, IP-10/CXCL10, I-TAC/CXCL11, macrophage inflammatory protein-1 alpha (MIP-1α/CCL3), MIP-1β/CCL4 and RANTES/CCL5, among others) have been found to be significantly increased in tears from DED patients. MMP-9 measurement in tears, specifically, has already been proposed as a sensitive method for DED severity determination. A

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commercial device by the name InflammaDry®, RPD has already been developed in the US to detect therapeutically relevant levels of MMP-9 (1) (9).

Different phenotypes of DED, like aqueous-deficient dry eye, (DRYaq), lipid-deficient dry eye (DRYlip) and a combination of the two (DRYaqlip) could also be differentiated on the basis of tear-proteomic analysis. Research groups using MALDI / SELDI-TOF analysis showed lower amounts of LPRR4 in both DRYaq and DRYaqlip patients, compared to healthy controls. Moreover, mammaglobin B, lipophilin A, S100A8/calgranulin A and β-2 microglobulin (B2M) precursor were increased in these subgroups. On the other hand, DRYlip patients deviated only slightly in terms of tear proteins, but were radically different from both DRYaq and DRYaqlip (1).

LC-MS/MS spectral-counting quantitative proteomics were used to analyse and differentiate between DED and MGD patients. Compared to healthy controls, DED patients showed over-expression of proteins like C3, S100A6/A8 (cytokines involved in immune responses), CP (concerned with iron metabolism), APOD (involved in lipid interactions and metabolism), ORM2 (ER proteins concerned with lipid homeostasis), TXN (involved in immune response, cell proliferation, cell–cell signalling, and oxidation–reduction processes), IGHG1 (associated with immune response), PLA2G2A (involved in defence and inflammatory responses and lipid catabolic processes), SERPINA1 (concerned with acute-phase response, proteolysis and platelet activation), and SLPI (involved in defence response), in contrast to MGD patients who showed a sharp decline in the levels of TXN, IGHG1, PLA2G2A, SERPINA1, and SLPI. LPO, which is a marker for oxidative stress, was severely under-expressed in DED patients. On the contrary, MGD patients showed an over-expression of proteins like ANXA1, CLU, ORM1, and LPO (8).

A study evaluating proteomic differences between SS-DED and non-SS-DED patients, identified a total of 435 proteins using 2D nano-LC-MS/MS, and among them, 56, including defensin-1, clusterin, lactotransferrin (LT), and Cathepsin S (being the most prominent biomarker with a ratio as high as 41:1 compared to healthy controls and 2:1 as compared to non-specific DED patients) were found to be unique to SS-DED patients; this established the importance of Cathepsin S as a single most crucial protein biomarker symbolising SS-DED. On similar grounds, a severe down-regulation of mucin (MUC) 5AC (concerned with mucus hypersecretion) was found in the tears of SS-DED patients. Anti-SS-A and anti-SS-B, as well as anti α-fodrin antibodies have been established as accessory markers for SS-DED patients (1).

A collective node of biomarkers in the likes of MMP9, JUN, RELA, STAT3, TLR4, ESR1, GNAI3, ANXA1, and TIMP1 proteins hold the key when it comes to DED analysis. A common observation in all these studies is that when compared and contrasted on the basis of tear proteomes, not only can a specific band of up-

regulated and down-regulated proteins be used to distinguish between the different phenotypic variants of DED, tracing the respective functions could also shed light to their underlying disease physiology and pathogenesis. The sensitivity and specificity of such processes was well above 90% on an average (8).

Age-related Macular Disintegration (AMD) characterized by a progressive loss of central vision as a result of degeneration of the macula, is a leading cause of blindness among the elderly. Though proteomic analysis of the aqueous humor and blood samples make it possible to detect biomarkers, the invasive nature of sample collection affects its efficacy and timeliness, alongside the difficulty to reproduce similar results. The need to screen for early signs of the disease and obtain an optimal set of biomarkers from accessible body-fluids make tear-proteomic analysis a go-to strategy for ophthalmologists (2).

A study using 2D electrophoresis and MALDI-TOF analysis identified a total of 342 proteins, most of which were previously described in various proteomic studies concerning AMD. Out of these, 138 were for wet AMD, 125 were for dry AMD and 126 for control group patients. Some of the specific biomarkers for wet AMD included signal transducer and activator of transcription 3 (STAT3), Ras GTPase-activating protein 1 (RASA1), Fibroblast growth factor receptor 1 (FGFR1), concerned with angiogenesis, Cyclin-G1 (CCNG1), Cyclin-dependent kinase 4 inhibitor D (CDKN2D) among those related to apoptosis, inflammatory proteins like Myosin-13 (MYH13) and some related to cytoskeleton motility, like Myosin-13 (MYH13) (2).

Dry AMD-specific proteins included those related to oxidative stress, inflammation and proteolysis, like Guanine nucleotide-binding protein subunit alpha-11 (GNA11), Myc proto-oncogene protein (MYC), Pyruvate kinase PKM (PKM), Endothelin-converting enzyme 1 (ECE1), Ribosomal protein S6 kinase alpha-3 (RPS6KA3) and Ubiquitin/ISG15-conjugating enzyme E2 L6 (UBE2L6), among others.

Common protein panels which could be linked exclusively to AMD included Shootin-1, histatin-3, fidgetin-like protein 1, SRC kinase signaling inhibitor, Graves disease carrier protein, actin cytoplasmic 1, prolactin-inducible protein 1, and protein S100-A7A (2).

Inferring from the aforementioned data, it can be said that since inflammation, oxidative stress, impairment of autophagy and apoptosis are some of the most noted pathologies in AMD, the presence of these proteins only ascertains their roles in ADM etiology (2).

Glaucoma is another ocular complication that causes blindness, especially among the elderly. With its many forms, namely, primary open-angle glaucoma (POAG, the most common form), primary angle-closure glaucoma, secondary glaucoma and developmental (or congenital) glaucoma, untimely detection leads to ghastly

consequences that cause irreversible damage. This makes early screening indispensable (1).

One study assessed the reduction of brain-derived neurotrophic factor (BDNF) in the tear fluids of normal-tension glaucoma (NTG) patients when compared against healthy controls. Another group studied tear profiles of patients with medically controlled POAG and pseudoexfoliative (secondary) glaucoma, using SDS-PAGE and MALDI-TOF MS, to find that levels of inflammatory proteins like Igs, PIP, lysozyme C, LCN-1 and protein S100, differed between the two patient subtypes, indicating the different inflammatory pathways that led to such conditions (1).

Another study focused on significantly higher levels of tear fluid homocysteine (Hcy, a homologue of the amino acid cysteine) in POAG patients against healthy controls. Another interesting observation made in this study was the correlation between DED and POAG patients; POAG patients with DED had significantly higher tear fluid levels than POAG patients without DED, indicating that Hcy might serve as a marker for increased risk of both POAG and DED in glaucoma patients (1).

Diabetes

Diabetes mellitus is a multifaceted malady with the most debilitating and life-threatening consequences. Diabetic retinopathy (DR), a leading cause of blindness, has been previously scanned for biomarkers via analysis of the aqueous and the vitreous humor along with plasma. With the advent of tear proteomics, the focus now lies on early screening of diabetes in high-risk patients.

One study compared tear fluid samples, with the help of ESI-Q-TOF MS/MS, from patients with diabetes mellitus without the retinopathy symptoms, and those with non-proliferative diabetic retinopathy, against healthy volunteers. Three proteins, LCN-1 (also known as Tear Lipocalin, concerned with binding to macromolecules, which regulate tear viscosity, and release of lipids, and are involved in endonuclease inactivation of viral DNA (10)), HSP27 (another popular serum biomarker, concerned with the crucial function of enabling cells to adapt to exposure to oxidative stress including the up-regulation of glucose-6- phosphate dehydrogenase and glutathione peroxidase and by decrease intracellular iron levels and inhibits apoptotic pathways (11)) and B2M (concerned with cell surface expression of MHC class I and stability of the peptide binding groove, often correlated to diabetic nephropathy and Alzheimer's disease (12)) were down-regulated. These could be taken as early screening biomarkers for patients with a risk of diabetes (13).

Another study successfully linked significantly increased levels of the cytokine tumour necrosis factor alpha (TNF-α) in diabetic patients with the severity of DR(1).

Neurodegenerative Disorders

Alzheimer's Disease (AD) one of the most common forms of age-related dementia, with mostly unknown etiology, affecting more than 25 million people worldwide, with new cases being continuously on the rise, in both developed and developing countries (5).

Selected reaction monitoring (SRM)-based targeted proteomics analysis with triple quadrupole-containing mass spectrometers, which allows for highly specific identification, is a versatile tool for biomarker screening in this department. It can analyse multiple molecules simultaneously from the same sample, paving the way for a more cost-effective analysis.

One study comparing 37 tear samples from 14 patients with AD and 9 healthy controls were analysed using the SRM technique, showed the difference between tear profiles of both patient groups; those with AD had a significant reduction in the amounts of lipocalin-1 (LCN-1), lactotransferrin (LTF), extracellular glycoprotein lacritin (LACRT), lysozyme-C (LYZ-C), and prolactin inducible protein (PIP), while the level of dermcidin was significantly elevated (5).

Statistical analyses calculating the significance of tear-biomarkers indicating future predictive biomarkers for AD achieved the highest sensitivity was obtained by combination of lysozyme-C and extracellular glycoprotein lacritin; yielding 91% sensitivity. The most balanced performance was achieved when lipocalin-1, dermcidin, lysozyme-C and extracellular glycoprotein lacritin were combined (5).

Parkinson's Disease (PD) is another progressively debilitating motor-neurone disorder affecting millions, globally, each year. Tear-biomarker analyses are still in their early stages, but one crucial biomarker deciphered by researchers at the University of Southern California, who studied 55 people with PD against 27 healthy controls, is the α-synuclein, the protein molecule responsible for forming Lewy bodies, the pathological toxic clumps that causes nerve damage, levels of which are significantly higher in tears of PD patients (14). TNF-α was also found to be considerably elevated in PD tear samples.(1).

Multiple Sclerosis (MS) is another neuro-inflammatory disorder that causes demyelination of the CNS. IgGs have been the most prominent biomarkers in serum and CSF samples; additionally, this has also been verified by tear proteomic analysis. Some studies have also resulted in elevated IgA and IgM alongside IgG levels. Other prominent studies have highlighted the possible role of α-1-antichymotrypsin (ACT) protein which was found to be significantly elevated in three different study-groups comparing MS patients with healthy controls (1).

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Cancer

Tear-fluid based biomarker testing in the field of oncology has found prominence over the past decade.

Breast cancer biomarker studies using two-dimensional gel electrophoresis (2DGE) technique or SELDI-TOF coupled with ProteinChip® Arrays successfully traced increased amounts of lacryglobin (mammaglobin, a low molecular weight protein) in breast cancer samples with bone metastasis, which was absent in healthy controls, highlighting the potential of lacryglobin in human tears as adependable biomarker for breast cancer. Later on, lacryglobin was also found to be upregulated in patients with metastasis in lymph nodes that further emphasized on the previous findings (15) (16).

A common observation from tear-fluid analysis of major cancers was that **lacryglobin** was present primarily in patients with **colon** (100 %) or **prostate** cancer (100 %), followed by cancers of the **breast** (88 %), **lung** (83 %) and **ovary** (33 %). Around 60% of the control subjects showed lacryglobin presence, wherein 40% of these subjects had a family history of breast and prostate cancer (1).

A panel of 20 biomarkers have been identified from tear analysis, that might indicate breast cancer in early stages, with a specificity and sensitivity of approximately 70% (15) (17) (18). A few proteins studied in trials conducted later on have shown a marked up-regulation of at least two-fold (some being much higher) several proteins likeExtracellular sulfatase Sulf-1 (SULF1), Cystatin SA, (CST2); 5-AMP-activated protein kinase subunit gamma-3, (PRKAG3); Triosephosphate isomerase, (TPI1); Microtubule-associated tumor suppressor 1, (MTUS1); Transferrin receptor protein 1, (TFRC) and Putative lipocalin 1-like protein 1(LCN1L1)and downregulation of others like DNA damage-binding protein 1,(DDB1); Protein S100-A9, (S100A9); and GTP-binding protein Di-Ras2, (DIRAS2)(19). Few other proteins to be identified through this process include lipocalin-1 (LCN1), lactotransferrin (TRFL), lysozyme c (LYSC) and aldehyde dehydrogenase (AL3A1) (20).

Keeping the success of breast cancer proteins in mind, studies have been conducted tear protein analysis of **Prostate Cancer** (CaP) patients compared against normal controls using SELDI-TOF-MS, using reversed-phase surface protein chip (H50) that showed there were two peptides missing (7110 and 14213 Da) in the sequence of CaP; this hinted the potential of tear proteome analysis in screening of prostate cancer patients as well (15).

With technological upgradation of analytical tools, MALDI/SELDI-TOF has been replaced by faster techniques to assess more complex proteomes and yield faster results. MRM-MS is one such highly specific, sensitive technology to immunoassays for quantification of target proteins or peptides, offering multiplexing capabilities,

allowing for the simultaneous quantification of numerous proteins in parallel. A recent, large National Cancer Institute–sponsored inter-laboratory study advocated the efficacy of MRM approach that was successful in quantifying target proteins in a background of unfractionated human plasma with highly robust and reproducible results. MRM also has the potential to enhance the measure of post-translation modifications (PTMs), which are otherwise difficult for antibody-based systems to assess. Dermcidin, another novel tear-protein, was identified by MRM which provides the groundwork for understanding the PTM of tear proteins and their subsequent applications in the search for CaP biomarkers in tears (20).

Conclusion

Starting from the convenience of tear collection, to the easy availability of tears, the patients' willingness to comply with the physician owing to non-invasive means of sample collection – everything adds up to emphasize on the immense therapeutic potential tear-biomarker analysis holds in early and effective screening, diagnosis and monitoring of ocular and systemic diseases alike. Apart from the aforementioned conditions, avenues for tear-biomarker testing are opening for ocular allergies, thyroid-associated orbitopathy (TAO), trachoma, peripheral ulcerative keratitis, and even rare disorders like cystic fibrosis and aniridia, to name a few (1).

Coming to the advancing technologies in analytical techniques, it would be soon possible to assess multiple samples at the same time, making the system more productive. One can easily visualise the huge impact that artificial intelligence might have when coupled with such fast analyses. With advanced machine learning algorithms and neural networks on the rise, tear-biomarker analysis can soon become as effective as AI-based ophthalmologic scanning techniques, which have taken the medical fraternity by storm already.

It might not be a far-fetched reality when a blood-test or a tissue-biopsy is replaced by a simple tear-test at clinics that yields fast, accurate and detailed results on a patient's health. This would be the much-needed boost for personalized-medicine to flourish on a genomic level, with exponentially-improved patient outcomes.

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