Proteomic Profiling Of Tear Fluid For Potential Biomarker Discovery In Dry Eye Syndrome

THESIS

Submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

VENKATA SAIJYOTHI ALURU

2006PHXF431p

Under the supervision of

Dr. N. ANGAYARKANNI

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CERTIFICATE

This is to certify that the thesis entitled "***Proteomic Profiling of Tear fluid for Potential Biomarker Discovery in Dry Eye Syndrome*"** submitted by **Ms. Venkata Saijyothi Aluru** ID. No. 2006PHXF431 for award of Ph.D. Degree of the Institute embodies original work done by her under my supervision.

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Date:

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ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
APS	Ammonium per sulphate
AVP	Arginine vasopressin
ANA	Anti nuclear antibody
AEBSF	Aminoethylbenzylsulfonylfuride or pefabloc SC
ACN	Acetonitrile
BCA	Bicinchoninic acid;
BD FACS	Calibur four-color flow cytometry
BSA	Bovine serum albumin
BVA	Biological variance analysis
BLAST	Basic local Alignment sequence tool
BRM	Body's reaction modifiers
CTD	Connective tissue Disease
Ca2+	Calcium
Cl^-	Chloride
CRP	c-reactive protein
CCP	Cyclic citrullinated peptide
CHAPSO	3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CGRP	calcitonin gene related peptide
$CuSO_45H_2O$	Copper(II) sulfate pentahydrate
DES	Dry Eye Syndrome
DCF	Dichlorodihydo fluorescence diacetate (DCF) method
DCFDA	2', 7' DichlorodihydrofluoresceinDiacetate
DEPC	Diethylpyrocarbonate
DIGE	Differential gel electrophoresis
DM	Diabetes mellitus
DMF	Dimethyl formamide
DEWS	Dry eye workshop

DTT	Dithiotheritol
DsDNA	Double stranded deoxyribonuleotide
DIA	Differential gel analysis
DAB	diaminobenzidine
DMARDS	disease modifying anti-rheumatic drugs
DAVID tool	**D**ata base for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery
EGTA	ethylene glycol tetraacetic acid
EDTA	Ethyl diamine tetra acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme inked immune sorbent assay
ESI	Electron spray ionization
EDA	Extended data analysis
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
FA	Formic acid
GSH	Glutathione
GSK3	Glycogen synthase kinase 3
GSSG	Glutathione disulfide (oxidized)
GC-MS	Gas chromatography- mass spectrometry
H_2O	Water
HCO^-_3	Bicarbonate ion
HPLC	High pressure liquid chromatography
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HCHO	Formaldehyde
HRP	Horse raddish peroxidase
IEF	Iso electric focussing
IPG	Immobilized pH gradient
IgA	Immunoglobulin A
IHC	Immuohistochemistry
IL	Interleukin 8
ICAM	Intracellular adhesion molecule
IP10	Inducible protein 10

IAA	Ido acetic acid
KCS	Keratoconjuctivitissicca
KCl	Potassium chloride
kDa	kilo Dalton
KH_2PO_4	Potassium di hydrogen phospatae
LASIK	Laser Induced keratoplasty
LG	Lacrimal gland
LFU	Lacrimal Functional unit
LPRR4	Lacrimal proline rich 4 protein
MSH	melanocyte stimulating hormone
MCP	Monocyte chemotactic protein
MGD	Meibomian gland dysfunction
MIP	monokine induced protein
mg	Milligram
$MgCl_2$	Magnesium chloide
MALDI	matrix assisted laser desorption ionization
Mg^{2+}	Magnesium ion
MQ water	Milli-Q-water
MTT	3-(4,5-dimethylthiazol-2-yl) 2,5diphenyl tetrazoliumbromide
MMP	Matrix metallo proteases
MS	Mass spectrometry
MUC	Mucin
MOPS	3-(N-morpholino)propanesulfonic acid
Na^+	Sodium ions
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
N SS	Non Sjogren’
NPY	neuropeptide Y
NF-K	nuclear factor-kappa
NSAIDS	Non steroidal anti-inflammatory drugs
OSDI	Oular surface Disease Index
OSC	Ocular surface clinic

pSS	Primary Sjogren's syndrome
PAGE	Polyacrylamide Gel electrophoresis
PMSF	Phenyl methyl sulphonylfluride
PBS	Phosphate buffered saline
PRR	Proline rich protein
pI	Iso electric point
PI	Propidium iodide
PDB	Protein Data Bank
RA	Rheumatoid Arthritis
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT-PCR.	Reverse transcription polymerase chain reaction
RF	Rheumatoid factor
RT	Room temperature
SDS	Sodium dodecyl sulfate
SJS	Stevens-Johnson syndrome
SS	Sjogren's syndrome
SSA	Skin sensitizing antibody A(Ro)
SSB	Skin sensitizing antbody B(La)
SP	substance P
SH3	Src homology 3
SOD	Superoxide dismutase
SLE	Systemic lupus erythematosis
SSC	Side scatter
TBUT	Tear breakup time
TMH	Tear meniscus height
TBS	Tris buffered saline
TBST	Tris buffered saline with tween 20
TSP	Tear specific prealbumin
TCA	Trichloroacetic acid
TEMED	N,N,N',N' tetra methyl ethylene dia amine
TGF	Transforming growth factor
TNF	Tumor necrosis factor

TFI	Tear function Index
TBP	tributylphosphine
TFA	Tri fluoro acetic acid
TRIS-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
TOF	Time of flight
IT	Ion trap
USP6NL	Ubiquin specific protease 6 N-terminal like
VCAM	Vascular cell adhesion molecule
V	Volts
VIP	vaso active intestinal peptide
VEGF	Vascular endothelial growth factor
α	Alpha
β	Beta
µ	Micro
µl	Microlitre
2DE	Two dimensional electrophoresis

ABSTRACT

Tear fluid comprises of 3 layers namely lipid, aqueous and mucous layers, is a complex biological mixture, contain electrolytes, proteins, lipids, mucins, some small organic molecules and metabolites. The functions of the tear film include lubrication, protection from disease and nutrition of the cornea. It is secreted from lacrimal gland, meibomian gland and also from other accessory glands. Normal tearscontain 6 to 10 mg/mL of total proteins. More than 100different tear proteins detected in tear. Major tearproteins include lysozyme, lactoferrin, secretory immunoglobulin A (sIgA), serum albumin, lipocalin and lipophilin. Tear proteins play a major role for the maintenance of ocular surface integrity. Dry eye syndrome (DES) isamultifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tearfilm instability withpotential damage to the ocular surface. Oxidative stress, inflammation plays an important role in the disease. The prevalence of DES is increasing and is ranging from 5.5 to 37.5 % word wide, and more in elderly persons. DES is classified as either aqueous tear deficiency or evaporative tear loss. Aqueous tear deficiency is due to aging, hormonal changes, due to certain drugs, and also with systemic disease Sjogren's syndrome which is either primary (pSS) or secondary to Rheumatoid Arthritis (RA). Tear protein alterations reported in non Sjogren's and in Sjogren's syndrome. Tear specific protein identification helps to understand the disease mechanism, to develop diagnostics and therapeutics. Tear proteomics plays an important role for the identification of proteins in DES. Therefore, the proposed study is to look for the differential expression of tear proteins in DES associated with non Sjogren's syndrome, primary and secondary Sjogren's to RA and identification of the same in DES.

The study was approved by the institutional ethical board. A written informed consent was obtained from all the cases recruited in the study. All the DES and RA cases were recruited according to the inclusion criteria. For the diagnosis of RA, blood investigations ESR, Rheuamtoid factor (RF), C-reactive protein (CRP), Anti-

SSA, anti-SSB, anti-dsDNA and anti-citrulinated peptide (CCP) were done using ELISA. As prospective case- control study,age and sex matched control and cased of 73 controls (mean age: 43 ± 12 y, 30 M, 43 F) and 129 DES (mean age: 45 ± 3 y, 51 M, 78 F) were recruited to look for the differentially expressed proteins. Of these, 2D gel electrophoresis was done in 39 healthy controls (mean age: 43 ± 12, 12M, 27F), 26 cases of Non sjogrens (NS) (mean age: 40 ± 17 y, 10M,16F), 15 cases of primary sjogrens (PSS) (mean age: 48 ± 11y, 5M, 10F), 26 cases of dry eye secondary to Rheumatoid arthritis (RA), (mean age: 48 ± 10 y, 6 M, 20 F). DIGE was done in 18 controls (mean age: 43 ± 12 y, 8M, 10F), 11 cases of Non sjogrens (NS) (mean age: 42 ± 16 y, 7M, 4F). 8 cases of primary Sjogrens (PSS) (mean age: 46 ± 11 y, 4M, 4F), 16 cases of dry eye secondary to Rheumatoid arthritis (RA), (mean age: 49 ± 8 y, 2 M, 14 F). for the validation of LPRR4 by ELISA, tear samples were prospectively collected from dry eye cases (mean age: 49 ± 16 y, n = 27) associated with Non sjogren's (mean age: 45 ± 20 y, n = 9), primary Sjogren's (mean age: 49 ± 20 y, n=7) and Rheumatoid Arthritis (mean age: 52 ± 9 y, n = 11) with age matched controls (mean age: 43 ± 10 y, n = 16, 10 M, 6 F). For validation Rn-tre was in tear specimen of 27 controls (mean age 44 ± 16 years) and in 34 DES samples (mean age 40 ± 15) that includes 17 cases of DES-pSS (mean age: 39 ± 10 years), 12 DES-RA(mean age: 45 ± 9 years) and 5 cases of DES-Non SS(mean age: 40 ± 10 years).For cytokines, 27 controls (mean age: 45 ± 11 y, 13 M, 14 F) and 32 DES (mean age: 42 ± 11 y, 28 F, 14 M) were recruited to look for the cytokine changes. Among the 32 DES cases, 22 were DES with RA, 3 were DES with primary Sjogren's (pSS), 5 were DES with non Sjogren's (NSS) and 2 were MGD without any DES. Tear samples were collected using Schirmer method from all the control and cases. Tear protein was extracted using 8M Urea buffer. Protein estimation was done using Bradford method. Tear protein was subjected to two dimensional gel electrophoresis (2DE) 30 µg of tear protein was separated on 17 cm IPG pH 3-10, and 2^{nd} dimension separation was done using 13 % SDS-PAGE. 2D-differential gel electrophoresis was done with the tear samples with the pooled tear samples to look for the similar changes observed in 2DE. Validation of protein was

done using ELISA. Structural and function relation of protein was done using *insilico* approach.

RA diagnosis parameters ESR, RF and anti-CCP increased in DES-RA with increase in DES grade as well as with increase in RA severity and also in severe DES-pSS.No change in parameters CRP and ANA was observes in all the DES cases. Anti-SSA and anti-SSB were increased in severe DES cases of DES-RA and DES-pSS. From 2DE, differentially expressed spots were identified in all types of DES. Total 63 differential spots were observed. From this the identified spots are lacrimal proline rich 4 protein (spot 1-8, 15, 16), lacritin precursor (spot 18, 19, 21 and 22) immunoglobulin J, dermicidin (spot 54, 55), were down regulated in all types of DES cases. Lipocalin (spot 35, 37), lipocalin precursor (spot 81), protein S100 A8 (spot 38) increased in DES-non SS but reduced in DES-pSS and DES-RA. S100 A10 (spot A), Methylenetetrahydrofolate containing domain protein (spot C) increased in DES all types, more in DES-RA condition. When compared DES- NSS and SJS, SJS showed differential expression of proteins more severe than other DES-Non SS. The differential expression of all the above proteins were also validated by 2D-DIGE. DES- RA showed 8 differentially expressed proteins, 6 down regulated namely Ribonuclease p protein subunit 20 and Protocadherin (spot 24), Heterogeneous nuclear ribonucleoprotein Q isoform 6 (spot 34), SHC transforming 1 isoform (spot 132), cystatin SN precursor (spot 21), lactotransferrin precursor (Spot and 2 up regulated proteins Ecto-ADP ribosyltranferase-5-precursor and Rho-related GTP-binding protein RhoQ precursor (spot 270), and cytoskeletal protein 10 (spot 258). One of the down regulated protein was transcription cofactor HES-6/ keratin cytoskeletal protein 13 in DES-pSS. The *insol* tryptic digestion of tear of control showed 37 proteins. 16 of the proteins were also seen in all types of DES. 19 of the proteins in DES-RA, 16 proteins in DES-NSS and 9 from proteins in DES-pSS were characteristically in the respective groups.2 proteins present in all types of DES. The down regulation of these proteins were seen in > 80 %. One of the down regulated protein in all types of DES was lacrimal proline rich4 protein (LRR4). This protein was validated in DES cases using ELISA. This protein levels were significantly reduced in all types of DES. LPRR4 was also

correlated with the disease severity, and also with the DES clinical parameters Schirmer value and TBUT. The structure of this protein was not available, and it was predicted using *insilico* approach, the plausible model was predicted. The interacting proteins of the LPRR4 was seen using STRING analysis. One of the up regulated protein in DES-pSS by *insol* analysis was similar to Ubiquitin specific protease 6 N-terminal like protein (USP6 NL/RN-tre). This protein was also validated in DES cases using ELISA, showed up regulation of USP6NL in DES-pSS than other DES condition. This protein functions when it combines with EPS8 with SH3 domain. The structure of both USP6NL and EPS8 was modeled and the possible interacting proteins at the binding site was predicted. Further functional analysis of LPRR4 and USP6 NL has to be done to understand its role in the disease mechanism. The validation of the DES-RA, DES-pSS and DES-NSS specific proteins need to be done.

CHAPTER 1: REVIEW OF LITERATURE

1.1 INTRODUCTION

1.1.1. **Eye:** To visualize the objects is dependent on the coordinated actions of several structures in and around the eyeball. Light rays are reflected from the object to the **cornea** which is**,** focused by the **lens**, on the **retina**. At the retina, the light rays are converted to electrical impulses which are then transmitted through the **optic nerve**, to the brain, in which the image is translated and perceived in an upright position. Ocular surface, tear fluid, iris, sclera, choroid, aqueous and vitreous humor supports the eye that functions as an optical system (Balasubramanian, Pye et al. 2012).

Current thesis discusses mainly the ocular surface components of the anterior segment such as cornea, conjunctiva and the physiology, properties, molecular changes of tear film in normal and disease condition.

The **ocular surface** is comprised of epithelia of cornea, conjunctiva, tear film, the lacrimal glands, accessory lacrimal glands, meibomian glands and the eye lids which together works as an integrated functional unit.

1.1.2. Cornea: Cornea is the transparent convex tissue in the front of the eye and is composed of several discrete layers namely corneal epithelium, stoma, and endothelium, which is a protective physical barrier and together with sclera, shields the inner eye from the external environment. It is able to protect itself from various types of damage that includes physical trauma and biochemical injury, to infections by pathogenic organisms, to the deleterious effects caused by long term exposure to light. The cornea also safeguards other under laying ocular structures from similar kind of damage. Cornea is the main refractive element of the visual system, directs the incoming light onto the crystalline lens. The cornea has unmyelinated nerve endings sensitive to touch, temperature and chemicals. The cornea does not have blood vessels and therefore, it receives nutrients via diffusion from the tear fluid at

the outside and from the aqueous humour at the inner side and from neurotrophins supplied by nerve fibers that innervate it. It gets oxygen directly through the air.

1.1.3. Conjunctiva: The conjunctiva lines the inner part of the eye lids, covers the sclera and is composed of non keratinized stratified columnar epithelium. The main function of conjunctiva is to lubricate the surface by producing mucus from goblet cells. It helps in the immune surveillance by preventing the microbe's entry into the eye. Conjunctiva is divided into 3 main parts namely Palpebral or tarsal conjunctiva which lines the eye lids, bulbar or ocular conjunctiva that covers the eye ball over the sclera and Fornix conjunctiva, the junction between palpebral and bulbar conjunctiva. Both cornea and the conjunctiva are covered by a thin tear film produced from the lacrimal and the accessory glands of the eye.

1.1.4 Tear film: Tear is a thin film that covers the ocular surface. The pre corneal and conjunctival tear film forms an interface between the air and ocular tissues.

Production and drainage of Tear: The lacrimal glands secrete lacrimal fluid as a result of neuronal stimuli, which flows through the main excretory ducts into the space between the eyeball and lids and during blinking of eyes, the lacrimal fluid is spread across the surface of the eye. Lacrimal fluid accumulates in the lacrimal lake, and is drawn into the puncta by capillary action, then flows through the lacrimal canaliculi to the inner corner of the eyelids entering the lacrimal sac, then to the naso lacrimal duct, and then finally into the nasal cavity. In strong emotional condition excess of tears form and thus cause the nose to run.

The tear film is a complex mixture of proteins, lipids, mucus, salts, enzymes and other metabolites produced from multiple sources such as the lacrimal gland, meibomian gland, goblet cells and accessory lacrimal glands of the ocular surface. Glands of Krause located in the lamina propria of the conjunctival fornices (superior and inferior)., Moll glands at base of the lashes anterior to the Meibomian glands and Wolfring present located above the superior border of the upper lid tarsus contributes the secretion of components into the lacrimal fluid (Lamberts, DW., Smolin, G., 1994).

Tear fluid has multiple functions that includes *

(i) Lubrication of the eyelids

(ii) Formation of a smooth and even layer over the cornea and conjunctiva for protection

(iii) Provides antibacterial systems for the ocular surface and nutrients for the epithelium

(iv) Severs as a vehicle for the entry of Polymorphonuclear leukocytes in case of injury and washes away the toxic irritants (Lamberts, DW., Smolin, G. 1994., Lemp and Blackman 1981)

Tear film has a trilaminar structure consisting of (figure 1.1)

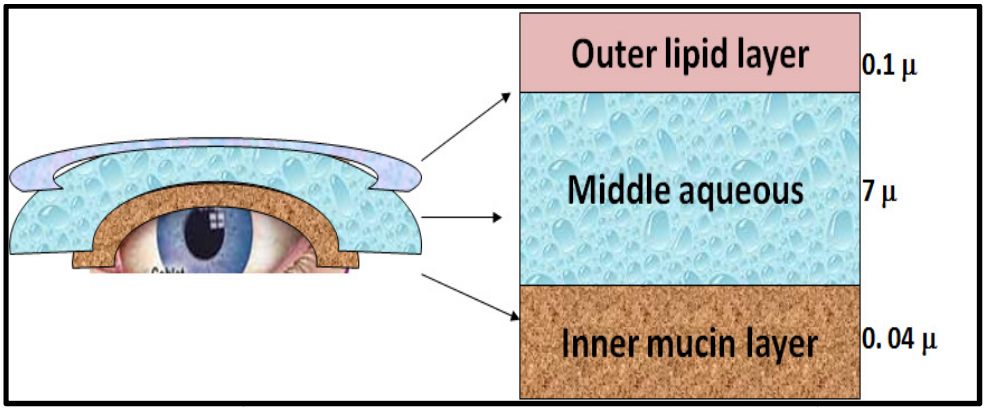
1. A thin anterior lipid layer (0.1 μ) originates from meibomian glands which contains both polar and nonpolar lipids

2. An intermediate aqueous layer (7μ) originates from lacrimal glands which contains water soluble substances include electrolytes, proteins, retinol, immunoglobulin's and enzymes

3. An innermost mucous layer (0.02 – 0.04μ) – secreted by conjunctival goblet cells and lacrimal gland acinar cells components loosely bound to glycocalyx of the corneal and conjunctival epithelial cells.

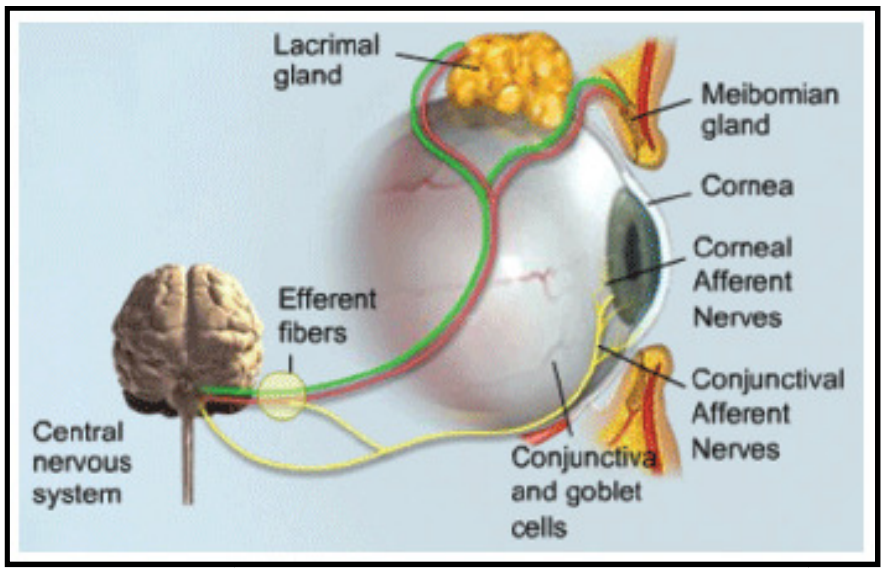
In mammals, the tear mucus layer is produced by goblet cells in the cornea and conjunctiva, while the aqueous layer is produced by the lacrimal glands and other accessory glands, and the surface lipid layer is produced by the meibomian glands.

Figure 1.1: The layers of Tear film



1.2. Anatomy of Lacrimal gland: The lacrimal gland (LG) is paired almond-shaped glands, one for each eye, that secrete the aqueous layer of the tear film. They are situated in the upper, outer portion of each orbit, in the lacrimal fossa of the orbit formed by the frontal bone (Walcott 1998). Lacrimal functional unit (LFU) is an integrated system including lacrimal gland, ocular surface, lids, sensory and motor nerves, to carry out its function as seen in figure 1.2.

Figure 1.2: Human lacrimal function Unit



Source: Henry D, Fery ***Am J Manag Care. 2008;14 :S79-S87***

The lacrimal glands consist of a tubular secretory epithelium organized into lobes that drain into ducts. These ducts open into larger ducts that finally drain onto the ocular surface. It is a multilobular tissue composed of acinar, ductal, and myoepithelial cells (Figure 1.3)

1. **Acinar cells:** The acinar cells account for 80% of the cells present in the lacrimal gland and are the site for synthesis, storage, and secretion of proteins. Several of these proteins like lysozyme, lactoferrin have antibacterial or growth factors such as epidermal growth factor, transforming growth factor and are crucial to the health of the ocular surface.

2. **Ductal cells:** These cells modify the primary fluid secreted by the acinar cells and to secrete water and electrolytes.

3. **Myoepithelial cells** – These cells contain multiple processes, which surround the basal area of the acinar and ductal cells. They contain a smooth muscle actin, that helps to contract and force the fluid out of the ducts and onto the ocular surface similar to salivary and mammary glands.

   Other cell types present in the lacrimal gland include plasma cells, B and T cells, dendritic cells, macrophages, bone marrow-derived monocytes, and mast cells. Immunoglobulin A (IgA) - positive plasma cells are the majority of the mononuclear cells in the lacrimal gland (Wieczorek, Jakobiec et al. 1988; Dua, Gomes et al. 1994). These cells synthesise and secrete IgA, which then transported into acinar and ductal cells and secreted by these epithelial cells as secretory IgA.

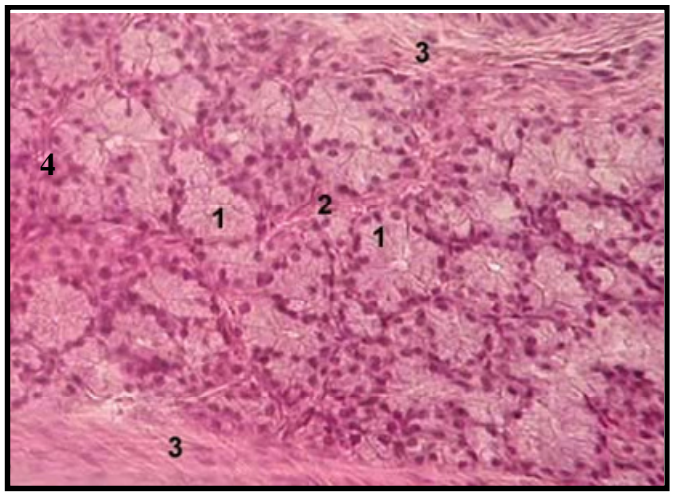
1.3. Regulation of lacrimal gland secretion (Zoukhri 2006)

1.3.1. Neural control

The lacrimal gland is innervated by the parasympathetic and sympathetic nervous system (Dartt 2004, Botelho, Hisada et al. 1966; Sibony, Walcott et al. 1988). Nerves are located in close proximity with acinar, ductal, and myoepithelial cells

and as well as blood vessels, and they control a wide variety of lacrimal gland functions (Botelho, Hisada et al. 1966; Sibony, Walcott et al. 1988). Stimulation of lacrimal gland secretion occurs through a neural reflex arc originating from the ocular surface (Botelho 1964). Stimuli to the ocular surface activate afferent sensory nerves in the cornea and conjunctiva that in turn activate efferent parasympathetic and sympathetic nerves in the lacrimal gland to stimulate secretion.

Figure 1.3.: Cross section of lacrimal gland



Source: www.histol.chuvashia.com

Figure 1.3: Cross section of lacrimal gland stained by haematoxylin and eosin. 1. Secretory unit, 2. Interlobular connective tissue 3, Capsule, 4. Intralobular excretory duct.

Neurotransmitters and neuropeptides released by the lacrimal gland nerves include acetylcholine, vaso active intestinal peptide (VIP), norepinephrine, neuropeptide Y (NPY), substance P (SP), and calcitonin gene related peptide (CGRP). These neuro mediators interacts with specific receptors present on the surface of lacrimal gland cells to initiate a specific response ((Hodges and Dartt 2003). Acetylcholine and norepinephrine are the most potent stimuli of lacrimal gland protein, water and electrolytes secretion (Hodges and Dartt 2003; Dartt 2004). Acetylcholine binds to cholinergic M3 muscarinic receptors and norepinephrine binds to α and β adrenergic receptors (Mauduit, Jammes et al. 1993; Hodges and Dartt 2003).

1.3.2. Hormonal control: Hormones from the hypothalamic–pituitary–gonadal axis has a profound impact on lacrimal gland structure and function. Adrenocorticotropic hormone (ACTH), a-melanocyte stimulating hormone (a-MSH), prolactin, androgens, estrogens, and progestins influence lacrimal gland functions (Leiba, Garty et al. 1990; Mircheff, Warren et al. 1992; Sullivan, Block et al. 1996). In addition, glucocorticoids, retinoic acid, insulin, and glucagon affect various aspects of the lacrimal gland (Petersen 1976; Ubels, Dennis et al. 1994; Rocha, de et al. 2000). Arginine vasopressin (AVP), a peptide produced in the posterior pituitary has physiologic role in fluid homeostasis, also present in the lacrimal gland acinar and ductal cells (Djeridane 1994). Androgens are potent hormones that stimulate the secretion of secretory IgA, an important component of the mucosal immune system of the eye (Sullivan, Block et al. 1996). Androgens accounts for many of the gender-related differences seen in the lacrimal gland (Azzarolo, Mircheff et al. 1997; Sullivan, Wickham et al. 1999).

1.4. Protein and water secretion (Walcott 1998) from lacrimal gland: A number of proteins are synthesized and secreted by the lacrimal gland acinar cells. The secretion of these proteins are stimulated by the neurotransmitters and neuropeptides found in the neurons that innervate the gland(Dartt 2004). The acinar cells have receptors for acetylcholine (muscarinic M3), VIP and norepinephrine and also receptors for peptides of the proenkephalin family as well as other peptides such as neuropeptide Y, adrenocorticotropic hormone (ACTH), and α-melanocyte-stimulating hormone (α-MSH). The acinar cells are extensively coupled by gap junctions. Second messengers produced by activation of those receptors, such as Ca^{2+} and inositol trisphosphate (IP3), diffuse from stimulated cells to adjacent non stimulated ones, causing them to become activated, which further leads to protein secretion.

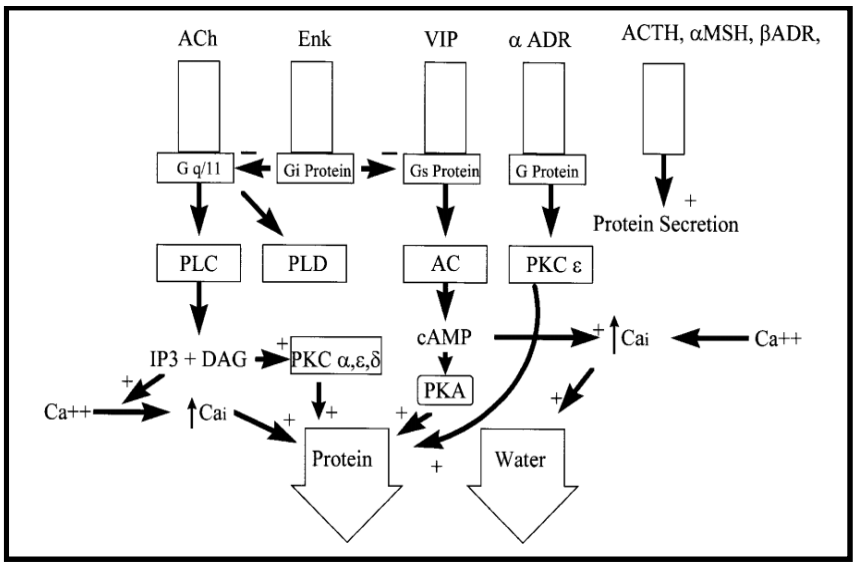
1.4.1. Secretion of water

One of the major secretory “products” of the lacrimal gland is water. This water is moved from the interstitial spaces of the gland into the lumen of the gland where it

is mixed with the other secretory products. This water movement is accomplished by osmosis, which depends on the movement of ions from the acinar cells into the lumen (Dartt 2009).

The acinar cell surface membrane is differentiated into basolateral and apical domain, which are separated by the junctional complex. The apical domain contains water channels (aquaporin 5), which facilitate the movement of water across the epithelium. In addition, Cl^- and K^+ channels are present to allow the movement of solute across the epithelium. The baso lateral membranes contain large numbers of Na^+ pumps (Mircheff 1989), the Na^+-K^+-ATPase, which actively move K^+ into the cell and Na^+ out of the cell, maintains the gradients. It is this gradient (more Na^+ outside and K^+ inside) that provides the motive force for the movement of ions and water across the epithelium. In addition, there are several coupled transport systems (porters) driven by the concentration gradients created by the Na^+ pump and by the activity of carbonic anhydrase. The basolateral membranes also have ion channels, specifically for K^+, Cl^-, and Ca^{2+} as well as more general cation and anion channels. This link between ion channels and their permeability and the movement of water may form the underlying mechanism of dry eyes.

Figure 1.4: Secretion of protein and water from lacrimal gland



Source: News Physiol. Sci. (13) 1998

1.5. Inflammatory disorders of lacrimal gland

In several pathological instances, the lacrimal gland can become a target of the immune system and show signs of inflammation. This can occur as a result of autoimmune diseases such as Sjogren's syndrome, sarcoidosis, bone marrow transplants and ageing. There are two major types of dry eye syndrome: aqueous-deficient dry eye due to lacrimal gland diseases and evaporative dry eye, which is mainly due to meibomian gland diseases (Lemp 1995).

1.6. Tear fluid properties

1.6.1. Physical properties of tears (Jordan and Baum 1980)

The physical properties of tears are listed in table 1.1. Tear is composed of 98% water and 2 % solids like proteins, lipids, carbohydrates, salts etc. The total tear volume produced as an average is 6.2 µl, ranging from 3.4 µl to 10.7 µl. The thickness of tear film is 6 -7 µm. pH of tear is slightly basic i.e. 7.5; the osmolarity is 300-334 mosm.

Table 1.1: Tear film physical properties.

Parameter	Value
Composition	98% water,2%solids
Volume	6.2 µl
Thickness	6.0-7.0 µm
Rate of secretion Unstimulated Anesthetized Schimmer	 1.2 µl/min 0.5-2.2 µl/min 1.8 (µl/min)
Turnover rate Normal Stimulated	 12-16% 300%/min
Osmolarity	300-334 mosm/K
PH	7.5

1.6.2. Chemical composition of tear fluid

Tear has salts like sodium, potassium, chloride, bicarbonate, Magnesium, calcium. The chloride concentration is more in tears compared to serum (Van Haeringen 1981). Tear has metabolites such as glucose, urea, lactate, pyruvate, ascorbate, retinol as seen in table 1.2. Tear also contains free amino acids and other metabolites like uric acid, bilirubin.The concentrations of electrolytes and organic solutes in human tears comparing with serum mentioned in table 1.2 (Van Haeringen 1981).

Table 1.2: Tear fluid components comparison with serum concentration.

Component	Tears	Serum
Electrolytes (mM)		
Na^+	120-165	130-145
Cl^-	118-135	95-125
HCo^{3-}	20-26	24-30
Mg^{2+}	0.5-0.9	0.7-1.1
K^+	**20-42**	3.5-5.0
Ca^{2+}	0.4-1.1	2.0-2.6
Organic solutes (mM)		
Glucose	0.1-0.6	4-6
Urea	3.0-6.0	3.3-6.5
Lactate	**2-5**	0.5-0.8
Pyruvate	0.05-0.35	0.1-0.2
Ascorbate	0.008-0.04	0.04-0.06
All-Trans retinal (µg/dl)	0.04-1.06	30-60

1.6.3. Tear Proteins

Normal tears contain 6-10 mg/ml total proteins and at least 80 tear proteins (Gachon, Richard et al. 1982) as reproted. Major tear proteins include lysozyme, lactoferrin, secretory immmunoglobulin A (sIg A), serum albumin(Li, Wang et al. 2005), lipocalin (Redl, Holzfeind et al. 1992; Glasgow, Abduragimov et al. 1995; Schoenwald, Vidvauns et al. 1998), lipophilin (Lehrer, Xu et al. 1998) and tear specific prealbumin (TSP) originates from lacrimal gland. Tear proteins reflect the

state of health of the ocular surface. Measured levels of proteins such as β2 microglobulin, cystatins, substance P, epidermal growth factor (EGF), Transforming growth factor β1 and β2 (TGFβ1 & β2), plasmin, tryptase, and α1 antitrypsin change depending on the conditions. Other proteins include glycoproteins, mucins, antiproteinases and bactericidal protein β-lysine (Ford, DeLange et al. 1976).

The glycoproteins include orosomucoid, α1-glycoprotein, transferrin, ceruloplasmin, Zn-α2-glycoprotein, mucous glycoproteins contribute to the highly viscous nature of ocular mucus. The viscosity of human tears is about 2.8 and the viscosity reduces during blinking but between each blink it raises and thus stabilizes the tear film. Growth factors, EGF, TGF-β1 and β2 are present in normal tear fluids and have been associated with corneal wound healing (Grus, Sabuncuo et al. 2001)

1.6.4. Enzymes: A large number of enzymes including glucose metabolizing enzymes, antiproteases, collagenases, matrixmetalloproteases, and other Proteolytic enzymes are present in tears. During infections of the eye or in corneal ulcerations some of these enzymes like α1-antitrypsin, α 2-microglobulin are increased (Ford, DeLange et al. 1976).

1.6.5. Lipids: It includes free fatty acids, free sterols, triglycerides, diesters, polar lipids, and hydrocarbons. Meibomian secretion functions as a barrier with its hydrophobic nature that prevents spill over of tears. Other important functions of lipid layer are that it reduces the rate of evaporation from the open eye, provides lubrication for the eyelid/ocular interface and contributes to the optical properties of the tear film (Nicolaides, Santos et al. 1989; McCulley and Shine 2001; 2003).

1.7. Control of tear secretion

Control of tear secretion is under constant neural regulation. Reflex tears are a result of neural participation and the normal tears result due to intrinsic lacrimal gland activity. Homeostatic regulation of ocular surface will be under control mechanism with CNS (Stern, Beuerman et al. 1998). Upon stimulation afferent nerves from the

cornea and other ocular surface tissues acts on central nervous system, from which efferent nerves comprises autonomic innervation to secretory tissues releases the products of neuro transmitter that stimulate lacrimal glands leads to lacrimal reflex which Contribute to tear film production (Wieczorek, Jakobiec et al. 1988).

1.8. Collection of tear

Two most important tear collections include capillary tear collection and Schirmer strip based tear collection. The glass capillary tubes that are calibrated are used to collect the tear. Reflex with chemicals, onions increases capillary tear collection. (Holly, Lamberts et al. 1982). Schrimer strip collection is a simple, inexpensive method for measuring tear volume in the clinic (Van Haeringen 1981). A strip of filter paper, usually 35mm long and 5mm wide is inserted into the lower conjunctival sac. The tears are absorbed by the strip, and the wetting of 5-6 mm paper in 5 mm is considered a normal value. Detailed studies on the capillary flow of tears in a series of human subjects indicate that the Schirmer's strip method for tear collection is basically more reliable under most of the disease conditions. Other tear collection methods are mentioned in detail in chapter 3.

1.9. Bactericidal Properties of Tears

Tear fluid contains bactericidal or bacterolytic activities. The main one, and most extensively studied is lysozyme (Van Haeringen 1981) A heat labile non lysozyme antibacterial factor also known as beta lysine is reported (Ford, DeLange et al. 1976). Lactoferrin, a bacteriostatic protein with its efficient iron binding capacity is known. Apart from its role in nonspecific defence against invading microorganisms, lactoferrin also plays a role in preventing complement activation in inflammatory conditions (Kijlstra, Jeurissen et al. 1983). Peroxidase protects eye from potential hydrogen peroxidase toxicity. Prostaglandin, complement C1 to C9 play important role in inflammatory conditions. The oxygen free radicals generated during the metabolism are eliminated by lactoferrin and calcium molecules (Kuizenga, van Haeringen et al. 1987).

1.10. Diseases associated with tear fluid component changes

Ocular surface disease can result from the abnormalities in one or more of the tear film components, ocular or systemic diseases, various drugs and even environmental conditions (Rolando and Zierhut 2001). Dry eye (DES) **or** Keratoconjunctivitis sicca (KCS) is a common disorder of the tear film that results from decreased tear production or excessive tear evaporation, or abnormality in mucin or lipid components of the tear film (2007). Dry eye syndrome is a leading cause of ocular discomfort affecting millions of people. Dry eye conditions shows varied etiology ranging from mild eye to very severe dry eyes with sight threatening complications (Unlu, Guney et al. 2012).

Dry eye exhibits mostly in elderly people and it increases with age (2007). DES is more common in females than males. Younger patients who work with computers can also suffer from dry eyes more often than elderly patients (Miljanovic, Dana et al. 2007). Dry eye condition is also aggravated in polluted conditions, in dry weather, decreased ambient humidity. Ocular diseases such as glaucoma (Leung, Medeiros et al. 2008), cataract surgery (Cho and Kim 2009), use of contact lens (Sengor, Aydin Kurna et al. 2012), and also rarely keratoconus associates with DES (De Paiva, Harris et al. 2003).

1.11. Other diseases associated with tear film changes

Tear film changes are also associates with other ocular diseases such as Blepharitis, Keratitis. Other conditions such as glaucoma medications, cataract surgery, corneal transplantation, Viral infections and systemic diseases like Sjogren's syndrome, Diabetes, thyroid disease (Herber, Grus et al. 2001; Koo, Lee et al. 2005; Ananthi, Chitra et al. 2008; Okrojek, Grus et al. 2009) cause changes in tear film. Sjogren's syndrome, which is an autoimmune disease, having chronic inflammation of exocrine glands i.e. salivary and lacrimal glands leads to dry mouth and dry eye. Sjogren's syndrome can be either primary or secondary. Primary SS is characterized by a chronic autoimmune attack involves both lacrimal and salivary glands (Chung, Kim et al. 2012). Secondary SS is marked by an autoimmune attack against the

lacrimal and salivary glands and also associates with another autoimmune disease, most often a connective tissue disease like rheumatoid arthritis (RA), systemic lupus Erythmatosus (SLE) or scleroderma (Haga, Naderi et al. 2012).

This study concentrates on DES alone, and DES associates with primary and secondary to RA. Therefore this is discussed in following sections.

1.12. Dry eye Syndrome

Definition: Dry eye is a multifactorial disease of the lacrimal functional unit, that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface.

1.12.1. Signs and symptoms of DES (Ormerod, Fong et al. 1988):

a. **Symptoms:** Ocular irritation of dry sensation, burning, itching, pain, foreign body sensation, photophobia, and blurred vision are common in patients with dry eye. Paradoxically, some patients with dry eye syndrome complain of too much tearing. When evidence of dry eye syndrome exists, this symptom often is explained by excessive reflex tearing due to severe corneal surface disease from the dryness (figure 1.10).

b. **Signs of a dry eye include the following:** It includes, Decreased tear meniscus, Irregular corneal surface, Decreased tear break-up time, Punctate epithelial keratopathy, corneal filaments, increased debris in the tear film, conjunctival pleating, superficial punctuate keratitis, with positive fluorescein staining, mucous discharge, bulbar conjunctival vascular dilation and corneal ulcers in severe cases. In severe DES cases, secondary infectious keratitis and corneal perforations may also develop in severe dry eye.

1.12.2. Prevalence of DES

(i) **International:** Dry eye is a very common disorder affecting a significant percentage (approximately 10.8 % - 57.1 % of the population, especially those older than 40 years.

(ii) **National:** Based on Ocular surface Disease Index (OSDI), the DES prevalence in India is 29.2 %in India. Prevalence varies with age and gender. Older age people have 41.2 % more prevalence than younger age group. Women are more prone to DES with 27%, where as men with 12% DES (Gupta, Prasad et al. 2010).

1.12.3. Mortality/Morbidity of DES (Murube, Nemeth et al. 2005):

Dry eye can get complicated by sterile or infectious corneal ulceration, particularly in patients with SS. Ulcer is typically less than 3 mm in diameter, and located in the central or paracentral cornea. Occasionally, corneal perforation may occur. In rare cases, sterile or infectious corneal ulceration in dry eye syndrome can cause blindness. Other complications include punctate epithelial defects (PEDs), corneal neovascularization, and corneal scarring.

Race: No known racial predilection exists.

Sex: Dry eye may be slightly more common in women. KCS associated with Sjogrens syndrome is believed to affect 1-2% of the population, and 90% of those affected are women

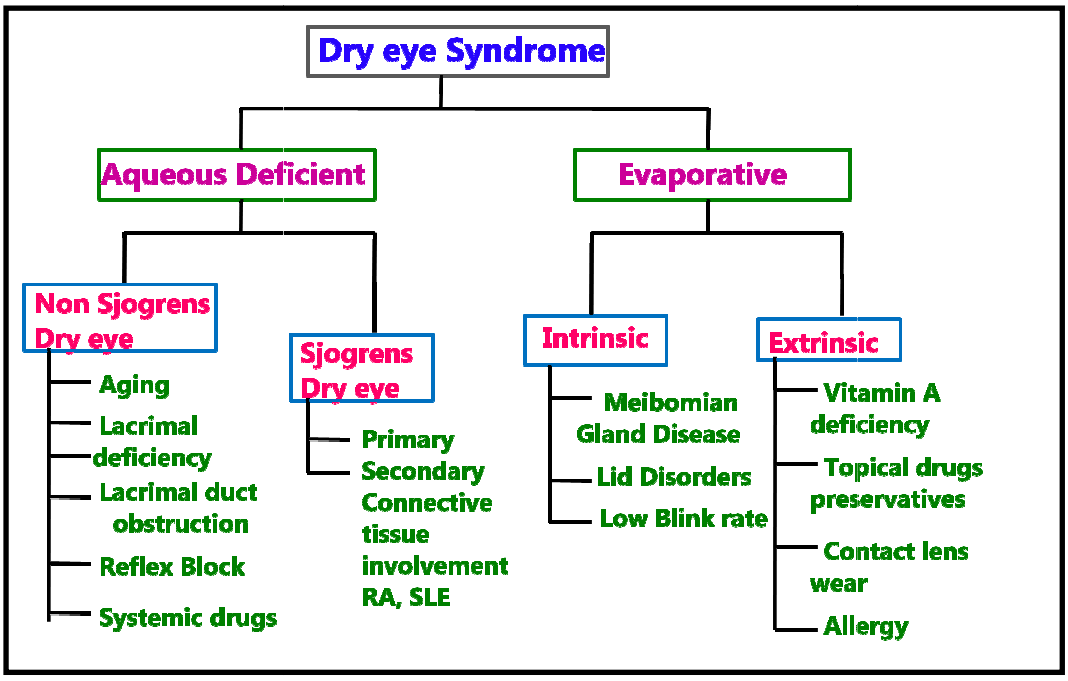
1.12.4. Classification of Dry Eye Syndrome

1.12.4. i. Classification of DES was modified in 2007 by the National Eye Institute**.**

A classification system by NEI distinguishes 2 main categories of dry eye states, an aqueous deficiency state and an evaporative state, as shown in Figure 1.5.

Evaporative dry eye may be intrinsic, where the regulation of evaporative loss from the tear film is directly affected, as by meibomian lipid deficiency, poor lid congruity and lid dynamics, low blink rate, and the effects of drug action, such as that of systemic retinoids. Extrinsic evaporative dry eye includes vitamin A deficiency, the action of toxic topical agents such as preservatives, contact lens wear and other ocular surface diseases like allergic eye disease. The aqueous tear deficiency is again classified into Non-Sjogren's and Sjogren's based on the auto immune component involved.

Figure 1.5: Classification of DES based on National Eye Institute



1.12.4. a. Deficient aqueous production

A. Non-Sjögren syndrome: This associates with the following causes,

(i) **Lacrimal disease (primary or secondary):** this may be due to Idiopathic, systemic vitamin A deficiency (xerophthalmia), malnutrition, fat-free diets, intestinal malabsorption from inflammatory bowel disease, bowel resection, or chronic alcoholism, Lacrimal ablation, Congenital alacrima (Riley-Day syndrome), Primary lacrimal deficiency, Graft-versus-host disease

(ii) **Infiltrative processes: associated with** Lymphoma, Amyloidosis, Hemachromatosis and Sarcoidosis leads to DES

(iii) **Infectious diseases: This includes** HIV diffuse infiltrative lymphadenopathy syndrome, Trachoma caused aqueous tear reduction.

(iv) **Lacrimal obstructive disease:** Trachoma, Ocular cicatricial pemphigoid, Erythema multiforme and Stevens-Johnson syndrome, Chemical burns, Endocrine imbalance, Post radiation fibrosis

(v) **Medications** such as Antihistamines, beta-blockers, phenothiazines, atropine, oral contraceptives, anxiolytics, antiparkinsonian agents, diuretics, anticholinergics, antiarrhythmics, topical preservatives in eye drops, topical anaesthetics, and isotretinoin can trigger DES

(vi) **Decreased corneal sensation** due to Neurotrophic keratitis, corneal surgery, Herpes simplex, Contact lens wear, Cranial nerve VII (CN VII) palsy, Diabetes and Aging causes DES.

B. Sjögren syndrome: Sjogren's syndrome (SS) is a systemic auto immune disease that mainly affects the exocrine glands and usually presents as persistent dryness of the mouth and eyes due to functional impairment of the salivary and lacrimal glands. In the absence of an associated systemic autoimmune disease, patients with this condition are classified as having primary SS. The histological hallmark is a focal lymphocytic infiltration of the exocrine glands, and the spectrum of the disease extends from an organ-specific autoimmune disease (auto immune exocrinopathy) to a systemic process with diverse extra glandular manifestations. **It can be either primary or secondary to other connective tissue disorders.**

(i) Primary (no association with connective tissue disease (CTD) (pSS)

(ii) Secondary (associated CTD) such as Rheumatoid arthritis (RA), Systemic lupus erythematosus (SLE), Progressive systemic sclerosis (scleredema), Primary biliary cirrhosis, Interstitial nephritis, Polymyositis and dermatomyositis, Polyarteritis nodosa, Hashimoto thyroiditis, Lymphocytic interstitial pneumonitis, Idiopathic thrombocytopenic purpura, Hypergammaglobulinemia, Waldenstrom macroglobulinemia and Wegener granulomatosis also leads to DES.

1.12.4. b. Evaporative loss. This is due to

(i) Blepharitis-associated - Obstructive meibomian gland disease, rosacea

(ii) Blink disorders

(iii) Disorders of eyelid aperture and eyelid/globe congruity – Exposure, lid palsy, ectropion, or lid coloboma Contact lenses.

The current study discusses with on the aqueous tear deficiency due to non-Sjogren's and Sjogren's syndrome.

1.12.5. Pathophysiology of Dry eye syndrome

The pathophysiology of DES is mostly due to tear hyperosmolarity and tears film instability. In DES, a cascade of inflammatory events occur at the ocular surface and release of inflammatory mediators such as interleukins, TNF alpha and MMPs into the tears. This further leads to cell death by apoptosis, loss of goblet cells, and disturbance in mucin expression finally leading to tear film instability. This instability causes ocular surface hyperosmolarity. Tear hyperosmolarity causes damage to the surface epithelium. Tear film instability can also be initiated without the prior occurrence of tear hyperosmolarity, by several etiologies, including xerophthalmia, ocular allergy, use of topical preservative and contact lens wear.

The major causes of tear hyperosmolarity are reduced aqueous tear flow due to lacrimal failure, increased evaporation of tear film. Increased evaporative loss is mostly by environmental conditions such as low humidity, high air flow and also unstable tear lipid layer due to meibomian gland dysfunction (MGD). In MGD, deficiency of androgens causes loss of the lipid layer by reducing specifically triglycerides, cholesterol, monounsaturated essential fatty acids and polar lipids. The loss of polar lipids leads to the evaporative tear loss, and the decrease in unsaturated fatty acids raises the melting point of meibum, leading to thicker, more viscous secretions that obstruct ductules and cause stagnation of secretions *(*Mantelli et al. 2013).

Reduced aqueous tear flow is due to impaired delivery of lacrimal fluid into the conjunctival sac. This could be a feature of normal aging, but also induced by certain systemic drugs, such as antihistamines and anti-muscarinic agents. The most common cause for aqueous tear deficiency is inflammatory lacrimal damage, seen in autoimmune disorders such as Sjogren syndrome and also in non-Sjogren syndrome dry eye (NSSDE). Inflammation causes both tissue destruction and also neuro secretory block.

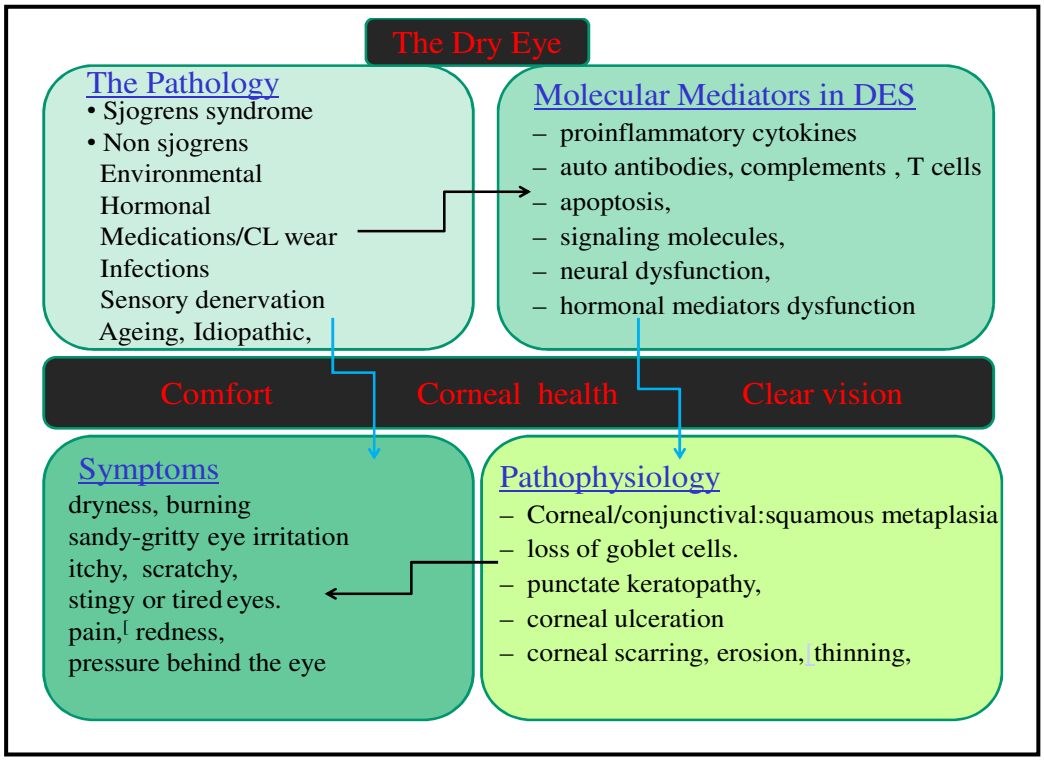
Mucin synthesizing genes, designated *MUC1-MUC17*, representing both transmembrane and goblet-cell secreted, soluble mucins, play important role in hydration and stability of the tear film. *MUC5AC* expressed by stratified squamous cells of the conjunctiva and is the predominant component of tear mucous layer. A defect in this and other mucin genes contributing factor for dry eye syndrome development (Mantelli, F., C. Moretti, et al. 2007, McKenzie, Jumblatt, J. E et al 2000). In addition to dry eye, other conditions, such as ocular cicatricial pemphigoid, Stevens-Johnson syndrome, and vitamin A deficiency, which lead to drying or keratinization of the ocular epithelium, eventually lead to goblet cell loss.

Various pro-inflammatory cytokines like including interleukin 1 (IL-1), interleukin 6 (IL-6) TNF-alpha activity increases with decrease in anti-inflammatory cytokines like interleukin 8 (IL-8), TGF-beta, and RANTES. IL-1 beta and TNF-alpha, in the tears of KCS patients causes dysfunction of the lacrimal gland, and loss of response to nerve stimulation and less reflex tearing leads to reduced tear production. Proinflammatory neurotransmitters, such as substance P and calcitonin gene related peptide (CGRP), are released, which recruit and activate local lymphocytes. Substance P also acts via the NF-AT and NF-Kβ signaling pathway leading to ICAM-1 and VCAM-1 expression, adhesions molecules that promote lymphocyte homing and chemotaxis to sites of inflammation. These cytokines, in addition to inhibiting neural function, may also convert androgens into estrogens, resulting in MGD. An increased rate of apoptosis is also seen in conjunctival and lacrimal acinar cells mostly due to the cytokine cascade. Elevated levels of tissue-degrading enzymes called matrix metalloproteinases (MMPs) are also present in the epithelial

cells. The epithelial injury in dry eye stimulates corneal nerve endings, leads to symptoms of discomfort, increases blinking, increases reflex lacrimal tear secretion.

A genetic predisposition in human leukocyte antigen B8 (HLA-B8) haplotype seen in SS associated KCS. In SS, chronic inflammatory state observed, with the production of auto antibodies, including antinuclear antibody (ANA), rheumatoid factor, fodrin (a cytoskeletal protein), the muscarinic M3 receptor, and SS-specific antibodies like anti-RO [SS-A], anti-LA [SS-B]), inflammatory cytokine releases cause focal lymphocytic infiltration mainly $CD4^+$ T cells but also B cells of the lacrimal and salivary gland which further leads to glandular degeneration and induction of apoptosis in the conjunctiva and lacrimal glands. Both androgen and estrogen receptors are located in the lacrimal and meibomian glands. SS is more common in postmenopausal women where in, a decrease in circulating sex hormones (i.e., estrogen, androgen) occurs, affecting the functional and secretory aspect of the lacrimal gland with infiltration of cells.

Figure 1.6: The pathophysiology of Dry eye syndrome



1.12.6. Diagnostic tests for Dry eye syndrome

Dry eye is multifactorial therefore requires a clinical diagnosis, combining from both the history and the examination by performing one or more tests. No single test is sufficiently specific to permit an absolute diagnosis of dry eye. The main diagnostic tests for DES are discussed in the following sections.

1. Schimmer's test: Tear secretion may be divided into basal and reflex secretion. The average basal tear volume ranges from 5-9 µl with a flow rate of 0.5 – 2.2 µl / min. (Cho and Yap 1993). This test measures the total (reflex and basal) tear secretion. A commercially available Whatmann No.41 filter paper strip of 5 mm X 30 mm, known as Schimmer strip used for this test. In this the patient is seated in a room with less light, and the filter paper strips are folded 5 mm from the end as showed in the figure 1.7. The folded end is placed gently over the lower palpebral conjunctiva at its lateral one -third. The patient asked to keep the eyes open and looks upward. After 5 minutes the strips are removed and the amount of wetting is measure from the folded end of the strips. Measurements greater than 30 mm at 5 minutes indicate the reflex tearing is intact but not controlled and, therefore, are of little diagnostic value. Between 10 and 30 mm of tear secretion may be normal. Values less than 5 mm on repeated testing indicate hypo secretion of basic tearing. To differentiate basic and reflex tearing, the basic secretion test should be performed (figure 1.7A)

1. **Schimmer test II:** The purpose of this test is to ascertain reflex secretion. The procedure is similar to the basal secretion test, but after the strips are installed, the anaesthetized nasal mucosa is irritated by rubbing with dry cotton tipped applicator. The amount of wetting of the filter paper is measured after 2 minutes. Less than 15 mm of wetting indicates failure of reflex secretion.

2. Basic Secretion test: This is most commonly used test, to measure the basal secretion by eliminating reflex tearing. Topical anesthetic is added into the conjunctiva. And the test is similar to the Schimmer test I and the interpretation of the result is also similar. The difference between the results of this test and those of

the Schimmer Test I is a measurement of reflex secretion. Less than 5 mm of wetting on the basic secretion test ensures a diagnosis of hypo secretion. (Lee and Tseng 1997)

3. Rose Bengal staining (Lee and Tseng 1997): The purpose of this test is to ascertain indirectly, the presence of reduced tear volume by the detection of damaged epithelial cells. The eye is anaesthetized topically with proparacaine 0.5%. One drop of 1% Rose Bengal solution or a drop from saline water. Rose Bengal strip is installed in each conjunctival sac. Rose Bengal is a vital stain taken up by dead and dying cells in the interpalpebral area (figure 1.7B**)**

This test is particularly useful in early stages of conjunctivitis sicca syndrome. A positive test shows triangular shape staining of the nasal and temporal bulbar conjunctiva in the interpalpebral area and possible punctate staining of the cornea, especially in the lower two third portions.

4. **Fluorescein Dye test:** This is a test for the stability of the tear film. After a certain time interval following blinking, the tear film normally ruptures and forms dry spots. Increased meibomian gland secretion possibly may act to decrease tear film stability. Deficiency of mucin and aqueous tears also decrease the tear film stability and shorten the time interval between the opening of the eye and the appearance of dry spots as fluorescein spots (figure 1.7C) **(Mengher, Pandher et al. 1986)**

Figure 1.7: The diagnostic tests of Dry Eye Syndrome

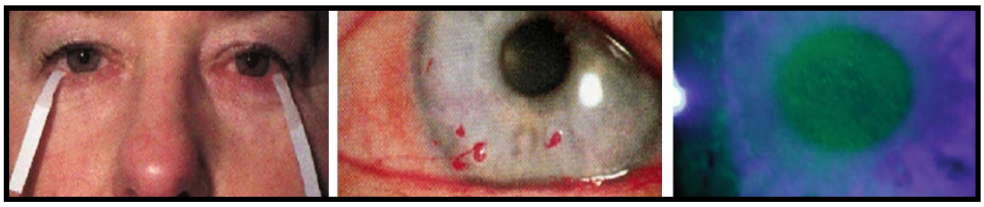


Figure 1.7: (A). Schirmer test (B). Rose Bengal staining (C) Fluorescein staining of the cornea - Central focal corneal epithelial fluorescein staining.

5. **Tear film break-up time (BUT):** No anaesthesia is used in this test. 1% Flourescein is installed in the lower cul-de-sac, or a dry Flourescein is touched to the inferior fornix when the patient is looking up. The cornea is scanned under low slit lamp magnification using blue cobalt filtered light. The patient is instructed to blink once or twice and then stare straight ahead without blinking. The time of appearance of the first dry spot formation (small black spots within the blue green field) from the last blink measures the tear film TBUT. (Lemp and Hamill 1973)

Flourescein staining can be graded from 0-3. (**Figure 1.8)**

0 – No staining of corneal epithelial surface.

1 – Mild staining occupying < 1/3 of corneal epithelial surface

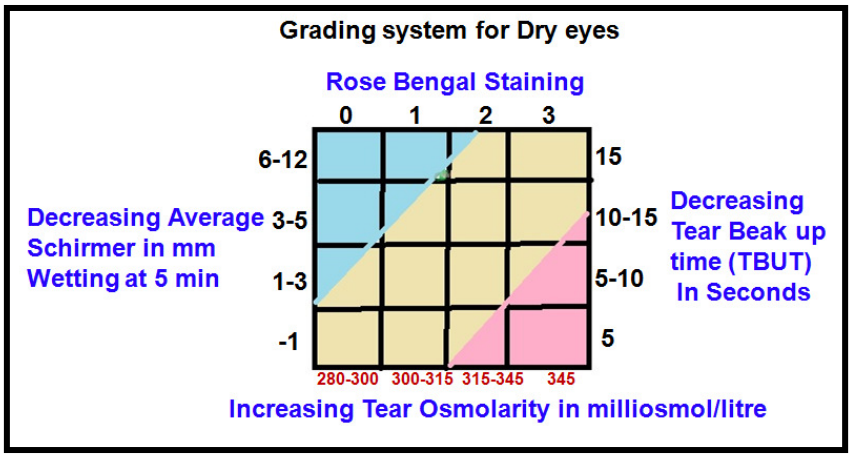
2 – Moderate staining occupying < 1/2 of corneal epithelial surface

3 – Severe staining occupying > 1/2 of corneal epithelial surface

The consistence appearance of dry spots in one area indicates an anatomic surface abnormality in that area. Abnormal BUT is invariably seen in clinically significant sicca and mucin deficient syndromes. The normal value is as long as 40 seconds.

Early or mild cases of keratoconunctivitis (KCS) are detected more easily with rose bengal than with fluorescein staining, and the conjunctiva is stained more intensely than the cornea. Interpalpebral staining of the nasal and/or inferior paracentral cornea is seen in KCS. A linear pattern of inferior conjunctiva and corneal staining by rose bengal is characteristic of MGD. The disease management is based on the grading of the dry eye

Table 1.8: Grading and interpretation of Schirmer, Rose Bengal and TBUT



1.10.6. Other diagnostic tests for dry eye

1. **Corneal residence time test or the Tear Clearance Rate (TCR):** To measure the time that preocular tear film and topical eye drops remain resident on the cornea. Patients with a normal lacrimal drainage system but abnormalities in tear production, the decay time of an indicator such as fluorescein or a radio isotope can be accurately measured. **(Font, Yanoff et al. 1967).** Patients with dry eye syndrome, the residence time of fluorescein or a radioisotope is prolonged because of decrease in the turnover of tears.

   Tear function Index (TFI): It is diagnostically superior to other tests in identifying dry eye and distinguishing dry eye that is associated with Sjogren's syndrome. TFI is the combination of both tear secretion and drainage test.

2. **Lissamine green staining** combines the advantages of fluorescein and rose bengal staining; it stains healthy epithelial cells that are not protected by a mucin layer (similar to rose bengal) and also stains degenerating or dead cells (similar to fluorescein). It avoids the pain, discomfort, and corneal toxicity associated with Rose Bengal but is less sensitive and more transient(Figure 1.9).

3. **Conjunctival impression cytology** can be used to monitor the progression of ocular surface changes, for confirming dry eye status. Conjunctival impression cytology is performed to determined goblet cell density of the bulbar or palpebral conjunctiva. A strip of filter paper is gently pressed against the bulbar or palpebral conjunctiva with a glass end. Following staining with Schiff's agent and counter staining with haematoxylin, the specimen is graded using a microscope. In dry eye decrease in goblet cell counts seen. (Bacman, Berra et al. 2001) (Figure 1.9).

Figure 1.9: DES diagnostic tests – Lissamine green staining and imprint cytology

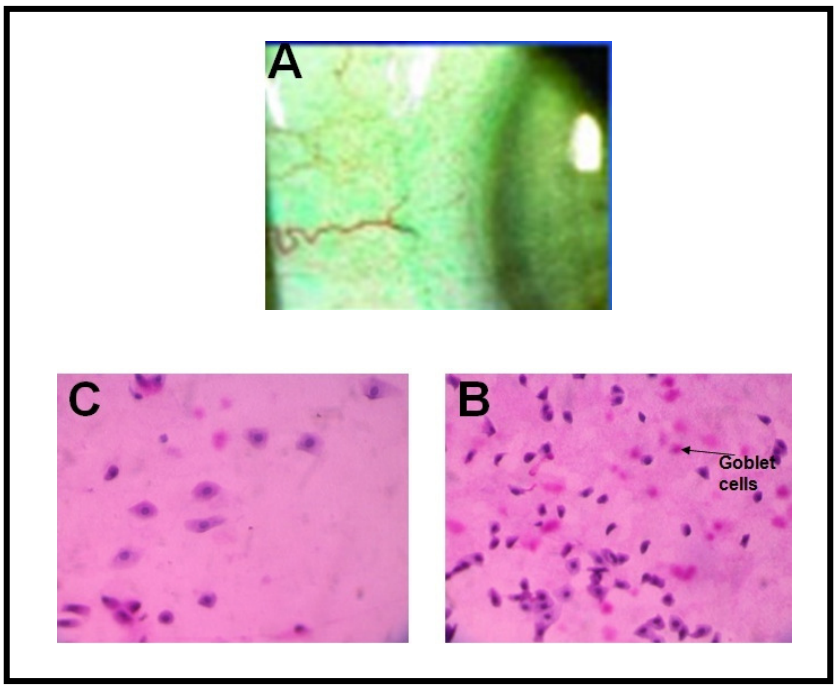


Figure 1.9: (A). Lissamine green staining. Green colour staining indicative of dry spots in DES. (B) and (C).Conjunctival impression cytology – B. Control C. DES. Reduction of goblet cells in DES.

4. **Serology for circulating auto antibodies:** Including ANA or SS antibodies (i.e., SS-A, SS-B), for the diagnosis of SS detection (Nakamura, Shibuya et al. 2003). Absence of nasal lacrimal reflex tearing, presence of serum auto antibodies, and severe ocular surface disease demonstrated by rose bengal or fluorescein staining argues strongly in favour of a diagnosis of SS associated KCS.

5. Additional tests that can be performed to quantify each individual tear component are lipid quantification from meibomian glands, mucins, checking tear osmolarity, tear lysozyme, tear lactoferrin, EGF, aquaporin 5, lipocalin, and IgA concentrations with ELISA, The tear turnover rate, Lacrimal gland or minor (salivary) gland biopsy performed to aid in diagnosing Sjogren's syndrome.

6. **Histologic Findings include** loss of goblet cells, cellular enlargement, and increase in cytoplasmic/nuclear ratio of the superficial conjunctival epithelial cells in KCS. The lacrimal gland and the conjunctiva are also heavily infiltrated by $CD4^+$ T cell (and B cell) lymphocytes.

1.12.7. Treatment for Dry eye syndrome

The treatment of dry eyes can be sub divided into

1. Pharmacological therapy
2. Supportive therapy
3. Therapy of the underlying cause
4. Surgical therapy

1.12.7. a. Pharmacological therapy: The main management of DES consists of tear replacement therapy with a variety of artificial tear solutions. Artificial tear solutions consist of inorganic electrolytes similar to natural tears to achieve tonicity and maintain pH to enhance their role in tear film substitution, various polymers that increase the wettability of hydrophobic corneal surface, stability of preocular tear film and increase conjunctival retention time because of their high molecular weight, which retards their drainage through the lacrimal puncta. The main stay of treatment of dry eye conditions is the instillation of a tear substitute in the eye several times a day.

Nutrients in the treatment of ocular surface disease

Topically Applied Vitamin A, Vitamin A-containing Artificial Tears, Vitamin $B_{12,}$ Lacrophilic Artificial Tears, Elevated Oncotic Pressure/Complete Wetting used for DES treatment.

1.12.7. B. Supportive Therapy

This includes reduce tear loss by evaporation.

A. Use of eye shields, glasses with side shields or swimmers goggles:

   In addition, moistened sponges may be attached to side shields. The water that released from these sponges provides an even moister environment and therefore more comfortable ocular environment.

B. Contact lens:

   For moderate dry eye conditions hydrophilic contact lenses may be useful in providing a tear reservoir as well as diminish tear evaporation. Silicone contact lenses useful as they have low fluid permeability and high oxygen permeability and thus conserve tears.

1.10.7. C. Therapy of underlying disease: As KCS is caused by various diseases, therapy should also be directed at the cause of the symptoms. Therefore it is necessary to find the new diagnostic tools for developing therapies for the Dry eye syndrome with various etiologies.

1. Chronic blepharitis which is frequently associated with ocular surface disorders is treated with eyelid hygiene and topical antibiotics.
2. In KCS associated with auto immune disorders especially in young women systemic and topical steroids and immuno suppressive drugs used in consultation with rheumatologist. Dry eye due to Sjogren's syndrome is markedly improved by using patient's autologous serum.
3. Epidermal keratinization and squamous metaplasia of mucous membranes, including cornea and conjunctiva respond to both oral and topical Vitamin A therapy. Topical use of retinal and systemic retinoic acid have been advocated for treatment of dry eye disorders.

1.12.7. D. Surgical Therapy. Punctal occlusion, blocks the drainage of tears so that tear evaporation as a result artificial tears solutions and whatever little secretion of natural tears that is present will be conserved in the eyes for longer time. It includes Laser Punctoplasty, Lid taping, Orbital decompression, lid constructive surgery.

1.12.7. E. Recent Studies –Development of Diagnostic Markers

In recent year's molecules such as Beta hydroxy butyrate which has anti apoptotic activity, Neurturin that is essential for the development of specific postganglionic parasympathetic neurons, Amphipathic polymers to enhance the stability of liposomes, n_3 and n_6 Fatty acids (FA) are potential molecules for DES therapy. Studies are still warranted for identifying therapeutic targets and for novel therapy. Differential tear proteomics can be a promising tool to identify such targets.

1.13. Approaches to study protein biomarkers by Proteomics

Molecular characterization of proteome is mandatory to understand the biological system completely. Proteomics helps for biomarker identification, protein structure function and relations and protein interactions. **Bottom up** or **Top down proteomic approach helps to understand protein system.**

Bottom-up proteomics: It is a common method to identify proteins and characterize their amino acid sequences and post-translational modifications by proteolytic digestion of proteins prior to analysis by mass spectrometry. The proteins first purified by gel electrophoresis resulting in one or a few proteins can be identified using proteolytic digestion.

Top down Proteomics: In this the complex biological sample can be separated using chromatography and then by mass spectrometry, from this significant candidate proteins selected, purified and then identification can be done. The advantage of this kind of approach is more structural identification.

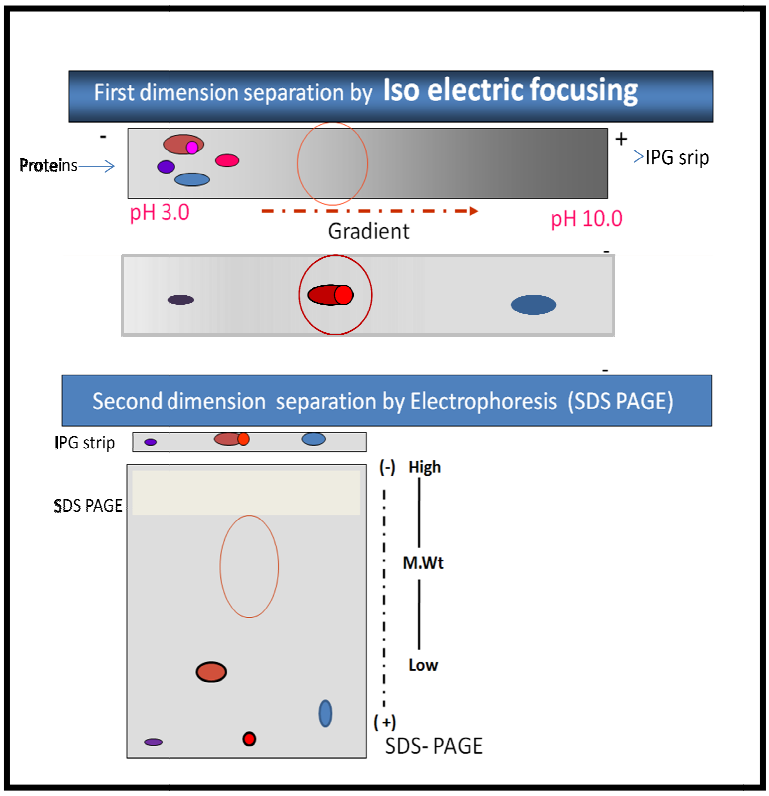
The current study used the "Bottom-up proteomics" using two dimensional gel electrophoresis based proteomics approach to identity the characteristic differential proteins in dry eye disease followed by identification by mass spectrometry (MS).

1.13.1. Gel based Proteomic approach

Two Dimensional Gel Electrophoresis (2DGE) (Gorg, Postel et al. 1988)

2DGE is a separation technique, which gives resolution of thousands of proteins. The high-resolution capability of 2DE comes from its two independent properties i.e. the first dimension separation using the isoelectric focusing (IEF), in which the proteins are separated based on their charge, in the second dimension, the proteins are separated according to their molecular weight as occurs in SDS-PAGE as shown in Figure 1.10.

Figure 1.10: Schematic representation of separation of proteins by 2Dimensional gel electrophoresis



1.13.1. A.

(i) Sample Preparation

Sample preparation is crucial for good 2-D results. This includes complete solubilisation of proteins using Chaotropic agents like urea, thiourea (Rabilloud 1998), zwitterionic Detergents like CHAPS, CHAPSO, ASB14 (Hermann, Finkemeier et al. 2000), carrier ampholytes, followed by disaggregation, denaturation, and reduction of the proteins using dithiotheritol (DTT) or tributylphosphine (TBP) (Herbert, Molloy et al. 1998). Sample enrichment of the protein is done by removal of nucleic acids and other interfering molecules like salt using dialysis spin dialysis, gel filtration, precipitation and Cut-off filters. (Macri, McGee et al. 2000). Protein degradation should be prevented during sample preparation using protease inhibitors like Phenyl methyl sulphonyl fluoride (PMSF), Aminoethylbenzyl sulfonyl fluoride (AEBSF), EDTA, Leupeptin, Pepstatin, Aprotinin, bestatin etc.

Protein Load and pH range of IPG strip

Optimum amount of protein should be applied to an IPG strip ranging from micrograms to 1 mg or more (Bjellqvist, Hughes et al. 1993) based on the length of the IPG used and the final detection used**.** Based on the protein pH in a solution the pH range of strip such as 3-10 or 3-8 and 7-10 etc. should be selected. While processing the sample and handling of IPG strips should be done carefully to prevent keratin contamination.

1.13.1. b. First-dimension Isoelectric Focusing (IEF)

Proteins are amphoteric molecules. Every protein has its specific pH at which its net charge is zero, called Isoelectric Point (pI) and is determined by the number and types of charged groups in a protein. Isoelectric Point (pI) Differences in proteins pI are the basis of separation by IEF. First-dimension separation requires pH range appropriate for the sample, as well as suitable sample application. A protein is

positively charged in solution at pH values below its pI and negatively charged at pH values above its pI. IEF, either using IPG strips or using carrier ampholytes in tube gels, may be used to resolve proteins in the first dimension (Garfin 2000). The IPG method has various advantages over the older tube gel method (Görg 1989, Görg 1991). The current study used IPG strips for protein separation and further 2DE experiments. A stable, linear, and reproducible pH gradient is crucial for a successful IEF. IPG strips are commercially available with pH gradients and are formed with acrylamide gels. These acrylamide derivatives are covalently incorporated into polyacrylamide gels at the time of casting to form pH gradient (Righetti 1990).

Sample Application

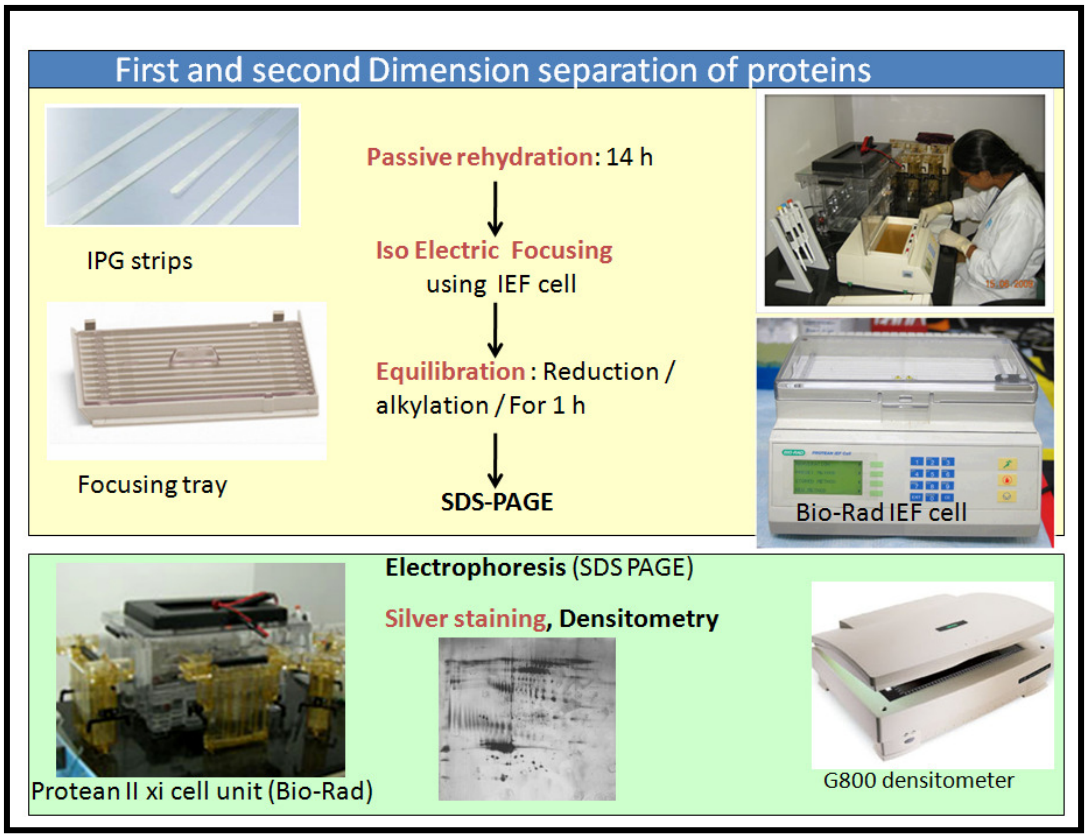
Commercial IPG strips are in dehydrated form therefore they must be rehydrated before use. This helps in flexibility for applying the sample to the strips. There are 3 methods for sample loading: passive in-gel rehydration with sample, active in-gel rehydration with sample, and cup loading of sample after IPG rehydration. For both active and passive rehydration methods, the sample is introduced to the IPG strip at the time of rehydration. As the strips hydrate, proteins in the sample absorbed and distributed over the entire length of the strip (Sanchez et al. 1997), then the focussing of proteins achieved at higher voltages.

1.13.1. C. The Second Dimension: SDS-PAGE

Second-dimension separation is by protein mass, or MW using SDS-PAGE. The proteins resolved in IPG strips in the first dimension are applied to second-dimension gels and separated by MW perpendicularly to the first dimension (Garfin 1995) (figure 1.11).

Imaging of 2D gels: The gel developed after 2D electrophoresis can stained using appropriate stain such as silver staining, coomassie staining and more recently florescent staining which is more sensitive for the detection of proteins in the 2D gels for visualization of protein spots for the analysis.

Figure 1.11. Processing of sample for 2DE

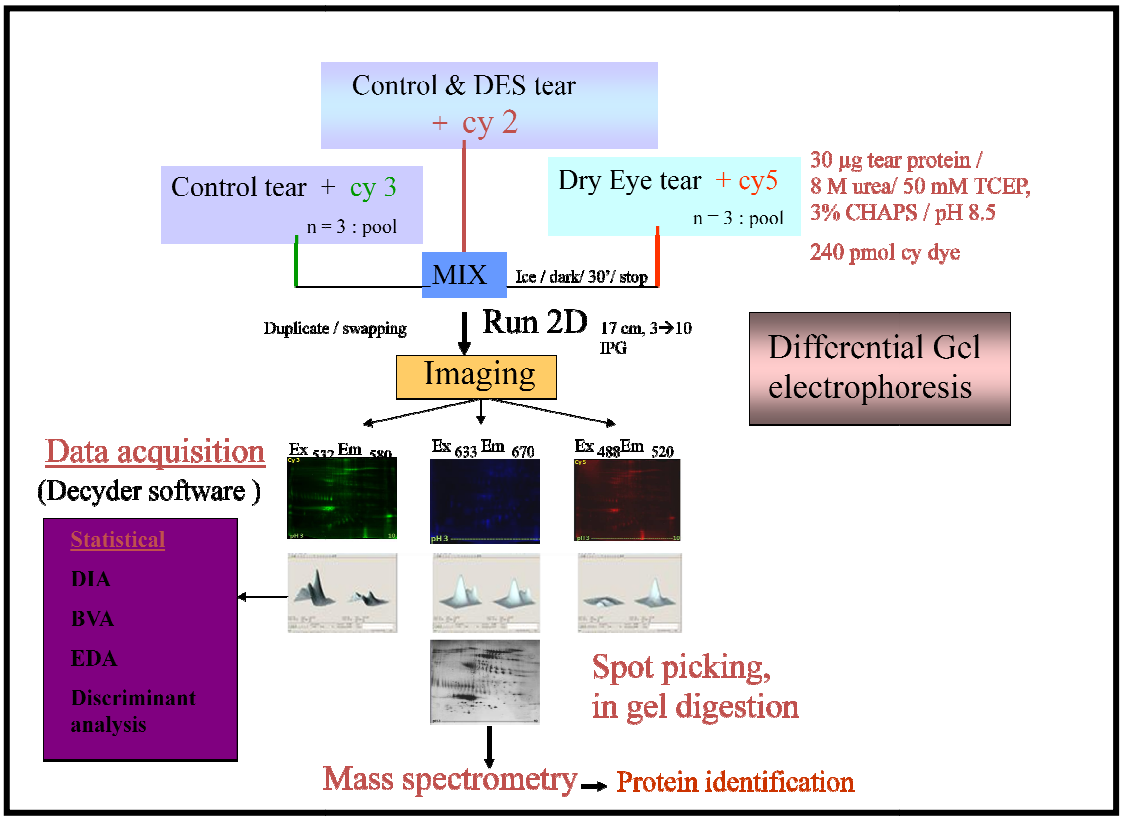


1.13.2. Two dimensional Differential gel electrophoresis (2D-DIGE)

2D-DIGE involves the pre electrophoretic labelling of samples with one of three spectrally distinct fluors, cyanine-2(Cy2), cyanine-3 (Cy3), or cyanine-5 (Cy5). The samples are prepared, each of which is first labelled with a different CyDye, and then combined. The sample containing the mixture is then subjected to gel electrophoresis and viewed individually by scanning the gel at different wavelengths, thus minimizing the problems with spot matching between gels. Image analysis programme then used to generate volume ratios for each spot. Volume ratios essentially describe the intensity of a particular spot in each test sample, and

thus enable expression differences to be identified and quantified (figure 1.12). CyDye DIGE fluors have a reactive N-hydroxysuccinimide ester group that forms a covalent bond with the ε-amino group of lysine side chains. The content of lysine is generally very high in most proteins such that an excessive amount of dye is required to direct the reaction to completion.

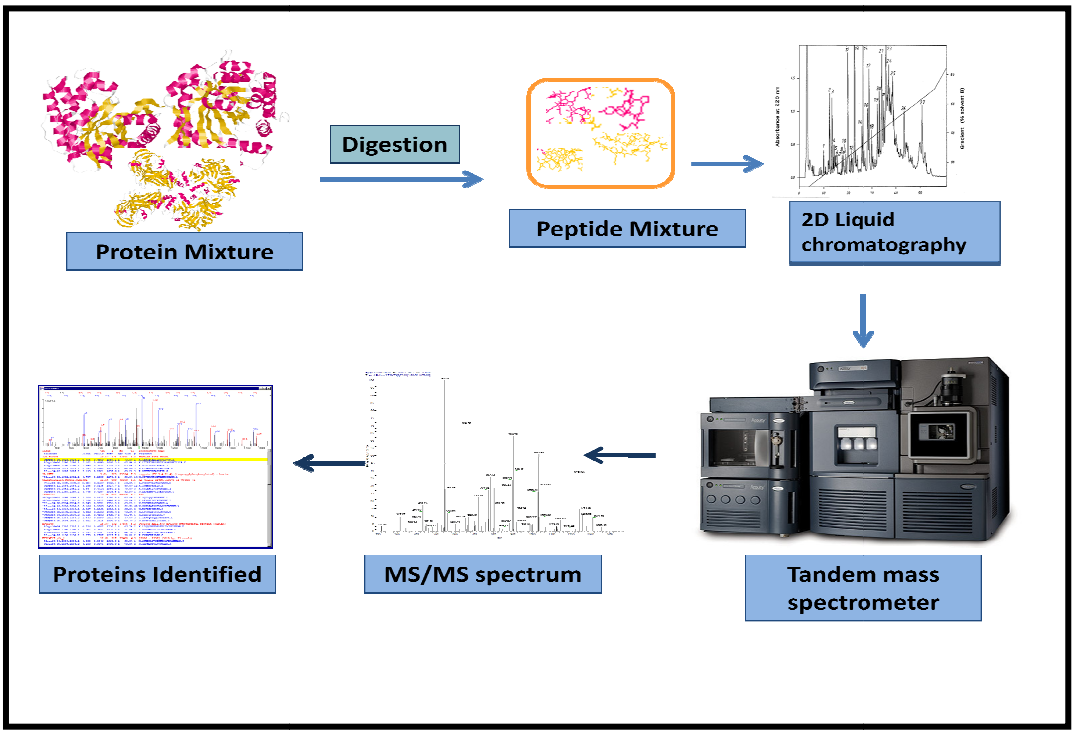
Figure 1.12: 2D Differential Gel Electrophoresis separation of Proteins



1.14.Mass spectrometry (MS): Mass spectrometry is a technique in which molecules are ionized and their mass to charge ratio is measured in order to determine the exact mass and then the molecule can be identified. In case of proteins, the sequence can be identified. The technique of mass spectrometry is a voluble tool in the field of proteomics. It can be used to identify proteins through variations of mass spectrometry techniques. In this, the protein is digested by a protease, such as trypsin and the peptides are then analyzed by peptide mass fingerprinting, collision induced dissociation, tandem MS and electro capture dissociation. Once the peptides masses determined the mass list can be sent to a

database, such as MASCOT, where the list is compared to the masses of all known peptides. Enough peptides should match that of a known protein to identify the protein. If the masses of peptides do not match a known protein, sequence the peptide by de novo sequencing using MS/MS methods. Another use of MS in proteomics is protein quantification. (Figure 1.13)

Figure 1.13: Mass spectrometry analysis of proteins – an overview



1.14.1. Components of MS: It is made up of three components: an ion source, mass analyzer and detector. After ionizing the sample, the ions of the sample are passed to the mass analyzer region where separation based on the mass-to-charge ratio occurs. Once separated by analyzer, then the ions enter the detector portion of the mass spectrometer. At this point the machine calculates the mass-to-charge ratio and the relative abundance of each of the different ions.

Ion source: Electron spray ionization (ESI), chemical ionization, thermal ionization, field desorption, matrix assisted laser desorption ionization (MALDI). MALDI and ESI are commonly used ionization methods in proteomics.

Mass analyzers: Mass analyzers are based on the principles of charged particles in an electric or magnetic field. Electric and/or magnetic fields are used for separating ions in gas phase. The mass analyzer is the part of the mass spectrometer. Mass spectrometers may be constructed with one or more analyzers, depending on the ionization to be used for. Instruments composed of two or more mass analyzers coupled together are known as tandem mass spectrometers. Different mass analyzers like scanning mass analyzers, Quadrapole mass spectrometers and time of flight (TOF) mass analyzers and ion traps (ITs) as a single or in tandem are commonly used in proteomic studies.

Liquid chromatography–mass spectrometry (LC-MS) is a chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used for applications in which very high sensitivity and selectivity required. Normally, it is used for the general detection and potential identification of chemicals in a complex mixture..

1.15. Clinical proteomics and Biomarker identification: Proteomics is the study of protein expression patterns, protein interactions and protein pathways in the blood, individual organ systems, tissue cells and also other body fluids. Proteomics accelerates the discovery and development of new biomarkers**.** Intracellular and extra cellular protein interactions can result in protein modifications, degradation, and protein coupling events that can be reflected in body fluids. A biomarker is a measurable indicator of a specific biological state. Biomarkers can be used clinically to screen for, diagnose or monitor the activity of diseases and to guide molecularly targeted therapy or assess therapeutic response.

The scope of clinical proteomics is to translate basic scientific knowledge into clinical applications for the benefit of the patient. The differentially expressed proteins that are either up regulated or down regulated significantly are picked up and validated further both in terms of sensitivity and disease specificity. The clinical proteomics involves translational proteomics and includes: (i) Early

detection/diagnosis of disease: The discovery and validation of novel protein biomarkers crucial for the early diagnosis and prognosis of many diseases that is essential for predictive diagnosis and preventive measures as well as monitor drug responses, (ii) Identification of novel targets for therapeutic intervention.

1.16. Tear proteomics in Dry eye Syndrome: Various studies to find out the biomarkers for DES are reported. Most of the proteomic studies using MS involve pooled tear samples and show alterations in major tear proteins like lysozyme, lactoferrin, lipocalins, lacritin, znic alpha glycoprotein, calcium binding proteins like S100 proteins, enolase. Characteristic DES proteins that play crucial role in the disease mechanism are not reproted. Since, DES is associated with Sjogren's syndrome, specific tear proteins characteristic to the type of DES are not reported. Therefore the current study focuses on the tear biomarker identification in dry eye syndrome associates with Non Sjogren's, Primary and secondary Sjogren's to RA, using 2DE based, mass spectrometry.

1.17. Gap In the Existing Research

Dry eye (DES) or keratoconjunctivitis sicca (KCS) could be due to various pathogenic mechanism. DES is also seen with co-morbidities like Sjogren's syndrome, rheumatoid arthritis and other autoimmune diseases, thyroid disease, diabetes mellitus. Moreover the heterogeneity of dry eye also challenges for better diagnosis and better treatment procedures for DES individual cases. The high degree of variability in patient populations creates data analysis much more complicate. Therefore, studies are required to understand the correlation between the disease severity and the possible tear component change. Because of this, there is necessity to look for new diagnostic tools that are specific and sensitive enough. Since DES associates with other co-morbidities such as Sjogren's syndrome (SS) and RA, the severity of DES also increases with these conditions, the proteins characteristic to these disease should also be identified.

Tear is the major protective barrier for ocular surface, and is reduced in DES. Therefore of the studies concentrated on tear fluid analysis to look for the variation

in the changes of proteins, lipids, mucins, small molecules metabolites. Proteins are the important molecules that play role in protection, stability and maintenance. Therefore altered tear protein profile in DES can give us the clue for the underlying pathology. Studies showed significant reduction in the major tear proteins that has antimicrobial role are lactoferrin, lysozyme, lipocalins, albumin, tear specific immunoglobulin, cytokines in DES. But DES disease specific and also DES-RA, DES-SS specific tear proteins are not identified so far. Therefore the current study may helps for better understanding the DES disease mechanism, by biomarker identification and for potential therapeutic applications. Apart from this biomarker identification may also helps to identify proteins that have prognostic value which can help in the dry eye management.

Thus this study aims to profile tear fluid protein, subjected to proteomic analysis using 2D DIGE based electrophoresis to find differentially expressed proteins in Dry eye, associated with primary, secondary Sjogren's and non Sjogrens syndrome.

CHAPTER 2: OUT LINE OF THE WORK

Human tears contain large number of proteins exerting significant influence on tear film stability, ocular surface integrity, and visual function. Proteins secreted by the lacrimal glands have been shown to contribute to the dynamics of the tear film in both health and disease. The possible mediators of lacrimal gland insufficiency in DES includes increased levels of pro inflammatory cytokines, production of auto antibodies, apoptosis, alterations in signaling molecules, hormonal imbalance and many others. Therefore alterations in the proteins profile are indicative of the disease mechanism and identification of marker protein can give clues on the disease severity as well as on the underlying pathology. Proteomic study using mass spectrometric analysis to identify protein biomarkers, further linking it to the disease activity as well as the treatment responses have been reported. However there are limited studies using tear as a specimen to identify such biomarkers. Dry eye syndrome (DES) is a multi factorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tears film instability with potential damage to the ocular surface. It is accompanied with increased osmolarity of the tear film and inflammation. Tear acts as good specimen to look for the tear protein changes in DES.

The proposed study collected tear using Schirmers strip in the recruited DES cases belonging to 3 different groups based on the aetiology namely non-Sjogren's (NSS), primary Sjogren's (PSS) and Sjogrens secondary to rheumatoid arthritis (RA-DES), as per clinical guidelines and criterions along with age and sex matched control group. Based on 2D-DIGE based proteomics, the samples were analysed for differentially expressed proteins. The DIGE analysis also allowed for the statistical analysis of the data to arrive at significantly differentially expressed spots, which were further validated for sensitivity and specificity in a set of 2D gels that were unpooled individual samples representing the various groups. Many of these proteins were then identified by mass spectrometry. Apart from these, further clues on differentially expressed protein were also arrived by in sol digestion of the tear

followed by LC/MS analysis and a Venn diagram shows the characteristic proteins in each group based on the identified ones though there are more yet to be identified. A few of these proteins were then validated by looking for clinical correlations, ELISA and *in silico* analysis for the interacting molecules and therefore validation by disease relevance. Two such molecules namely LPRR4 characteristic of all types of DES which is significantly lowered and RN-tre protein characteristic of primary Sjogrens syndrome were validated.

This is a comprehensive study that attempts to look for potential biomarkers of DES based on proteomics approach in tear that has come out with 2 novel proteins associated with DES that comprises all the 3 major types of DES using tear as a specimen.

2.1. OBJECTIVES

1. To compare the method of tear collection using Capillary tube and Schirmer strip based on the protein profile in 2D electrophoresis.

2. To profile the proteins in the tear of the Dry eye syndrome patients associated with

   a. Non-Sjogren's syndrome,

   b. Primary Sjogren's syndrome and

   c. Secondary Sjogrens due to Rheumatoid Arthritis and compared with the tear protein profile of healthy controls who are age and sex matched using two dimensional gel electrophoresis (2D) and 2D-Differential Gel Electrophoresis (DIGE) based proteomics approach and identify the differentially expressed proteins .

   and To identify the differentially expressed tear proteins by mass spectrometry using LC-MS/MS analysis

3. To validate potential biomarkers by

   a. estimating the protein in the disease by ELISA

   b. look for disease correlation in terms of alterations based on severity

   c. structure function analysis of the potential biomarker and identify the interacting proteins based on *in silico* analysis so as to identify the role of the biomarker in the disease mechanism

4. To study the tear specific chemokines involved in various types of dry eye syndrome

CHAPTER 3: METHODOLOGY

3.1. Patient recruitment

This study involved use of clinical specimens for the clinical, biochemical and proteomic analysis. Therefore recruitment of patients played an important role. A prospective case controlled study for a period of 3 years since 2008 was conducted at the cornea services at Sankara Nethralaya , a tertiary care hospital at Chennai so as to recruit patients with Dry Eye Syndrome and they were grouped along with age matched healthy volunteers as control.

This study involved the patient recruitment at 2 Centres A and B as given below.

3.1.1.

A. Dry eye (DES) patient recruitment at Ocular surface clinic (OSC) of the eye hospital, Chennai

- DES cases were recruited based on the inclusion criteria, after the complete DES investigations, diagnosis by the Ophthalmologist.
- Query cases of DES with Sjogren's were sent to RA clinic for the confirmed diagnosis as DES with primary Sjogren's syndrome (pSS) or secondary to RA, by the Rheumatologist
- For the Diagnosis of primary or secondary Sjogren's to RA, 8 biochemical tests were done at Biochemistry research Department. The 8 tests were antibodies to Rheumatoid factor (RF), Anti nuclear antibody (ANA), C - reactive protein (CRP), antibodies to double stranded DNA (dsDNA), SSA and SSB, cyclic citrullinated peptide (anti-CCP), alpha fodrin.
- Tear samples collected from the Confirmed diagnosed cases of DES and DES with RA, DES-pSS for the biochemical and proteomic analysis
- Clinical proforma of all the recruited cases was filled by the clinician at the clinic with all the details after detailed investigations for DES.

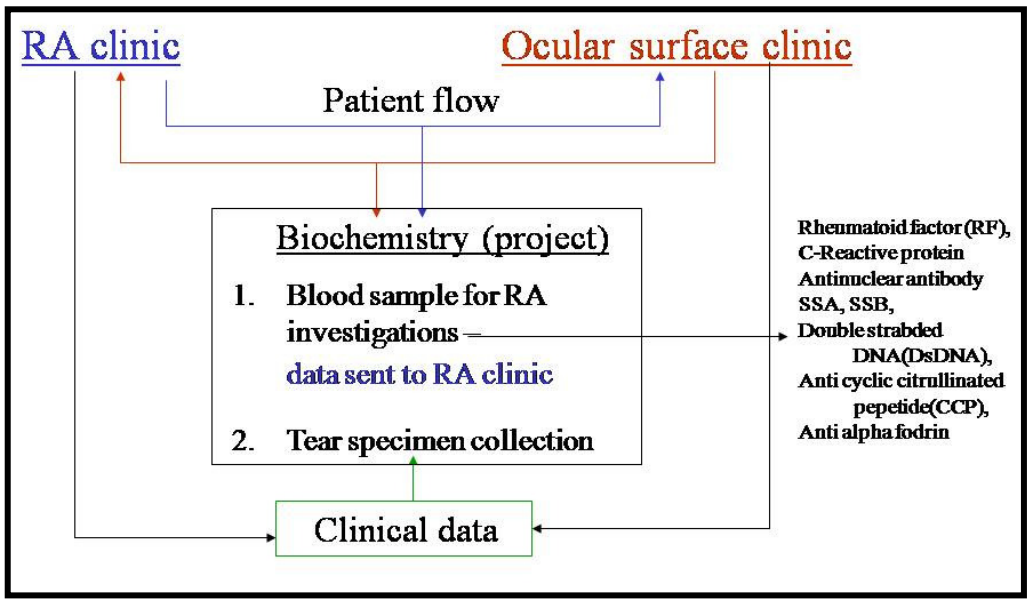
B. Rheumatoid Arthritis (RA) cases at RA clinic, Chennai

- RA cases were recruited based on the inclusion criteria by the Rheumatologist and the RA cases were sent to Ocular surface clinic for DES diagnosis by doing a detailed work up which is a panel of clinical investigations. Clinical proforma of RA cases were completed by the rheumatologist at RA clinic.

- Tear samples were collected from RA diagnosed cases as reported by the Rheumatologist at the Biochemistry department in the hospital.

  Research Department received from the RA clinic and the patient recruitment flow shown in figure 3.1.

Figure 3.1: Patient recruitment for tear sample collection



C. Study design: After patient recruitment they were grouped as seen in table 3.1

Table 3.1: Number of patients recruited during the study according to the study design.

	Group 1 RA with DES	Group II RA without DES	Group III DES alone	Group 1V Primary SS	Normal Controls
Sample size planned	30	30	30	30	
Samples collected	34	4	40	26	47
Mean age/sex	50 ± 10 yrs 12M,22F	50 ± 10 yrs 4 F	39 ± 17 yrs 16M,24F	49 ±11 yrs 12 M,14 F	49 ± 10 yrs 18M,29F

3.1.2. Inclusion and exclusion criteria to recruit DES and RA patients and Grading

A. For diagnosis of DES : slit lamp examination, Schirmers test, TBUT, Corneal staining were done and documented in clinical proforma used

(i) Inclusion Criteria

Based on DEWS 2007 diagnostic criteria for Sjogren's syndrome the DES grading was done as mentioned below.

Grading	Schirmers	TBUT	Corneal staining
Level 1 Mild to Moderate	>10mm	> 10 sec	1
Level 2 Moderate to severe	5-10mm	5 -10 mm	2
Level 3 Severe	2 -5mm	< 5 sec	3
Level 4 Severe	2 mm	< 5 sec	4

(ii) Exclusion Criteria: patients with the following were excludes from the study.

Surgical Intervention, Intraocular surgery, Chemical injury, Less than 18 years, Other connective tissue diseases (other than RA), Diabetes mellitus, Contact lens wearers, Hyperthyroidism, Anti histamine/anti depressant/ Anti hypertensive, Parkinson's disease.

B. For diagnosis of RA

(i) Inclusion Criteria**:** Based on the ACR Classification Criteria of Functional Status in RA (2008) as given below

Class I:	Completely able to perform usual activities of daily living (self-care, vocational, and avocational)
Class II:	Able to perform usual self-care and vocational activities, but limited in avocational activities
Class III:	Able to perform usual self-care activities, but limited in vocational and avocational activities
Class IV:	Limited ability to perform usual self-care, vocational, and avocational activities

RA patients recruited in the study were classified in two different methods. But in both cases the clinical presentation / bone deformities were taken into consideration for the diagnosis. (Baillieres Clin Rheumatol.1996; 10:435-53)

- *Based on the Rheumatoid factor (RF)* – A. Seropositive RA: RA cases positive for RF; B. Seronegative RA: RA cases negative for RF.

- *Based on the radiological joint damage*: Mostly, this parameter is used to monitor the degree of bone erosion in, progression of the disease, drug effect during the disease condition. This may be A. Erosive B: Non erosive

Since this study is to look for the biomarkers from tear fluid and not to study the effect of drug monitoring on RA prognosis, the radiological scoring was not done.

C. For differentiating primary from secondary sjogrens and to recruit the primary sjogrens

- The criteria for the diagnosis of primary and secondary sjogrens according to the American-European Consensus Group includes the presence of at least 4 rules out of 6 as mentioned below (Vitali et al 2002).

  Rule 1: ocular symptoms
  Rule 2: oral symptoms
  Rule 3: oral signs
  Rule 4: presence of anti Ro (SSA) anti La (SSB) antibodies
  Rule 5: histopathology
  Rule 6: salivary gland involvement

In this study first 4 rules for the diagnosis of sjogrens syndrome were taken and not histopathological staining (salivary gland biopsy) was done.

Exclusion Criteria: With other connective tissue disorder other than RA Other systemic diseases like Diabetes, Thyroid disease.

3.1.3. Clinical proforma of both DES and RA were enclosed as appendix I and II end of this Chapter 3.

3.1.4. Consent letter used to recruit the patients enclosed at as appendix III end of this chapter 3

3.2. Tear fluid Collection

3.2.1. Materials and Buffers

- 10 µl calibrated capillary tubes
- Sterile Schirmer strips
- Sterile vials

- Composition of Buffers

1. **10 mM Phosphate buffered saline (PBS- pH:7.4)**

0.13M NaCl (m.wt:58.5)	-	8 g
2.6 mM KCl (m.wt: 74.5)	-	0.2 g
8 mM Na_2HPO_4 (m.wt : 141.96	-	1.15 g
1 mM KH_2PO_4 (m.wt : 137.2)	-	0.24 g
Ultrapure Water	-	1000 ml

2. **10mM HEPES Buffer (pH: 7.0)**

10 mM HEPES (m.wt:238.3)	-	11mg
1 mM EDTA (m.wt:372.24)	-	1.8 mg
1mM DTT (m.wt:154.2)	-	0.7 mg
Ultra pure Water	-	5 ml

3. **8M Urea Buffer (pH: 7.6)**

8 M urea (M.wt: 60)	-	2.4 g
3 % CHAPS (M.wt:614.6)	-	0.2 g
25 mM DTT (M.wt: 154.2)	-	19.3 mg
Ultrapure water	-	5 ml

4. **Protease Inhibitor Cocktail: commercially available**

   1 mg/ml in ultra pure water

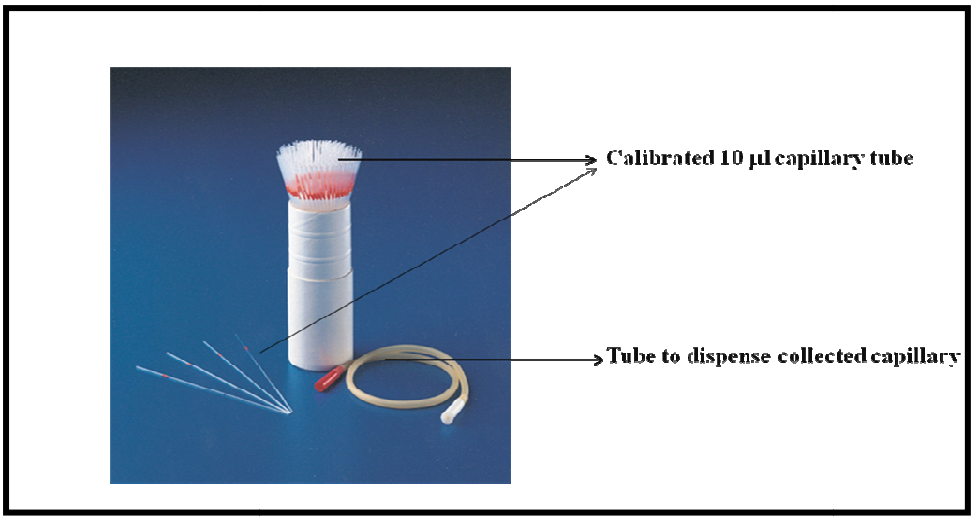
5. **Amicon 3 kDa desalting cut-off filters**

3.3. Collection of tear fluid.

3.3.1 Capillary tear collection: cases or control subjects were seated, with eyes widely open and capillary tears collected from the cul de sac or fornix region as mentioned below.

10 µl calibrated capillary tubes were placed in the cul-de sac region for 10-15 min to get the capillary tears. The collected capillary tear then placed in a sterile vial, and dispensed using the tube provided with the capillary tubes as seen figure 3.2, capillary tear in the sterile vial was centrifuged at 5000 rpm for 10 min at 4°C, supernatant was stored at – 80° C until processing the sample.

Figure 3.2.: Calibrated Capillary tubes for tear collection



3.3.2. **Schirmer tear Collection: Reflex tear:** Sterile 35 X 5 mm Schirmer strips were placed in lower lateral conjunctival region of the eye for 5 minutes in closed eye condition. After 5 min the strip was removed and placed in the sterile vial, stored at -80 °C until the analysis.

3.3.3. Precautions during capillary and Schirmer Tear collection: Subject was seated comfortably in resting position, no direct light was allowed to fall during the collection of tear samples. Other reflex tearing including emotional, such as laugh or any other chemical reflexes were avoided.

3.3.4. Samples were Stored at -80°C until analysis

3.4. Tear protein extraction from the Schirmer strip: Tear protein from Schirmer collected tear was extracted using buffers such as PBS, HEPES and Urea buffer. To the Schirmer strip with tear, added 300 µl of buffer and 30 µg of protease inhibitor cocktail, left at 4 °C for 3 hrs with an intermittent mixing. After 3 hrs of incubation, the Schirmer strip was properly drained and removed. The extracted proteins were centrifuged at 5000 rpm for 10 min at 4 °C. Then the supernatant was separated into new sterile vial.

3.5. Desalting of extracted tear protein: The extracted proteins were then subjected to desalting using 3kda cut-off filters. The cut-off filters were initially washed with double distilled water at 4 °C with 10,000 rpm for 5 min. Whole supernatant after extraction from the Schirmer strip was added to the cut-off filter, centrifuged at 4 °C with 10,000 rpm for 30 min. Then the desalted, concentrated tear protein was collected in another sterile vial. Protein estimation was done using Bradford method, aliqoted and stored at – 80 °C until processing for the analysis.

3.6.Tear protein Estimation:

3.6.1. **Lowry's method (Lowry et al 1951): Principle:** The amino acids tryptophan and tyrosine reacts with folins reagents in the presence of alkaline copper reagent and forms blue colored complex which was read at 660 nm in a spectrophotometer.

Reagents:

1. Standard Bovine serum albumin (BSA) – 0.1 % of BSA in 100 ml water
2. 2 % Sodium carbonate ($Na_2 CO_3$) in 0.2 N sodium hydroxide (NaOH) solution
3. 0.5 % $CuSO_4$ in 1 % tri sodium citrate
4. Alkaline copper reagent: Mix of 49 ml of 2% Na_2CO_3 + 1 ml of 0.5 % $CuSO_4$ - should be prepared freshly before the addition
5. Folins reagent - – commercially available. 1:1 dilution with water - should be prepared freshly before the addition to the standards/ samples.

Table 3.2: Protocol for Lowry's method

Reagents	Blank	S1	S2	S3	S4	S5	Test
Std BSA (ml)	---	0.05	0.1	0.15	0.2	0.25	---
Std BSA amount (µg)	---	50	100	150	200	250	---
Water (ml)	1.0	0.95	0.9	0.85	0.8	0.75	0.9
Test Sample	---	---	---	---	---	---	0.1
Alkaline reagent (ml)	5	5	5	5	5	5	5
INCUBATE FOR 10 MIN AT ROOM TEMPERATURE							
Folins reagent	0.5	0.5	0.5	0.5	0.5	0.5	0.5
INCUBATE FOR 20 MIN AT ROOM TEMPERATURE							
OD at 660 nm							

3.6.2. **Bradford method(Bradford 1976): Principle:** Coomassie dye binds to the basic amino acids like lysine, arginine of protein in an acidic medium, an immediate shift in absorption maximum occurs from 465 nm to 595 nm with a concomitant color change from brown to blue, based on the amount of protein present which can be read at 595nm.

Reagents

1. Normal saline – 0.9% NaCl in 100 ml distilled water.
2. 0.1 % BSA standard in 100 ml deionised water
3. Bradford reagent (commercially available) – Coomassie brilliant blue G250

Table 3.3: Protocol for Bradford protein estimation

Reagents	Blank	S1	S2	S3	S4	S5	Test
Std BSA (µl)	---	2	4	6	8	10	---
BSA amount (µg)	----	2	4	6	8	10	---
Normal saline (ml)	0.5	0.48	0.46	0.44	0.42	0.4	0.45
Test Sample (µl)	---	---	---	---	---	---	5
Bradford reagent (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
INCUBATE FOR 10 MIN AT ROOM TEMPERATURE							
OD at 595 nm							

3.6.3. **Bicinchonic acid method (Smith et al 1985): Principle**: this reaction combines the well-known reduction of Cu^{+2} to $Cu^{+1}$by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562nm that is nearly linear with increasing protein concentrations over a broad working range. The macromolecular structure of protein, the number of peptide bonds and the presence of four particular amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for color formation with BCA.

Reagents:

1. BCA Reagent A: Containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide
2. BCA Reagent B, 25mL, containing 4% cupric sulphate
3. Preparation of the BCA Working Reagent: 50 ml of BCA reagent A + 1 ml of BCA reagent B just before use
4. Standard albumin 1mg/1 ml

Table 3.4: Protocol for BCA protein estimation

Reagents	Blank	S1	S2	S3	S4	S5	Test
Std BSA (µl)	---	2	4	6	8	10	---
BSA amount (µg)							
Normal saline (ml)	0.5	0.48	0.46	0.44	0.42	0.4	0.45
Test Sample (µl)	---	---	---	---	---	---	5
Bradford reagent (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
INCUBATE FOR 10 MIN AT ROOM TEMPERATURE							
OD at 562 nm							

3.7. SDS-PAGE(Weber & Osborn 1969)

3.7.1. Principle: This is method of electrophoresis. Polyacrylamide gels are prepared by the free radical polymerization of acrylamide and cross-linking agent N N' - methyl bis acrylamide with Chemical polymerization controlled by initiator catalyst system - APS and TEMED.

3.7.2. Reagents:

- 30% Acrylamide: 29.2g of acrylamide and 0.8 g of bis –acrylamide in 100 ml of deionized water
- 1.48 M Tris-HCl pH 8.8: 18 g of Tris base dissolved in 100ml of deionised water. Adjust the pH to 8.8 using conc. HCl (m.wt of Tris base: 121.14)
- 0.49 M Tris-HCl pH 6.8: 6 g of Tris base dissolved in 100ml of deionised water. Adjust the pH to 6.8 using conc. HCl
- 10 % Sodium Dodusyl sulphate : 1 g of SDS in 10 ml of deionised water
- 10 % Ammonium Per Sulphate: 1 g of APS in 10 ml of deionised water
- TEMED
- Saturated Butanol: to 1-butanol add distilled water till it forms saturated solution.
- **SDS Sample buffer**

Tris HCl (pH:6.8)	-	1.0 ml
10 % SDS	-	1.6 ml
d H2O	-	4.0 ml
Glycerol	-	0.8 ml
Beta mercaptoethanol	-	0.4 ml
Bromo phenol blue	-	0.2 ml

Sample buffer: sample is mixed in the ratio 1:3. And then boiled for 3 minutes before loading.

- **SDS-PAGE running buffer (pH: 8.6)**
  Tris base – - 3.1 g
  Glycine - - 14.4 g
  SDS - 1 g
  Deionised water - 1000 ml

3.7.3. Preparation of PAGE gel

Table 3.5: preparation of PAGE separating and staking gel

Reagents	13 % separating gel for 10 ml solution	13 % separating gel for 300 ml solution	Staking gel preparation for 10 ml solution
30% Acrylamide (ml)	4.33	129.9	1.33
Tris HCl (pH 8.8) (ml)	2.5	75	------
Tris HCl (pH 6.8) (ml)	------		2.5
Deionised H_2O (ml)	3.045	91.35	6.0 15
SDS (µl)	100	3000	100
APS (µl)	50	1500	50
TEMED (µl) (to be added at the end)	5	150	10

3.7.4. Casting of the gel

- Clamped the glass plates, sealed the plates at the bottom end with agarose. Left it for 5 min.
- Poured the prepared, required 13% acrylamide gel into the clamed plated, overlaid with saturated butanol, left for 30 – 40 min at room temperature**.**
- After polymerization of separating gel removed the saturated butanol, washed with distilled water. Then poured the stacking gel and then place the combs immediately, left for polymerization for 10 min at room temperature. After polymerization remove the combs, wash the wells with distilled water.
- Boiled the sample of 5 µg with 1 µl of sample buffer, boiled for 3 min, cooled and loaded in the wells, added the running buffer Tris-Glycine-SDS buffer and kept for focussing with 120 Volts at RT for 2 hrs. Then the gel stained with silver staining.

3.8. Protocol for Silver staining (Rabilloud et al 1992)

Principle: after electrophoresis, proteins in the gel were fixed using methanol:acetic acid:water. Then it was thoroughly washed and exposed to silver nitrate solution. Silver ions bind with sulphydryl and carboxyl group of proteins. This was enhanced by the use of sodium thiosulphate. When the gel was developed in an alkaline medium, with formaldehyde, in which silver ions were reduced to metallic silver and form black precipitate indicating the presence of protein bands

Reagents:

1. Fixative: 40 % methanol + 10 % Acetic acid made up to 100 ml with deionised water
2. Activator : 0.02 % Sodium thiosulphate (hypo) (20 mg / 100 ml) deionised water
3. Staining reagent: 0.2 % Silver nitrate in 100 ml deionised water + 75 µl of 40 % formaldehyde to 100 ml of silver nitrate solution. Formalin should be added freshly just before adding to the gel.
4. Developing solution: 2 % Sodium carbonate in deionised water + 50 µl of 40 % formaldehyde to 100 ml chilled sodium carbonate solution.
5. Stopping solution: 10% acetic acid in deionised water.

Procedure:

1. After the PAGE, the gel was removed from the plates, placed in the fixative for 30 min or up to 12 hrs at RT.
2. Washed the gel with deionised water for 2 hrs at RT, with 3 water wash changes.
3. Added hypo for 30 sec at RT immediately washed the gel twice with deionised water.
4. Added staining solution left in dark for 20 min at RT.
5. Washed the gel thrice with deionised water.

6. Added chilled developing solution to the gel, left the gel in the developing solution till the black/brown spots are seen on the gel.
7. Stopped the reaction with 10 % acetic acid when the desired spot intensity occurred.

3.9. Two Dimensional gel Electrophoresis (2DE) (Gorg et al 1988)

3.9.1. Principle: Two-dimensional electrophoresis (2D electrophoresis) is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. This technique sort's protein according to two independent properties in two discrete steps: the first-dimension step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (pI); the second-dimension step, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), separates proteins according to their molecular weights (MW). Each spot on the resulting two-dimensional array corresponds to a single protein species in the sample.

3.9.2. REAGENTS

1. Rehydration Buffer: proteins to be focussed rehydrated with the buffer components

8M Urea
3% CHAPS
25 mM DTT
0.2% ampholytes,
Bromophenol blue (0.1mg/mL) few drops

2. Immobilized pH Gradient (IPG) strips (Commercially available):

(i) 7 cm IPG strips pH: 3-10
(ii) 11 cm IPG strips pH 3-10 and pH 3-6
(iii) 17 cm IPG strips pH 3-10, pH 3-6 and 7-10

3. 20 % Trichloroacetic acid (TCA) in deionised water

4. Mineral Oil (Commercially available)

5. **Equilibration buffer I:** to equilibrate the IPG strip with the PAGE buffer, that was focussed with urea buffer and keep the proteins in the reduced state by using DTT.

6M Urea
2% SDS,
0.375M Tris-HCl,
20% Glycerol
2% DTT

6. **Equilibration buffer II:** to equilibrate the IPG strip with the PAGE buffer, this was focussed with urea buffer, so by alkylating, to stabilize the reduced proteins, iodoacetamide was used which prevents further oxidation.

6M Urea
2% SDS
0.375M Tris-HCl
20% glycerol
2% Iodoacetaamide

7. **Over laying agarose:** 0.5 % low melting agarose in 100 ml of SDS-PAGE running buffer with 0.01% bromophenol blue.

8. **SDS-PAGE running buffer** (pH: 8.6)

Tris base – - 3.1 g
Glycine - - 14.4 g
SDS - 1 g
Deionised water - 1000 ml

3.9.3. Sample Preparation for 2DE

- The tear protein extracted and then desalted from Schirmer strip used for 2DE. Table 3.6 shows the amount of protein added on various lengths of IPG strips both for silver and coomassie staining.
- The protein after quantified from tear sample was less and therefore silver staining was done after 2DE.Therefore the amount of protein used for 7 cm and 11 cm IPG strip was 50 µg initially, then standardized to 20 µg which gave good resolution of protein spots on the gel. For 17 cm IPG 30 µg of tear protein showed good resolution (table 3.6).

- The required amount of protein was mixed with the rehydration buffer and then buffer containing sample was added to the IPG strips. The volume of total rehydration buffer with the sample to be added to the IPG strip varies with its length. This is mentioned in table 3.7.

Table 3.6: Amount of Protein loads for IPG strips (Bio-rad manual)

IPG Strip Length	Amount of protein for Coomassie staining Recommended (µg)	Amount of protein for Silver staining Recommended (µg)	Amount of tear protein standardized for silver staining (µg)
7 cm	200 – 500	10 -100	15 - 20
11 cm	250 - 1000	50 – 200	20
17 cm	1000 - 3000	100 - 300	30

Table 3.7: Volume of rehydration buffer to be added to IPG strips

IPG Strip Length	Volume of sample + Rehydration Buffer Recommended (µl)	Volume of sample + Rehydration Buffer standardized (µl)
7 cm	125	125
11 cm	185	180
17 cm	300	285

3.9.4. Passive Rehydration

- Brought the IPG strips from – 20 °C to room temperature. **Note:** should not be kept at RT more than 20 min after removed from – 20 °C.

- Meanwhile, the IPG strips were getting thawed, the sample for rehydration can be prepared and kept ready for loading. The required amount of protein sample was mixed with the rehydration buffer gently, centrifuged at 5000 rpm for 3 min. The supernatant was used for rehydration.

- The sample was added to the rehydration tray without any air bubbles, in the specified sample loading well. The plastic sheet of IPG at lower end was removed gently without touching the gel and the IPG strip was placed over the sample carefully in such a way that the sample was spreaded over the IPG strip uniformly without any air bubbles and left it at RT for 10 – 15 min to absorb the sample by IPG strips. After 15 min 3 - 4 ml of mineral oil was added to the strips, left it at RT for 14 - 16 hours

  Note: Used non powdered, nitrile glove during 2DE procedure to avoid keratin contamination

3.9.5. Isoelectric focussing of IPG strips

- After 14 – 16 hrs of passive rehydration, the IPG strips were removed from rehydration tray and the mineral oil was drained out.
- Kept it on the IEF focussing tray (figure 3.4) by gel side facing upwards. Placed sterile paper paper wicks (commercially available) at the end of the IPG strips. Added 8 µl of nano pure water to the filter papers placed on 7 and 11 cm IPG strip/10 µl of nano pure water to the filter paper wicks placed on 17 cm strips. Placed the movable electrodes on the filter paper wicks at the end of the IPG strips. Added 3 – 4 ml of mineral oil to the IPG strips. Applied the voltage for focussing of proteins based on pI in the IPG strips. Focussing voltage varies with the length of the strip. The recommended voltage mentioned in table 3.8.
- The current per strip was set as 50 µA. This current limit per strip should not be exceeded while focussing the proteins.
- After isoelectric focussing, the strip was removed from the focussing tray, drained the mineral oil and processed for 2^{nd} dimensional SDS-PAGE immediately.

Note: The strip was stored at - 80°C for a week then subjected to PAGE, when it not proceeded for the second dimension immediately.

Table 3.8.: Focussing conditions for IEF.

IPG strip length (cm)	Stage 1 (Conditioning step - linear Voltage ramping)		Stage 2 (Focussing step Linear voltage ramping)		Stage 3 (Final Focussing -Rapid voltage ramping)
	Volts	Time (min)	Volts	Time (hours)	(Volt hours)
7	250	20	4,000	2.00	10,000
11	250	20	8,000	2.30	20,000
17	250	20	10,000	2.30	40,000

Figure 3.3: Isoelectric focussing (IEF) apparatus.

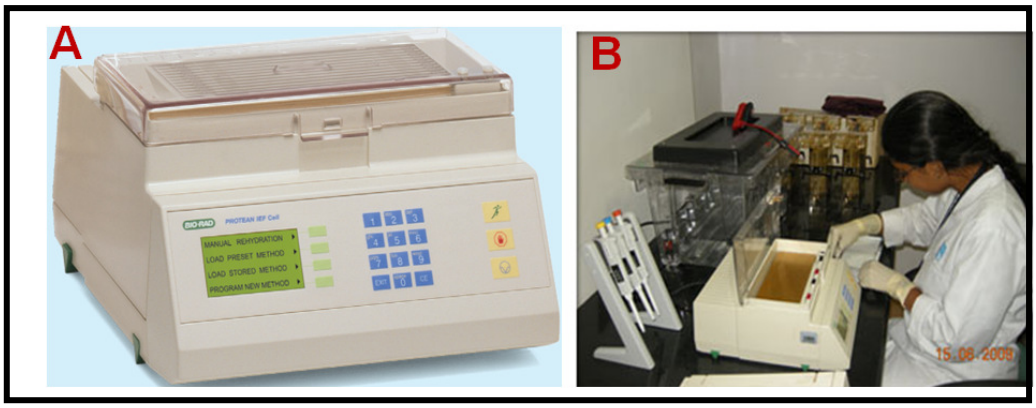


Figure 3.3: A. IEF focussing tray protean IEF cell for focussing of IPG strip. B. Settings for focussing IPG strip and placing of the tray.

3.9.6. Equilibration of the IPG strip

- The focussed strip was then, placed in the rehydration/equilibration tray, added 3-4 ml of equilibration buffer I, and kept for rocking for 30 min at RT.

- After 30 min, buffer was drained out, then added equilibration buffer II, and kept for rocking for 30 min at RT.

3.9.7. SDS-PAGE of focussed IPG strip

For 7 and 11 cm IPG, normal PAGE apparatus was used. The casting of apparatus and pouring of gel mentioned in section 3.7.3 and 3.7.4.

3.9.8. SDS-PAGE of focussed 17 cm IPG strip using Bio-Rad Protean II Xi unit

(i). Assembling of protean Xi unit for PAGE

Protean II Xi unit is PAGE apparatus for 17 cm IPG strips. " This unit allows 18.5 cm wide x 20 cm long gels to run on Bio-Rad's PROTEAN II xi Cell thus accommodating commercially available immobilized pH gradient gel strips for 2-D second dimension protein separations. This unit contains

- Wide sealing gaskets for the upper buffer chamber (2)
- 20 cm glass plates (2 sets)
- 20 cm notched clamps (2 sets)
- Foam gaskets for the PROTEAN II xi Casting Stand (2)
- Alignment card
- Narrow spacers (4) 2 mm, Prep Combs (2) 2 mm

The PROTEAN II xi multi-gel casting chamber was used to cast twelve 1.5 mm gels simultaneously. Acrylamide monomer was poured from the top prepare identical uniform percentage gels.

(ii). Preparation for casting of gels

Before use, the casting chamber parts cleaned with soap water, rinsed the parts thoroughly with deionized water, and allowed them to dry completely. During casting the chamber with glass plates was placed on an even surface and poured the prepared 300 ml of 13 % acryl amide gel for 2^{nd} dimension PAGE for protean II Xi unit. Over layered with 3 ml of deionised water and left it at RT for polymerisation for 30 – 40 min. Once the gels polymerised used immediately for 2^{nd} dimension PAGE or stored at 4°C if not processed the IPG on the same day. Protean II Xi accommodated 6 gels to run at a time with 3 multi cells. Each cell accommodates 2 gel sandwiched glass plates as seen in the figure 3.4..

Figure 3.4: Protean II XI multi cell for focussing 2nd dimension gels

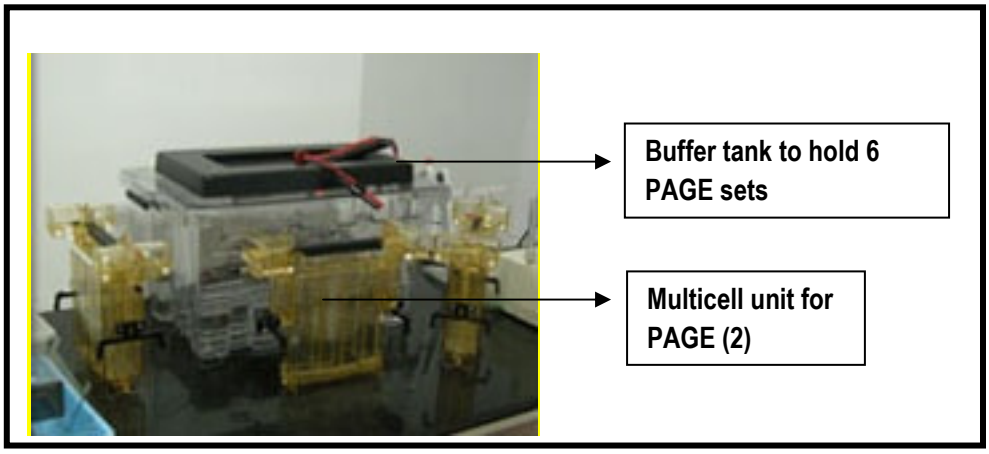


Fig 3.4: Each multi cell as shown in the picture accommodates 2 slab gels with acrylamide gel, and a total of 6 gels at a time in the buffer tank.

(iii) Equilibration of the focussed IPG Strip

The focussed IPG strip was equilibrated using 3 ml of equilibration buffer I and then with equilibration buffer II for 30 min each at RT before 2nd dimension SDS-PAGE.

(iv) Placing of IPG over the Gel for 2nd dimension

After the equilibration, the strip was dipped in SDS-PAGE running buffer for 3 times, then placed over the 13% polymerised acrylamide gel. The strip was placed such that, the IPG strip was in contact with the PAGE gel. 0.05 % low melting agarose with bromophenol blue was layered over the strip to avoid formation of gap between the PAGE gel and IPG the strip. Then the multi cell with the focussed IPG strips was placed in the SDS-PAGE unit, electrophoresis was carried at temperature maintaining below 16°C with focussed current per gel was 16 µA initially for 30 min, then increased the current to 20 µA per IPG strip for rest of the focussing for 5hrs. Once the tracking dye reached the end of the gel, current was stopped, the gel was removed from the plates then preceded for staining using silver stain.

(viii) Staining of gels – silver staining done as described in this chapter in section 3.8

(ix) Gel documentation

Once the gel was stained using silver stain, the developed gels were scanned and documented using Bio-Rad's densitometer GS800.

(x) Settings for scanning of the gel and analysis:

The gel was placed on the scanning area, and then the resolution option 127.0 X 127.0 for the large gels was selected, scanned and saved the image. Prior to PD quest analysis, the images were transformed with specific measurements of high slider as 4, low slider 0.96 and gamma slider as 1.5. the spot intensities and differential spots were analysed using PD Quest soft ware.

3.10.Two - Dimensional Differential gel electrophoresis (2D-DIGE) (Marouga et al 2005)

3.10.1. **Principle:** Two-dimensional DIGE is based on fluorescence prelabeling of protein mixtures before 2D gel electrophoresis. Protein samples are labelled with up to three spectrally distinct, charge and mass-matched fluorescent dyes known as CyDye DIGE fluors. The labelled proteins are then mixed and separated simultaneously on the same 2D gel. The different protein extracts labelled with different CyDye DIGE fluors can then be visualized separately by exciting the different dyes at their specific excitation wavelengths. This is achieved by use of an imager containing appropriate laser wave lengths for exciting the different dyes and filters for collecting the light emitted. Each dye generates digital images of each individual sample. The sensitivity of CyDye DIGE fluor minimal dye Cy2 is 0.075 ng, for Cy3 0.025 ng, and for Cy5 0.025 ng. Compared with silver staining. Major advantage of this technique is its ability to substantially reduce the effects of gel to gel variation on the quantitation of a protein spot on a gel.

3.10.2. Reagents for 2D-DIGE

1. Cy dye minimal labelling kit 2 nmol and 5 nmol– contains Cy 3, Cy 5 and Cy 2
2. 30 mM Tris-HCl buffer (pH:8.5)
3. 0.5 mM Tris (2-carboxyethyl) phosphine (TCEP)
4. 8M Urea
5. 3 % CHAPS
6. Extraction buffer: 30 mM tris-buffer containing 8M urea, 3 % CHAPS, 0.5 mM TCEP and finally added 30 µg of protease inhibitor cocktail.
7. N, N-Dimethyl formamide (DMF, fresh chemical. < 3 months old)
8. IPG strips pH 3-10
9. Protease inhibitor cocktail: 1 mg / ml

3.10.3. Extraction of Tear protein for DIGE

The tear collected using Schirmer strip were extracted for the proteins using 30 mM Tris-HCl extraction buffer (pH: 8.5) with 8M urea buffer, and the extraction procedure was similar as in section 3.3. Pooled tear protein of both control and dry eye tear protein was used for DIGE. Pooled 3 of the controls and then separately 3 of the DES groups desalted using 3 kDa filters. The retentate was used for protein estimation, and then used for labelling with Cy dyes

3.10.4. Reconstitution Cy dyes

The Cy dyes of 2 nmol and 5 nmol minimal labelling kit contains Cy 3, Cy 5 and Cy 2.The Cy dyes were supplied in powdered form in amber colour vials to protect from light. To 2 nmol Cy dye added 3 µl of DMF, cyclomixed for 1 min, spun for 30 sec at RT. To 5 nmol Cy dyes added 6 µl DMF , cyclomixed for 1 min, spun for 30 sec at RT. From this stock, the required amount was diluted in DMF itself and used for labelling. For 30 µg of tear protein 240 pmol of each Cy dye was used. The stock Cy dyes were stored at – 20 °C for 3 months. But the diluted Cy dyes were stored at – 80 °C for 1 week.

3.10.5. Labelling of tear proteins with Cy dyes

30 µg of control and 30 µg dry eye tear protein was used for label with Cy dyes for DIGE. 15 µg of each control and the diseased protein was pooled, labelled with Cy 2 which acts as an internal standard. The combination of tear protein labelling with Cy dyes showed in table 3.9 to compare the groups such as Control vs. DES-Non SS, control vs. DES-primary SS and control vs. DES-RA, DES-Non SS vs. DES-RA, DES-pSS vs. DES-RA, RA alone vs. DES-RA to look for the differential proteins expressed between these groups. Also done the swaping of labelling look at the differences. This swaping showed no significant differences. Table 3.10 showed the labelling of tear protein in various groups of DES.

Table 3.9: combination of Cy dyes labelling in control vs. DES groups for differential protein expression

1	Control	Non SS -DES
2	Control	Non SS -DES
3	Non SS -DES	control
4	Control	Primary SS-DES
5	Control	RA SS-DES
	Sub group	
	RA without DES	RA with DES (severe)
	RA mild DES	RA severe DES
	Primary	RA-DES

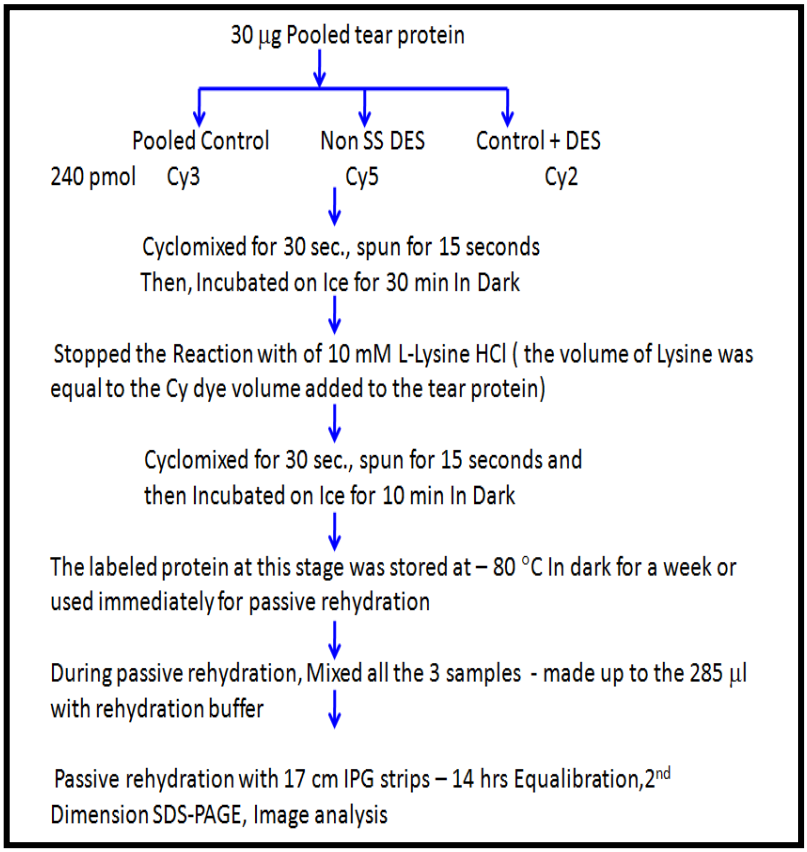
The protocol of labelling of tear protein was shown in figure 3.11. The labelling of protein with Cy dye carried out at RT in dark conditions to prevent the leaching of fluorescent Cy dye. To 30 µg of tear protein, added 240 pmol of either Cy 3, Cy 5 or Cy 2 (from the diluted stock) separately. Cyclo mixed for 30 sec, spun for 15 sec, then kept on ice for 30 min in dark. After 30 min of incubation the reaction was stopped by adding 10 mM L-lysine to the reaction mixture. The volume of L-lysine added to the each mixture was equal to the volume of 240 pmol Cy dye added initially to the tear protein for labelling. Cyclo mixed for 30 sec, spun for 15 sec, then kept on ice for 10 min in dark. After 10 min of incubation, the labelled proteins can be stored at – 80 °C for a week or can proceed for passive rehydration.

Table 3.10: Labelling of tear protein with Cy dyes and its combination.

S.No	240 pmol Cy 3 + 30 µg tear protein	240 pmol Cy 5 + 30 µg tear protein	240 pmol Cy 2 + 15 µg of respective each tear protein (total 30 µg)
1	Control (pool of 3)	DES non SS (pool of 3)	Control + DES non SS
2	Control (pool of 3)	DES non SS (pool of 3)	Control + DES non SS
3	DES Non SS (pool of 3)	Control (pool of 3)	DES Non SS + Control
4	DES Non SS (pool of 3)	RA DES (pool of 3)	DES Non SS + RA DES
5	RA – DES (severe) (pool of 3)	RA without DES (pool of 3)	RA – DES + RA alone
6	RA without DES (pool of 3)	RA Severe DES (pool of 3)	RA alone + RA Severe DES
7	RA Severe DES (pool of 3)	RA mild DES(pool of 3)	RA Severe DES + RA mild DES
8	Control (pool of 3)	RA –DES (pool of 3)	Control + RA –DES
9	Control (pool of 3)	mild Non SS DES (pool of 3)	Control + mild Non SS DES
10	Control (pool of 3)	DES-SJS (pool of 3)	Control + DES-SJS
11	DES non SS (pool of 3)	DES-SJS (pool of 3)	DES non SS + DES-SJS
12	Primary SS - DES (pool of 3)	RA DES (pool of 3)	Primary SS - DES + RA DES
13	Primary SS – DES (pool of 3)	Control (pool of 3)	Primary SS – DES + Control
14	RA alone (pool of 3)	Control (pool of 3)	RA alone + Control
15	RA –DES (pool of 3)	RA alone (pool of 3)	RA –DES + RA alone

For passive rehydration, all the 3 Cy dye labelled tear protein was mixed together, and the final volume of the sample was made up to 285 µl with the rehydration buffer. This final sample was used for passive rehydration. And the remaining protocol of focussing of IPG, equilibration, SDS-PAGE was similar to normal 2DE as discussed in this chapter section 3.9. The imaging of gels was done using typhoon scanner, analysis was done using decyder software. After the imaging by typhoon scanner, the gels were stained using silver stain as discussed in the section 3.8.

Figure 3.5: Schematic representation of labelling of tear protein with Cy dyes.



The labelled tear protein was separated using 17 cm IPG with pI 3-10, focussed using 1st dimension IEF, and then equilibrated the strip followed by SDS-PAGE of the focussed proteins.

The IEF, SDS-PAGE was carried out, similar to normal 2DE but maintained the dark conditions to prevent the leaching of fluorescent dye from light. Then the gels were scanned using typhoon scanner, the images were then analysed using Decyder software.

3.10.6. Imaging of Focused gels using Typhoon scanner

Imaging was done using Typhoon scanner with 500 V PMT, 100 microns pixel. The laser wavelength for each Cy dyes were Cy3 Ex 532 Em 580 nm, Cy 5 Ex 633 Em 670 nm and for Cy 2 Ex 488 Em 520 nm. Gels were analyzed using Decyder 2D version 7.0 software.

3.10.7. Analysis of Cy dyes using Decyder software

De cyder 2D version 7.0 software used for the DIGE gel image analysis for identifying differentially expressed tear proteins/peptides and also to confirm the proteins that were differentially expressed by 2DE as describes in the Decyder manual.

3.11. Tryptic digestion of tear proteins.

3.11.1. *In gel* Tryptic digestion of 2D tear protein/ peptide spots (Li et al 1997)

Materials:

1. 100 mM ammonium bicarbonate(NH_4HCO_3)(M. Wt: 79.056): prepared in nanopure water
2. 50 mM ammonium bicarbonate in nanopure water
3. 100 mM sodium thiosulphate (M.wt:248.2): prepared in nanopure water
4. 30 mM potassium ferricyanide (M.wt: 329.24): prepared in nanopure water
5. Destaining solution: 1:1 ratio of 100 mM sodium thiosulphate : 30 mM potassium ferricyanide. Should be prepared freshly before use.
6. 100 mM dithitheritol (DTT) (M.wt: 154.4) in 100 mM ammonium bicarbonate
7. 55 mM Iodoacetamide (IAA) (M.wt:184.96) in 100 mM ammonium bicarbonate
8. Sequencing grade Trypsin
9. Digestion buffer: 12.5 ng/µl trypsin in 50 mM NH_4HCO_3 (per one gel spot. Accordingly added the digestion buffer)
10. Acetonitrile
11. Nanopure water
12. 5 % Formic acid(FA)
13. Extraction solution: 50% ACN:5% Formic acid: 45% water
14. Gel spots

Procedure: The protocol for ingel tryptic digestion is mentioned in Fig 3.7.

Figure 3.7: Ingel tryptic digestion of silver stained spots from 2DE/2D-DIGE gels

Excised protein/peptide spot using sterile blade under laminar hood, and made into tiny pieces

↓

Washed the gel pieces with MQ water for 5 min - 2 times

↓

Silver stained gel pieces
Added Destaining solution (Mix equal Volume of 30 mM Potassium Ferricyanide +100 mM sodium thiosulphate)

↓

Waited for the discoloration of silver stain (appx. 2 min)

↓

Removed the destain

↓

Washed with MQ water 1 min each - 3 times

↓

Added 35 µl of 100% ACN – Incubated for 10 min at RT

↓

Removed ACN, Dried the gel pieces in Speed vac for 10 min

↓

Added 150 µl of DTT (prepared in 100mM NH_4HCO_3)

↓

Incubated for 1hr at 56°C

↓

Cooled, to this added 150 µl of 55 mM Iodoacetamide (prepared in 100mM NH_4HCO_3)

↓

Incubated for 45 min in Dark at RT with occasional vortexing

↓

Removed the solutions, washed with 50 µl of 100 %ACN, Incubated for 10 min at RT – repeated this 2 times

↓

Washed with 100mM NH_4HCO_3, Incubated for 10 min at RT – repeated 2 times

↓

Added 50 µl Digestion Buffer – 50 mM NH_4HCO_3 + 12.5 ng / µl Trypsin to the gel pieces
(Trypsin should be kept in ice while adding to gel pieces, the pH of the reaction 7-9)

↓

Incubated overnight at 37 °C

↓

After incubation, spun at 14,000 rpm for 1 min, Saved the supernatant in another vial

↓

To the gel pieces added 150 µl Nanopure water, Incubated for 10 min at RT

↓

Spun at 14,000 rpm for 1 min; added the supernatant to the previous solution

↓

Extracted the peptides into 50 µl of 50% ACN + 5% Formic acid

↓

Incubated at RT for 30 min, repeated the above 50% ACN + 5% Formic acid extraction for 3 times

↓

Pooled all the extracts, dried in Speed vac maintaining 18 ° C till it concentrated.

↓

Store at – 20°C until analysis

3.11.2. *Insol* tryptic digestion of tear protein

Materials:

1. Tear protein extracted using 8M urea buffer (discussed in section 3.2)
2. 100 mM ammonium bicarbonate(NH_4HCO_3)(M. Wt: 79.056): prepared in nanopure water
3. 50 mM ammonium bicarbonate in nanopure water
4. 100 mM dithitheritol (DTT) (M.wt: 154.4) in 100 mM ammonium bicarbonate solution
5. 55 mM Iodoacetamide (IAA) (M.wt:184.96) in 100 mM ammonium bicarbonate solution
6. Sequencing grade trypsin
7. Digestion buffer: 1:20 ratio in 50 mM ammonium bicarbonate. i.e. to 20 µg of protein 1µg of trypsin was added.

Procedure

Figure 3.8: *Insol* tryptic digestion of Schirmer tear protein

30 µg of total protein, after desalting

↓

Added 25 µl of 100mM NH_4HCO_3

↓

Added 25 µl DTT incubated for 1 hr at 56 °C

↓

Cooled, then added 25 µl of iodoacetamide

↓

Incubated for 45 min at RT in dark

↓

Added digestion buffer – Trypsin 1:20 ratio (1µg of Trypsin for 20 µg of Protein)

↓

Incubated for 1 hr at 37 °C

↓

After an hour, again added Trypsin and incubated at 37 °C overnight

↓

Stopped the reaction by keeping the reaction mixture at – 20 °C or at – 80 °C

↓

Looked for the digestion of proteins by running on PAGE/ HPLC

↓

If proper digestion has not occurred repeated the digestion with Trypsin as mentioned previously

↓

Concentrated the digested solution using speed vac, stored at Store at – 20°C until analysis

3.11.3. Mass analysis of the tryptic digested spots

Solvents used:

Buffer 1A: 98% water: 2% Acetonitrile: 0.5 % formic acid
Buffer B: 98% Acetonitrile: 2% water: 0.5 % formic acid for washing of the column
Buffer 2A: 98% water: 2% Acetonitrile: 0.5 % formic acid
Gradient flow with 2A buffer during the analysis.

- **Column**: Michrome (530) magic C18 column, 5 µ, 100 °A with 0.1X150 mm.
- **Flow rate during the analysis:** 400 nl/min
- **Pressure during the run** – 1100-1200 psi
- **Run time for MS:** total of 60 min. 0-10 min sample pick up, after 10 min separation of peptides for

Processing of the Tryptic Digest

To the tryptic digested extract
↓
Added 30 µl of solvent A. 98 % water: 2 % ACN: 0.5 % Formic acid
↓
Mixed well and centrifuged at 4000 rpm for 5 min
↓
From the supernatant of tryptic digest 10 µl + 15 µl of solvent A. mixed properly and injected 25 µl of sample into LC for separation of peptides

3.11.4. nano LC – MS/MS Analysis of Tear Proteins

Peptide mixtures were loaded on to a nano LC reverse phase column of internal diameter 75 mm, packed with C18 particles of size 5 mm (Michrom) and eluted into a ESI – Quadra pole Time of Flight Mass Spectrometer (Q STAR Elite, MOS, Geiex – Applied Biosystems) with a 60 min gradient. Fragments ion spectra were recorded using information dependent acquisition (IDA). Data was analyzed using Protein pilot 2.0 Software with All Entries Database**.**

For the peptide analysis –To look at the best hit for the peptide or protein identification the following points were considered

- At least one peptide, with 5 consecutive y or b ions
- The peptide confidence > 95%
- % coverage more than 10 % in case of low abundant proteins

3.12. RNA extraction from Lacrimal gland and corneal epithelium using trizol method (Chomczynski and Sacchi, 1987)

3.12.1. Principle: TRI Reagent has phenol and guanidine thiocyanate in a monophase solution to facilitate the immediate and most effective inhibition of RNase activity. Biological samples are homogenized or lysed in TRI Reagent; the Subsequent addition of bromochloropropane or chloroform results in the separation of the homogenate into aqueous and organic phase. RNA partitions to the aqueous phase, DNA to the interphase, and protein to the organic phase. The RNA can then be precipitated from the aqueous phase with the addition of isopropanol. The isolated RNA is suitable for any downstream application, including RT-PCR

3.12.2. Materials

1. Diethyl Pyrocarbonate (DEPC, commercially available)
2. 0.1 % DEPC water – To 1000 ml of deionised water added 1 ml of DEPC. Mixed till it become clear solution. Left the solution at RT overnight. Then autoclaved the water.
3. DEPC vials/ tubes – socked the vials, tubes in DEPC water, Left at RT overnight. Then autoclaved the vials and tubes.
4. Trizol Reagent (commercially available)
5. Chloroform
6. Isopropanol
7. 70 % Ethanol in DEPC treated water
8. 200 mM 3-(N-morpholino) propanesulfonic acid (MOPS- MWt: 209.26) in DEPC water
9. 50 mM sodium acetate (MWt:82.04) in DEPC water
10. 10 mM EDTA(MWt:372.04) in DEPC water

11. 10 % (10 X) MOPS buffer

MOPS	–	8 g
Sodium acetate	–	8.2 g
EDTA	–	0.74g
DEPC water	–	200ml

Adjust the pH to 7 using 1N NaOH.

12. 1% Running Buffer

10XMOPS buffer	–	100 ml
Formaldehyde (HCHO)	–	20 ml
DEPC water	–	880 ml

13. 5 % RNA loading buffer

DEPC water	-	10 ml
10 X MOPS buffer	–	4 ml
Formamide	–	3.084 ml
100 % glycerol	-	2 ml
HCHO	–	0.72 ml
500 mM EDTA	–	80 µl
Saturated butanol	–	16 µl

14. **2 % Agarose in 1 X MOPS buffer.** When agarose was in molten stage, to this added 10 µl of ethedium bromide (Etbr, from 2mg /ml), mixed well and then poured in the sealed plates.

15. **Sample preparation to visualize RNA**: To 2 -3µl of RNA made up to 15 µl with DEPC water, then added 15 µl of RNA loading buffer, boiled the solution at 65° C for 10 min. Cooled in ice for 2 min.

3.12.3. Lacrimal gland homogenization

Lacrimal gland after receiving was very slimy. Therefore for homogenization, the tissue was freeze thawed in liquid nitrogen repeatedly 3 times of1 min each time. To this, 2 ml of trizol was added, grounded for 5 min using mortar and pestle and preceded for RNA extraction procedure as in section 3.14. Corneal epithelium was small tissue. To this added 1ml of trizol, cyclomixed and then proceeded as discussed in the RNA extraction procedure.

Procedure

1. Both the tissues after mixing with trizol, were incubated at room temperature for 5 min.
2. To the trizol solution, 200µl of chloroform was added and was mixed well for 15sec.
3. It was incubated at room temperature for 5 min. Centrifuged at 12,000 RPM for 15 mins.
4. The aqueous layer was transferred to a new vial.
5. To the aqueous layer 500 µl of isopropanol was added, gently mixed for 5 times, and incubated at room temperature for 10 min. Centrifuged at 12,000 RPM for 10 min.
6. The supernatant was discarded. To the pellet added 1 ml of 70% ethanol gently mixed, centrifuged at 12,000 RPM for 5 mins.
7. The supernatant was discarded, 70 % ethanol wash was repeated 1 more time.
8. Final pellet was dried for few min. To the pellet 15 µl of DEPC treated water was added.
9. 2 µl of extracted RNA was quantified by Nanodrop (Spectrophotometer)
10. 2 µl of extracted RNA was electrophoresed on 2 % agarose prepared using MOPS Buffer.

3.12.4. Electrophoresis of RNA using MOPS Buffer

- 2% agarose was dissolved in 1X MOPS buffer, boiled, when it was in molten state added 10 µl of EtBr, mixed well and poured in the horizontal casting apparatus, then placed the combs. Once the gel was solidified, removed the combs from the gel.
- The gel was electrophoresed using 1X MOPS running buffer, by applying 5V/ cm i.e. 40 V was used for 10 min.
- Then the RNA, that was boiled with RNA loading buffer was loaded in the wells

- When the tracking dye reached 3/4th of the gel, it was removed from the tank, visualized under Bio-Rad's gel doc system, with a wavelength of 254 nm.
- The RNA was visualized as 3 distinct bands were 8S, 18S (~1900bases) and 28S (~4800bases) RNA molecules which indicated the quality of RNA was good for further work.

3.12.5. cDNA conversion from RNA

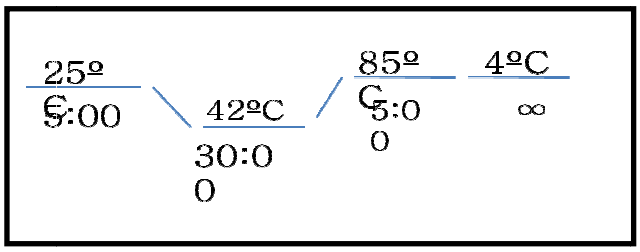
RNA was converted to cDNA using iScript RT-PCR Kit (Biorad) following the manufacturer's protocol using the reagents provided in the kit.

Procedure

1. The following reaction mix was prepared

Reagents	**Volume**
RNA Template	2 µg
Nuclease free water	13 µl
5x Iscript Reaction Mix	4 µl
Reverse Transcriptase	2 µl
	20 µl

2. The following protocol was followed:



3. The cDNA was quantified by using Nano drop (spectrophotometer).

3.12.6. RT-PCR for Lacrimal proline rich 4 protein (LPRR4) and Housekeeping gene-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

Materials

1. cDNA
2. Primers for LPRR4 and GAPDH

(i) LPRR4: primers were designed using Genscript website.

Forward primer: 5'TGCTCTCAGTGGTCCTTCTG3'

Reverse Primer: 5'CTTCAGGAGGAGGTCTCTGG 3'

500 ng of both forward and reverse primer was used for RT-PCR

Product base pair size - 144bp

(ii) GAPDH: Used these primers from the literature

Forward Primer: 5'GCCAAGGTCATCCATGACAAC3'

Reverse primer: 5'GTCCACCACCCTGTTGCTGTA3'

100 ng of both forward and reverse primer was used for RT-PCR

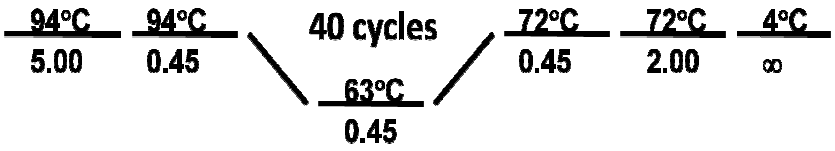
Product base pair size - 498bp.

3. 3 Units/ µl Taq polymerase enzyme (commercially available) – 1.2 units/ reaction
4. 25 mM dNTPs (commercially available) – 0.16 mM used during RT PCR
5. RT-PCR buffer with 15 mM $MgCl_2$(commercially available) – during RT-PCR buffer contains 1.5 mM $MgCl_2$
6. Autoclaved nano pure water
7. 10 X TBE buffer: Tris – 57.1 g
   Borate – 27.4 g
   EDTA – 3.68 g
   Deionised water – 500 ml
   pH of the buffer – 8.4
8. 1 X TBE buffer – 10 ml made up to 100 ml with water
9. 2 % agarose in 1X TBE buffer – boiled, when it is in molten state added to 100 ml of agarose 10 µl of Ethedium bromide was added.
10. Tracking dye: 0.1 % Bromophenol blue in 1X TBE buffer + equal volume of 40% sucrose.

Procedure:

For GAPDH:Reagents	1ReactionVolume
cDNA	200 ng
dNTPs	4 µl
10X PCR buffer	2.5 µl
100 ng Forward Primer	1 µl
100 ng Reverse Primer	1 µl
Taq Polymerase	0.3 µl
	8.8 µl

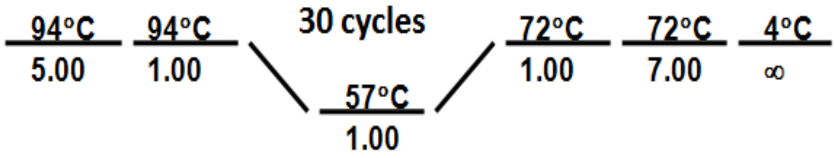
PCR Settings for GAPDH:



(i) For LPRR4:

Reagents	1ReactionVolume
cDNA	200 ng
dNTPs	4 µl
10X PCR buffer	2.5 µl
500 ng Forward Primer	1.8 µl
500 ng Reverse Primer	2.0 µl
Taq Polymerase	0.3 µl
	10.6 µl

PCR Settings for LPRR4:



(ii) After RT-PCR of both LPRR4 and GAPGH, 10 µl of PCR product was mixed with 10 µl of tracking dye, loaded on to 2 % agarose, the voltage set was 100V for 1 hr. The product was visualized using Bio-Rad's gel doc system.

3.13. ELISA for LPRR4

Principle: The microtiter plate provided in the kit was pre-coated with an antibody specific to PRR4. Standards and samples were then added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for PRR4. Next, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain PRR4, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in colour. The enzyme-substrate reaction was terminated by the addition of sulphuric acid solution and the colour change was measured spectrophotometrically at a wavelength of 450 nm ± 10 nm. The concentration of PRR4 in the samples was then determined by comparing the O.D. of the samples to the standard curve.

Materials

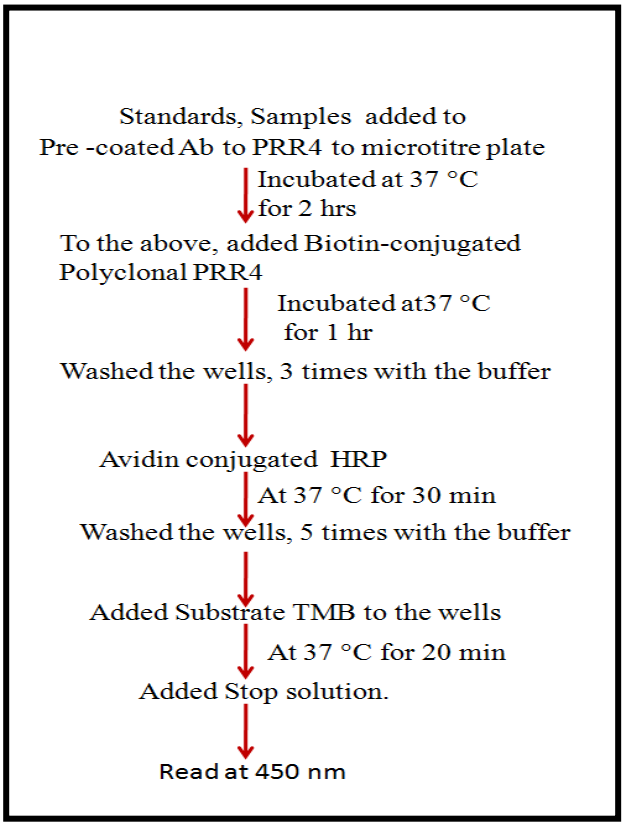
1. Tear samples
2. Phosphate buffered saline (PBS, pH:7.2)
3. Protease Inhibitor cocktail: 1 mg/ml
4. ELISA kit for LPRR4
5. Deionised water
6. Micropipettes

Procedure

1. Extraction of tear protein for LPRR4 ELISA

To the Schirmer collected tear, added 200 µl of PBS, and then 20 µl of protease inhibitor cocktail. Mixed and incubated at 4 °C for 3 hrs with an intermittent mixing. After 3 hrs of incubation, squeezed and removed the strip, centrifuged the solution at 5000 rpm for 10 min at 4 °C. The supernatant was used for LPRR4 determination by ELISA and also for protein estimation.

Figure 3.6: schematic representation of ELISA for LPRR4



3.14. ELISA for USP6NL

Principle: The microtiter plate provided in the kit was pre-coated with an antibody specific to USP6NL. Standards and samples were then added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for USP6NL. Next, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain USP6NL, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in colour. The enzyme-substrate reaction was terminated by the addition of sulphuric acid solution and the colour change was measured spectrophotometrically at a wavelength of 450 nm ± 10 nm. The concentration of USP6NL in the samples was then determined by comparing the O.D. of the samples to the standard curve.

Materials and the protocol were similar to LPRR4 ELISA as Discussed in chapter 2, section 3.13.

3.15. Periodic acid Schiff's (PAS) Staining of PAGE gel

Principle:

This stain was used to look for the glycoprotein. Proteins separated by either SDS-PAGE or 2DE, were first oxidized by periodic acid. The oxidative process results in the formation of aldehyde groupings through carbon-to-carbon bond cleavage. Free hydroxyl groups should be present for oxidation to take place. Oxidation is completed when it reaches the aldehyde stage. The aldehyde groups were detected by the Schiff reagent. A colorless, unstable dialdehyde compound was formed and then transformed to the colored final product by restoration of the quinoid chromophoric grouping.

Reagents:

1. Tear protein
2. 0.8 % Periodic acid in deionized water – prepared freshly
3. 95% alcohol in deionized water
4. 100% alcohol
5. Schiff's reagent:

   Dissolved 0.5 g pararosaniline (chloride) in 15 ml 1N hydrochloric acid and dissolved 0.5 g sodium disulfite in 85 ml distilled water. Mixed both solutions and allowed to stand for 24 h at room temperature. Added 0.3 g activated charcoal, shaked vigorously for 15 sec and then filtered. The solution appeared light pink at the beginning, then turned colorless within a short time. Stored the solution in a brown glass bottle in the refrigerator.

6. Destaining solution: 5% sodium metabisulfite solution in 5% acetic acid
7. Storage solution: 0.1% acetic acid

Procedure

- After 2DE, the gel was fixed in 1% acetic acid or in the methanol(50):acetic acid(5):water(45) fixative for 30 min to overnight
- Oxidized the gel using periodic acid for 10 min.

- Washed the gels in deionized water 2 times
- Stained the gel with Schiff's reagent for 20 -30 min till the pink colour spots developed
- Excess stain was removed using sodium metabisulfite twice, 2 min each.
- Stored the gels in acetic acid

3.16. Immuno Histochemistry (IHC) of Lacrimal gland for LPRR4

Lacrimal gland was stored in acid-formalin for tissue processing, immediately after collection.

TISSUE PROCESSING

Stored lacrimal gland was processed, embedded and sectioned for further Immunohisto- chemistry

3.16.1 Steps in Processing: The tissue was removed from acid –formalin and processed in the following reagents in the similar order mentioned below.

- 100 % alcohol - 30minutes
- 100 % alcohol - 30 minutes
- 100 % alcohol - 30 minutes
- Xylene - 4 minutes
- Xylene - 4 minutes
- Paraffin wax - 30 minutes.
- Paraffin wax - 60 minutes
- Wax in vacuum - 30minutes

3.16.2 Steps in Embedding

- Orientation of tissues was decided.
- Molten paraffin wax 2º or 3ºc above the melting point was dispensed into the mould.
- It is the process of placing the tissue in a support medium (paraffin wax)
- Thin film of solid has formed on surface.
- Gently pressed the tissue into the wax in the oriented plane.

3.16.3 Vacuum impregnation

- Transferred the cleared tissues to a heated, sealed container of molten wax.
- Degree of vacuum should not exceed 400-500 mm of Hg.

3.16.4 Sectioning the tissues

The sections of thin microtome 5 µm was taken on the charged slides (fisher scientific) and slides were kept for drying and next day deparffinisation was done

3.16.5 Deparafinisation: Deparaffinaisation of sections was carried in the following order as mentioned below.

- xylene 1 - 4 minutes
- xylene 2 - 4 minutes
- xylene 3 - 4minutes
- 100 % Isopropyl alcohol - 4 minutes
- 100 % Isopropyl alcohol - 4 minutes
- 95 % Isopropyl alcohol - 4 minutes
- 80 % Isopropyl alcohol - 4 minutes
- 70 %Isopropyl alcohol - 4 minutes
- 60 %Isopropyl alcohol - 4 minutes
- Distilled water - 4 minutes

3.17. Immuno histochemistry (IHC) for LPRR4

Principle: The qualitative identification by light microscopy of antigens in sections of formalin-fixed, paraffin-embedded tissue is studied by immunehistochemisty (IHC). The sections were subjected to epitope retrieval prior to staining. Endogenous peroxidase activity is neutralized using the NovocastraTM peroxidase block. This was followed by application of the protein block to reduce non-specific binding of primary and polymer. The sections were subsequently incubated with optimally diluted primary antibody. Post primary block is used to enhance penetration of the subsequent polymer reagent. The polymer recognizes mouse and rabbit immunoglobulins, it detects any tissue-bound primary antibody. Sections were further incubated with the substrate/chromogen, 3,3' - diaminobenzidine (DAB). Reaction with the peroxidase produces a visible brown precipitate at the antigen site.

3.17.1 Reagents

1. Peroxidase Block - 3 % Hydrogen peroxide.

2. Protein Block - 0.4 % Casein in phosphate-buffered saline, with stabilizers, surfactant, and 0.2 % Bronidox L as a preservative.

3. Post Primary Block - Polymer penetration enhancer containing 10 % animal serum in tris-buffered saline/0.09 % ProClin™ 950.

4. NovoLink TM Polymer. Anti-mouse/rabbit IgG -Poly-HRP (each at 8 µg/ml) containing 10 % animal serum in tris-buffered saline/0.09 % ProClin™ 950.

5. DAB Chromogen. 1.74 % 3,3' - diaminobenzidine, in a stabilizer solution.

6. NovoLinkTM DAB Substrate Buffer (Polymer) - Buffered solution containing 0.05 % hydrogen peroxide and preservative.

7. Haematoxylin - 0.02 % Haematoxylin.

3.17.3 Protocol

- Lacrimal gland sections were fixed in 10 % neutral buffered formalin, processed, embedded and 5 µm sections were done as per **section 3.16**.
- The deparafinized sections were treated with trypsin for 10 min for antigen retrieval.
- Washed slides in de-ionised water.
- Neutralized the endogenous peroxidase using Peroxidase Block for 5 minutes.
- Washed in TBS for 2 x 5 minutes.
- Incubated with Protein Block for 5 minutes.
- Washed in TBS for 2 x 5 minutes.
- Incubated with optimally diluted primary antibody (biotin conjugated LPRR4 Ab 1:10 dilution).
- Washed in TBS for 2 x 5 minutes.
- Incubated with Post Primary Block for 30 minutes.

- Washed in TBS for 2 x 5 minutes.
- Incubated with NovoLink TM Polymer for 30 minutes.
- Washed in TBS for 2 x 5 minutes with gentle rocking.
- Developed peroxidase activity with DAB working solution for 5 minutes.
- Rinsed the slides in water.
- Counterstained with Haematoxylin.
- Rinsed the slides in water for 5 minutes.
- Dehydrated, cleared and mounted the sections.

3.18. FACS analysis for Chemokines using multiplex beads (Cook et al 2001)

Principle: CBA assays provide a method of capturing a soluble analyte or set of analytes with beads of known size and fluorescence, making it possible to detect analytes using flow cytometry. Each capture bead in the kit conjugated with a specific antibody. The detection reagent is a mixture of phycoerythrin (PE)–conjugated antibodies, which provides a fluorescent signal in proportion to the amount of bound analyte. When the capture beads and detector reagent were incubated with an unknown sample containing recognized analytes, sandwich complexes (capture bead + analyte + detection reagent) are formed. These complexes can be measured using flow cytometry to identify particles with fluorescence characteristics of both the bead and the detector.

Five bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for CXCL8/IL-8, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1, and CXCL10/IP-10. The five bead populations are mixed together to form the bead array that is resolved in a red channel (e.g., FL3 or FL4) of a flow cytometry.

3.18.1. Materials and reagents

1. Tear samples
2. PBS pH:7.2
3. Protease Inhibitor cocktail:1mg/ml
4. Chemokine kit contains

(i). Capture beads: (a) CXCL8/IL-8 (A1), CCL5/RANTES(A2), CXCL9/MIG(A3), CCL2/MCP-1(A4), CXCL10/IP-10 (A5).

(ii) Human Chemokine PE Detection Reagent

(iii) Human Chemokine Standards

(iv) Wash Buffer

(v) Assay diluents

(vi) Cytometer Setup Beads

(vii) PE Positive Control Detector

(viii) FITC Positive Control Detector

3.18.2. Procedure:

1. Extraction of cytokines from Schirmer tear : To the Schirmer collected tear, added 200 µl of PBS, and then 20 µl of protease inhibitor cocktail. Mixed and incubated at 4 °C for 3 hrs with an intermittent mixing. After 3 hrs of incubation, squeezed and removed the strip, centrifuged the solution at 5000 rpm for 10 min at 4 °C. The supernatant used for desalting using 3kDa cut-off filters. 25 µl of retentate was used cytokine assay.

2. Chemokine assay using tear specimen done according to the Kit protocol

(i) Standard Chemokine preparation

The lyophilized powder of cytokine standard, to this added 4 ml of assay diluent. Mixed gently and left at RT for 15 min. Serial dilution from this standard was done to get concentrations ranging from 10 – 2,500 pg/ml.

(ii) Mixing of Chemokine capture beads

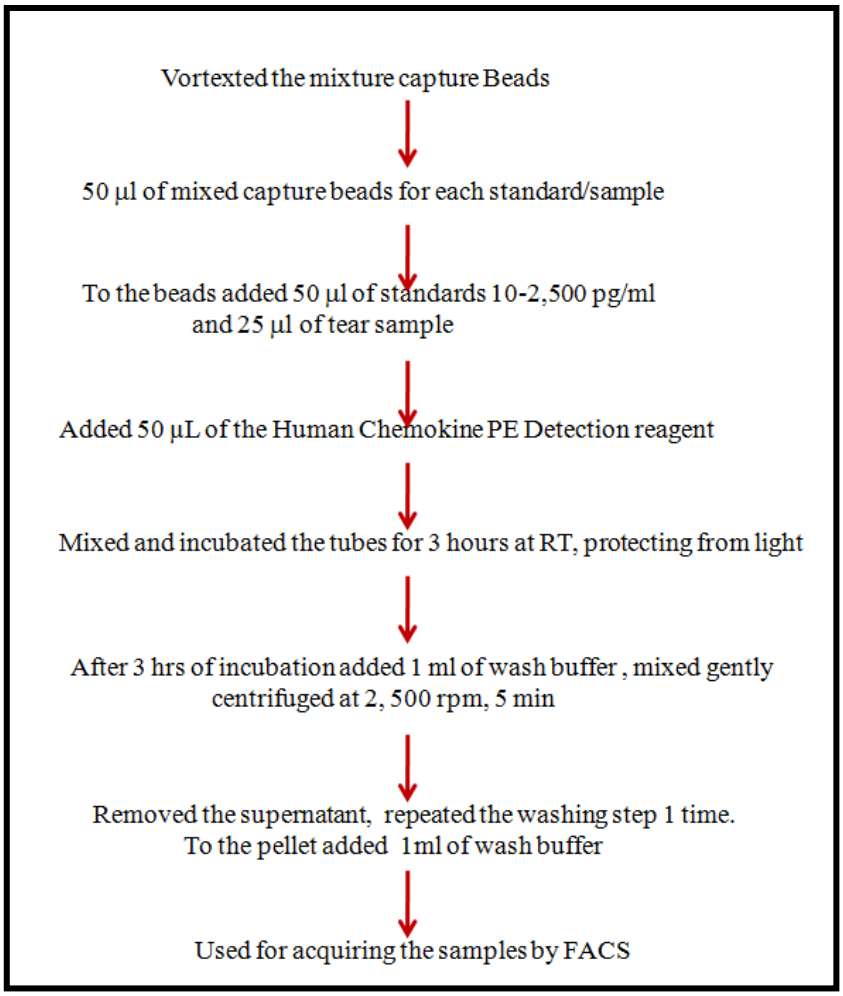
Based on the number of assay tubes including standards, controls and unknown samples the capture beads prepared. Mixed vigorously 3-5 times each capture bead.

10 µl of each chemokine capture bead (i.e. 5 beads) per 1 sample was mixed into another vial, vortexed vigorously.

(iii) **Sample dilution:** No sample dilution done.25 µl of desalted tear sample was used directly.

(iv) **Performing the Human Chemokine Assay**

Figure 3.7. Protocol for processing samples for FACS analysis



3.18.3 FACS Instrument setting for the assay:

Chemokine flex array was analysed using the FACS calibur instrument. The assay instrument setting was done according to the manufacture instruction. The acquiring of the individual sample was done as follows.

For acquiring two dot plots Forward Scatter (FSC) vs. Side scatter (SSC), FL2H vs. FL3H) and one Histogram (FL3H) was used. Initial 2000 (400 events for each cytokine) events were captured in R1 gate FSC vs. SSC plot. This R1 gate was

applied to the FL2H vs. FL3 H dot plot and FL3H histogram to visualize the intensity of the each peak. The discrimination and acquired data was saved as FCS file.

The BD™ CBA Flex Set system provides an open and configurable menu of bead-based reagents designed to make it easy to create multiplex assays. Available specificities include soluble protein assays for detection of human, mouse, or rat cytokines, chemokines, and growth factors; human immunoglobulins; and cell signalling assays for the detection of phosphorylated cell signalling proteins. Up to 30 analytes can be measured simultaneously using the BD CBA Flex Set system on a flow cytometer equipped with 488-nm or 532-nm and 633-nm lasers. The assays have been formulated to be mixed into any size plex.

Chemokine cytometric analysis was performed on a BD FACS Calibur four-color flow cytometer (BD Biosciences) equipped with two air-cooled lasers: a 15 mW Argon laser (488 nm) and a Red Diode laser (632 nm). The emission of fluorochromes was recorded through specific band pass filters: 525 nm for fluorescein isothiocyanate. 632 nm for PE, The flow cytometer was regularly checked by using standard BD Calibrated 3 beads (BD Biosciences) for photomultiplier tube (PMT) voltage adjustment, colour compensation setup, and sensitivity test. The cytokine coated coated were gated on a forward scatter (FS) versus side scatter (SS) plot based on their concentration. On FL2 versus FL3 side scatter (SS) dot plot where chemokines were was used to trace for its concentration change. Red fluorescence (beads) was displayed on FL3 single histogram gated on the previous two plots. An appropriate discriminator was set on the FS parameter to exclude small debris and other cell fragments. Data acquisition and analysis were performed using the software (BD Biosciences, USA). The data are expressed as change in the concentration of chemokines as pg /ml for 2000 events gated which is over and above the fluorescence seen both in the control and test, plotted against standards used in the experiment.

3.18.4. Data analysis: The quantification of the each cytokine was done using the software used respective FCS file as the raw data along with the standard file.

3.19. REACTIVE OXYGEN SPECIES (Hempel et al 1999)

Principle: Reduced form of 2, 7 dichlorodihydroflurescein diacetate which is non-fluorescent compound gets oxidized when exposed to reactive oxygen species which spectrophotometrically detected. The oxidized form is fluorescent and is measured at Ex 485 and Em 535.

REAGENTS

1. Dichlorodihydrofluorescein diacetate: Stock 1 mg/ml prepared in dimethyl formamide (DMF), from this 2.5 mM was used for the assay.

2. Phosphate buffered saline (pH:7.2)

3. Tear samples control and DES condition

4. Hydrogen peroxide (H_2O_2) – positive control

5. Extraction of ROS from tear sample: similar procedure as discussed in this chapter section 3.15.

PROCEDUE:

1. 200 µL of the PBS buffer served as blank.

2. 30 µL of the tear sample and 180 µL of PBS buffer were added.

3. H_2O_2 (1, 2, 3, 4, 5 µM), made upto 200 µL using PBS

4. 2.5 µM (5 µL) of DCDF DA was added to all the wells

5. Readings were read spectrophotometrically at Ex 485 and Em 535 for 2 hrs in a kinetic mode using fluorescent plate reader spectromax instrument.

6. Analysed the data from comparing with controls.

3.20. *In silico* structural and functional analysis of Lacrimal proline rich 4 protein (LPRR4) and Ubiquitin specific protease 6 N-terminal like protein (USP6NL)

3.20.1.a. 3D structure prediction of LPRR4

As a first step, the amino acid sequence of LPRR4 was retrieved from Uniprot (Acc.no: Q16378) and was BLASTp analyzed against Protein Data Bank (PDB) database. Since no suitable templates were found in PDB, fold recognition method based modelling was performed through I-Tasser server. Based on overall C-value and stereo chemical quality (PROCHECK) the best model was selected and was further refined using Modefiner.

Molecular Dynamics Simulation was performed on OPEN DISCOVERY Linux Platform with pre-installed GROMACS 4.3 software to analyze stability of the protein. GROMOS96 4.3 force field was used during the optimization and simulation process. Periodic boundary conditions were applied and 0.9 nm cubic box was used. The protein was solvated with SPC water model which added 7893 SOL molecules to the system. System was neutralized by replacing water molecules with Na^+ counter ions. Steepest Descent algorithm was used for energy minimization which converged in 283 steps. Equilibration of the position restrained ensembles was conducted for 100 ps at 300 k and with 2 fs of integration time step. And then final MD step was performed for 2 nanoseconds timescale. Finally, the trajectory files were analyzed through GROMACS utilities in order to obtain the Root-Mean-Square Deviation (RMSD) and Root-Mean Square Fluctuation (RMSF) value. The trajectories were visualized using X mgrace (Bandello et al 2001). The final simulated model was validated by Q mean server (Benkert et al 2009).

3.20.1.b. Data mining to identify interacting partners of LPRR4

STRING is a Search Tool meant for Retrieval of information on protein-protein Interactions based on direct (physical) and indirect (functional) associations(Szklarczyk et al 2011). Some of the interacting partners of LPRR4 were identified through querying the STRING (Search Tool for the Retrieval of Interacting Genes) database, wherein, consensus prediction protocol was adopted to achieve high confidence score.

3.20.2. 3D structure prediction of USP6NL/RN-tre

a. Data sets & homology Modelling: The protein sequences of RN-tre (Ac.No: Q92738) and EPS8 (Ac.No: Q12929) were retrieved from uniprotKb, the Sequences were Basic local Alignment sequence tool (BLAST) analysed against PDB database for identifying suitable structural templates for homology modelling. The results showed that EPS8 (SH3 domain) of human to be sharing 95% sequence similarity with SH3 domain Mus musculus EPS8 (1AoJ) and was chosen as a most suitable template for homology modelling by Modeller9v7(Sali & Blundell 1993). Since no suitable templates were found for RN-tre, the structure was predicted using fold recognition method by implementing Zhang's I-TASSER Server, which was ranked as the No.1 server for protein structure prediction in recent CASP7, CASP8 and CASP9 evaluations (Roy et al 2010).

b. Model refinement and validation: The selected models have been subjected to loop refining using "loop.py" script of MODELER9v7. The refined models were subjected to energy minimization using steepest descent carried out in vacuum with OPLS Force field set through GROMACS MD package (Abraham & Gready 2011; Xu & Zhang 2011). The stereo chemical properties of the optimized models were checked by Ramachandran Plot using PROCHECK (Laskowski et al 1996)

c. Protein-protein docking: HADDOCK web server (de Vries et al 2010) was used for docking RN-tre and EPS8. The active site region of EPS8 (SH3) domain is experimentally proven to be most favoured binding region for Rn-tre (725-729) in wet lab studies. Hence, this was given as additional input to the HADDOCK server which shall enhance the predictive accuracy. Docked complex was analyzed using Pymol Visualization tool (De Vera et al 2010).

3.21. ELISA for RA Blood Investigations

Sample used: Serum.

The sample was processed according to the manufacturer's instructions as mentioned in the manual for the assays of ANA, DsDNA, anti-SSA, anti-SSB, Rheumatoid Factor, C-reactive protein, anti-Cyclic citrullinated peptide.

CHAPTER 4: COLLECTION METHOD OF HUMAN TEARS IN DRY EYE SYNDROME FOR TWO DIMENSIONAL ELECTROPHORETIC ANALYSIS

4.1. Introduction

Tear is considered as a valuable specimen as it is available by non-invasive procedures. The up or down regulation of tear proteins can be indicative of the underlying pathology (ter Rahe and van Haeringen 1998; Ohashi, Ishida et al. 2003; Zhou, Huang et al. 2004; Grus, Podust et al. 2005; Koo, Lee et al. 2005; Tomosugi, Kitagawa et al. 2005). Analysis of the changes in these proteins can give insights into the disease process especially at the local ocular environment which helps in either diagnostics or prognostics of the disease. Various techniques such as two dimensional gel electrophoresis (2DE) (Molloy, Bolis et al. 1997), surface enhanced laser desorption ionization-TOF (Grus, Podust et al. 2005) and Mass spectrometry (de Souza, Godoy et al. 2006) have been used for the tear proteomic analysis.

4.1.1: **Collection of Tear fluid**

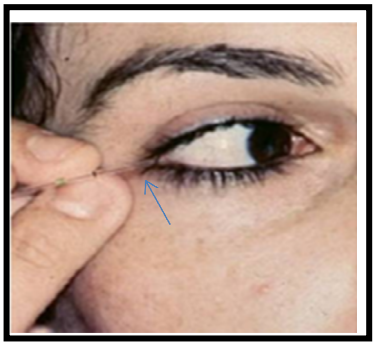
Tear collection and processing is an important step that can determine the suitability of the method for the clinical diagnostics or prognostics. The comfort conditions during their application both for the patient and for the operator are of primary importance considering the low rate of tear flow (0.5-1 μL/min) and its basal volume in the cul-de-sac (5 -15 μL). Few procedures for tear fluid collection and further biochemical analysis have been used in the past years. Those procedures consist basically of the absorption of tear fluid by cellulose sponges(van Agtmaal, van Haeringen et al. 1987), Schirmer paper strips, or porous polyester rods(Jones, Monroy et al. 1997), followed by its recovery from them or, alternatively the direct aspiration of the tear fluid by glass capillary micropipettes(Jones, Monroy et al. 1997). The tear collection methods are available as listed below.

A. **Capillary tear collection (Jones, Monroy et al. 1997)**: Direct aspiration of tears for the analysis (figure 4.1), It may be basal or reflex.

Basal tear collection: Calibrated capillary tubes are used for collection. Local anaesthesia will be used to collect basal tears. The capillary tube is placed in the lower conjunctival region, wait for the tear to rise in the tube by capillary action with an intermittent closing of the eye. This may take from 5 min to 30 min based on the tearing from the individual.

Reflex tear collection: Stimulation of tearing can be done by irritation of nasal mucosa using chemicals like ammonia and then collect the tears by capillary tubes.

Figure 4.1: Collection of tear fluid using calibrated capillary tubes



B. Schirmer tear collection (Stuchell, Feldman et al. 1984): Schirmer strips are small filter paper strips for collecting tears (Figure 4.2).

Basal tear collection: Local anaesthesia is added to the eye, followed by placing the strip in the lower lid conjunctival region for 5 min to collect tears.Reflex tear collection: stimulation tearing is done, and then tear is collected by filter paper strips for 5 min.

Figure 4.2: Collection of tear fluid using sterile Schirmer strips.

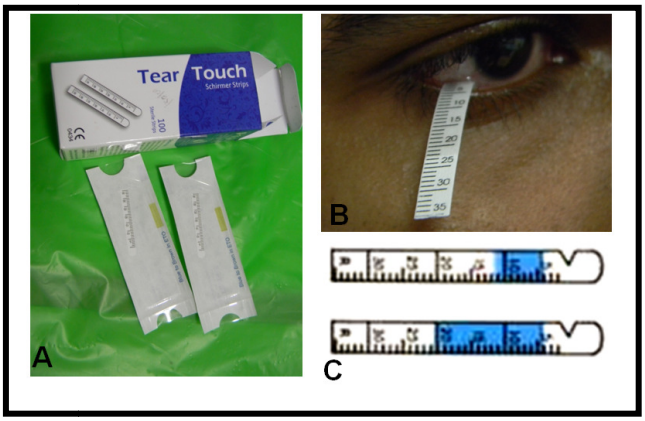
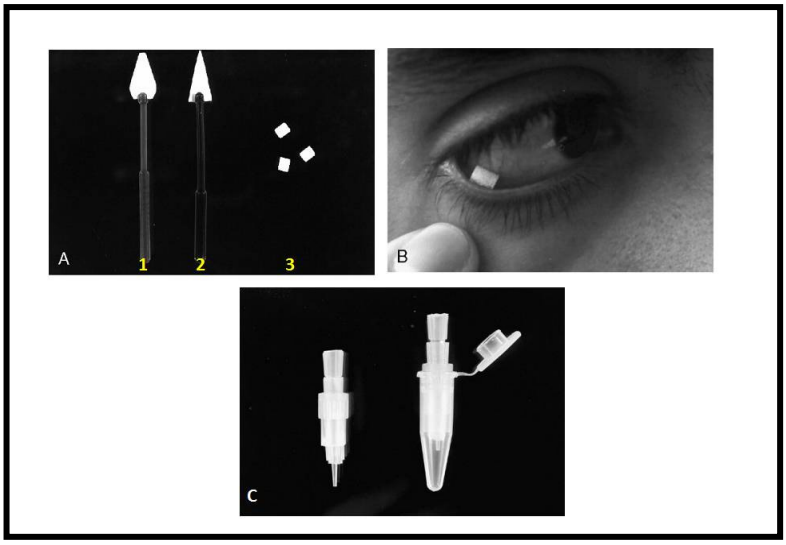


Fig 4.2: A. Sterile Schirmer's strip B. Collection of tear using Schirmer's strip placing at lateral conjunctival region. C. After collection the absorption (tear wetness) on strip in mm.

C.Polyurethane mini sponges (Lopez-Cisternas, Castillo-Diaz et al. 2006): These are absorbing or aspiring devices for tear fluid collection. Dry mini sponges are placed on the margin of the lower lid and after an average period of less than 10 minutes, the wet sponges are retrieved and compressed by centrifugation to transfer the tear fluid to a collection tube as seen in figure 4.3.

Figure 4.3: collection of tear fluid using polyurethrene sponges



4.1.2. Selection of tear fluid collection method

Most of the above mentioned procedures allow tear fluid collection in volumes that are sufficient to carry out studies using current sensitive analytical techniques. However, based on the aim of the study the method of collection is appropriately decided.

This study focuses on the proteomic profiling of tear proteins in dry eye conditions. In such condition where the tear production will be < 10 mm to almost < 2mm depending on severity of dry eye syndrome (DES), collection of the tear by routine capillary methods or cellulose sponge is tedious and almost impractical. Many of the studies have used pooled capillary tears and Schirmer based tear collection method. But pooling of the tear specimen in disease condition may mask the potential variations with respect to the severity/stages of the disease.

Therefore method for tear specimen collection was standardized during this study to meet the study objectives. As a part of standardization the capillary and Schirmer collection methods of tear was compared by protein profiling. The appropriate protein extraction buffer was standardized to extract tear protein from shirmer strip after collection. In addition, considering the extraction buffer constituents, which may hinder the protein estimation, the suitable protein estimation method for 2D electrophoresis was also optimised

4.2. Objectives

1. Standardize appropriate tear protein extraction buffer for Schirmer tear protein that gives maximum protein yield.

2. Standardize suitable protein estimation method for extracted tear proteins with its interference from the buffer used and to optimize the tear protein profile by 2D electrophoresis

3. To see if the proteins extracted from Schirmer's strip is comparable to the capillary collected tear profile and

4. To see if the differential protein profile is picked up in the dry eye syndrome.

4.3. Methods

4.3.1. Collection of Tear sample

4.3.1. a. Capillary tear fluid collection

The capillary tear (reflex tear) was collected using sterile 10 µl capillary tubes, placed in lower cul-de-sac region without any contact with the lower lid, the cornea or conjunctiva. The person was seated comfortably, head raised and against any direct source of light or flow of air. Then tear fluid was dispensed into a sterile vial. Centrifuged and supernatant was stored at -80°C until analysis.

4.3.1. b. Schirmer tear protein extraction

Reflex tears were collected by using a sterile schirmer strip. The schirmer strip placed in the lower cul-de-sac region and allowed to absorb the tear for 5 min in the open eye condition from the inferior prism without any contact with the rest of th regions of the eye. The tear absorbed on to the strip was removed after 5 min, placed in sterile vial and stored at -80°C until analysis.

4.3.2. Sample size details for capillary and Schirmer tear comparison

Tear specimen was collected from 9 control subjects (3 males and 6 females). The volunteers had no complaints of ocular pain or discomfort and had no recent history of

ocular diseases. Their Schirmer values were normal with a value of > 35mm in 5min in all the cases. Informed consent was obtained according to guidelines of the Institutional Review Board. Contact lens wearers were excluded from the study. Both capillary and Schirmer tear sample was collected from the same eye of the control to compare the protein profile.

4.3.3. Extraction of tear proteins

4.3.3. A. Extraction of tear proteins from Schirmer strips

Schirmer tear protein was extracted using 3 different buffers for comparison, namely PBS, 10 mM HEPES buffer and 8 M Urea buffer. To the tear absorbed Schirmer strip, added 300 µl of respective buffers. 30 µg of protease inhibitor cocktail was added and left at 4°for 3 hrs with intermittent mixing. After 3 hrs, the strip was removed, centrifuged and the supernatant was subjected to desalting process using 3 kDa cutoff filters. Then the desalted extracted tear was used for protein estimation. Protein was estimated using Lowry method. Urea extracted protein was not given satisfactory results with Lowry as well as with BCA method, Bradford method of protein estimation was used for urea extracted tear protein.

4.3.3. B. Extraction of tear proteins from capillary tear

50µl of collected capillary tear was made up to 300µl with 8M urea buffer, 30 µg of protease inhibitor cocktail was added, then subjected to desalting using 3 kda cutoff filters. Desalted tear was used for protein estimation using Bradford.

4.3.4. SDS-PAGE of tear proteins

5 µg of both capillary and schirmer tear protein was subjected to SDS-PAGE on 13% gel for 1.30 hrs and developed using silver stain. Band intensities were analysed using Bio-Rad's Quantity one software.

4.3.5. 2D electrophoresis of tear proteins

30 µg tear protein rehydrated passively for 14 hrs, focussed using IEF focussing instrument, equilibrated. 2nd dimension separation done on 13% SDS-PAGE for 5 hrs at 4 - 6°C. Spots were visualized using silver stain, and analysis for protein changes were done using Bio-Rad PD quest software.

4.3.6. Statistical analysis

The Bland-Altman tool was used for analysing the agreement between the Capillary and Schirmer collection methods. A graph was plotted based on the data on the spot density, using the mean of the Schirmer and Capillary method in the x-axis and the mean difference of both the methods in the y-axis. The mean differences of spot intensity between the two methods and the 95 % confidence interval were used as limits of agreement and > 75 % confidence interval was considered as significant agreement between the two methods. Intra assay correlation coefficients were used to find the agreement within the method. All the above statistical analysis was performed using the SPSS software version 14.1 and Med calc software for Bland-Altman analysis.

4.4. Results

4.4.1. Quantitation of Tear protein from various Schirmer tear extract

PBS extracted protein showed poor yield. HEPES and Urea buffer extracted protein showed maximum protein yield (Table 4.1).

Table 4. 1: Tear protein extraction from schirmer strips with various buffer

S.No	Protein (mg/ml)		
	PBS pH:7.0	10 mM HEPES Buffer pH:7.0	8M Urea Buffer pH:7.6
Mean	**0.52**	**1.32**	**1.68**
SD	**0.21**	**0.38**	**0.66**

4.4.2. SDS-PAGE of Schirmer tear proteins

1D PAGE of tear protein using PBS, HEPES and Urea Buffer showed 6 major proteins as shown in the Figure 4.4. Urea buffer extracted protein showed better protein profiling with good intensity bands and more number of protein bands.

4.4.3. 2D electrophoresis of Schirmer extracted tear protein

A. Effect of various buffer for extraction tear protein

2D profile of Schirmer tear protein extracted from 3 buffers was separated using 7 cm IPG strip with pH 3 – 10. The amount of protein used for separation was 30µg. Urea buffer showed good protein separation as shown in Figure 4.5. Therefore, further extraction from Schirmer absorbed tear protein was carried out using 8M urea buffer.

Figure 4.4: 1D tear protein profile of Schirmer extracted tear.

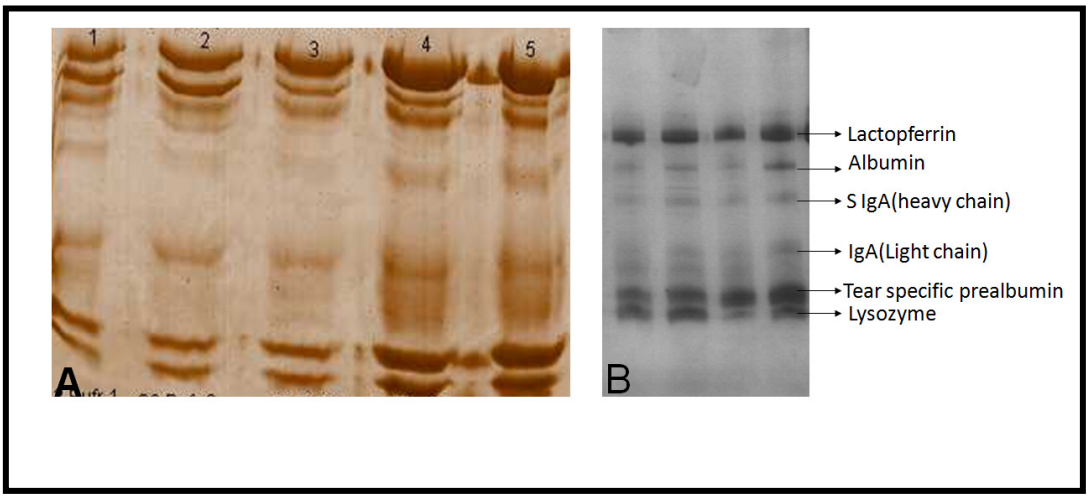


Fig 4.4: 5 µg of tear protein was separated on 13 % SDS-PAGE and silver staining was done to detect the bands. Figure 1A: Schirmer tear protein extracted using 3 buffers. Lane 1- PBS extraction. Lane 2&3 – 10mM HEPES buffer extraction. Lane 4 & 5: 8 M urea buffer extraction. Figure 1B: major 6 tear proteins shown by 1DE.

Figure 4.5: 2D electrophoresis of Schirmer tear protein using various buffers

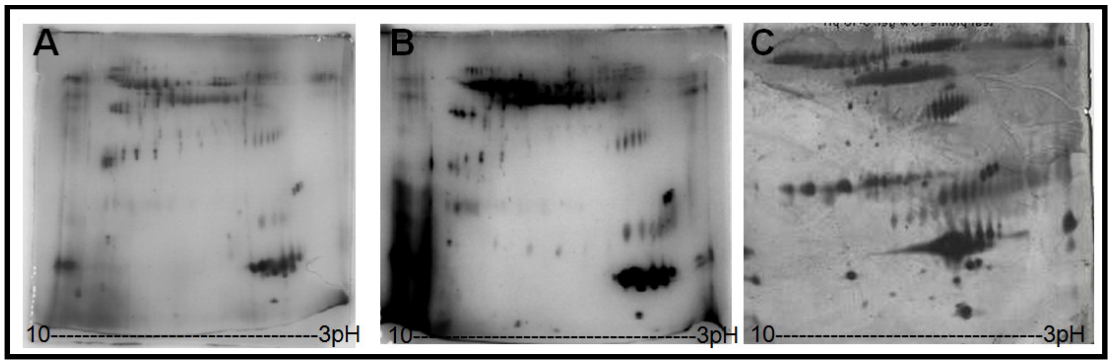


Fig 4.5: 2D tear protein profile of (A). PBS (B).10mM HEPES buffer (C). 8M Urea buffer extraction. 30 µg of total tear protein was used for separation on 7 cm NL IPG strip with ph 3-10 in 1st dimension, and 13 % SDS-PAGE separation in 2nd dimension.

4.4.3.B. Effect of tear protein concentration for 2D electrophoresis

Initially 50 µg of tear protein extracted from was used for 2D electrophoresis on 7 cm IPG as seen in Figure 4.6. This protein amount was very high, showed vertical streaking. Therefore the protein amount was reduced to 20 µg which showed good profile with protein spots as shown in Figure 4.6B. Further tear profiling of samples was done using 20 µg of tear protein when used 7 cm IPG strips.

To see the effect of precipitation, 50 µg of tear protein was precipitated using 10 % TCA and profiled using 7 cm IPG strips. It showed, majority of the proteins were lost during the process (Figure 4.6 C). Therefore TCA precipitation was not used further.

Figure 4.6.: 2D profile of tear protein with varied concentration

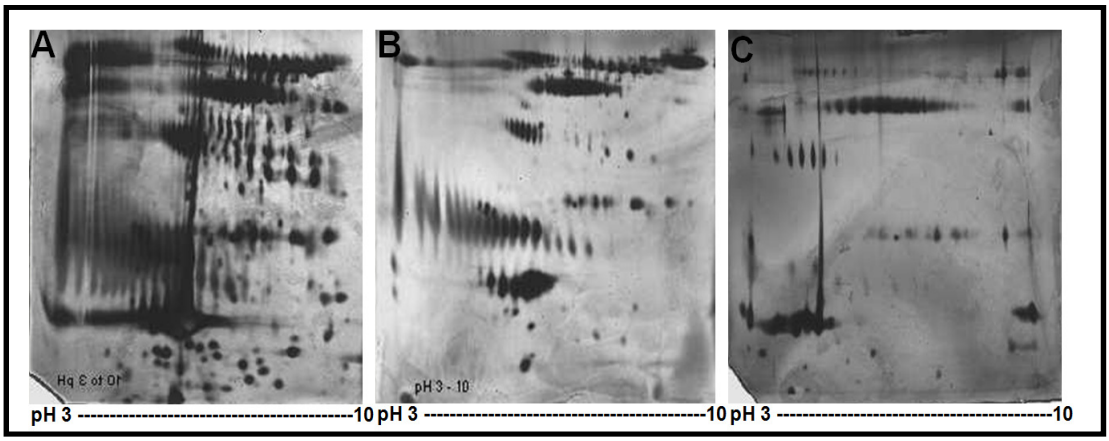


Fig 4.6: Tear protein was separated on 7 cm NL IPG strip pH 3-10 in 1st dimension, 13 % SDS-PAGE in 2nd dimension with various amount of tear protein extracted using 8M urea buffer. (A) 50 µg tear protein (B) 20 µg tear protein and (C) 100 % TCA precipitated 50 µg tear protein.

4.4.3. C. Effect of varying IPG strip length for tear protein separation

The resolution of Tear protein profiling on 7 cm IPG, did not give good resolution of proteins for quantification. IPG strip length of 11cm and 17 cm IPG with pH 3-10 range was therefore tried. Protein separation on 17 cm showed good resolution as seen in Figure 4.7. Therefore, further studies using tear proteins from various samples were profiled using only 17 cm, pH 3 – 10 IPG strips.

Figure 4.7: 2D tear protein profile on various lengths of IPG strip.

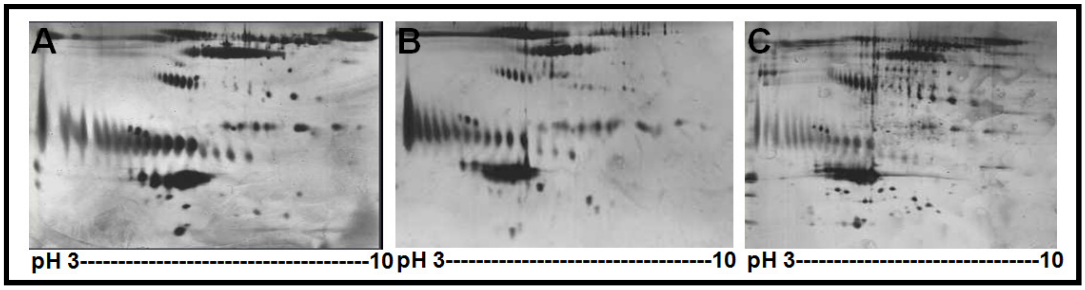


Fig 4.7: 30 µg tear protein was separated on various lengths of IPG strip. (A). 7 cm IPG (B). 11cm IPG and (C). 17 cm IPG in 1st dimension, then on 13% SDS-PAGE in 2nd dimension good protein separation.

4.4.4. Comparison of Capillary and Schirmer tear protein

Tear protein of capillary and Schirmer was compared using both 1D and 2D electrophoresis to look for the protein profiling differences. Since, dry eye syndrome in which tear production was reduced, capillary tear collection is highly impossible. In such cases, Schirmer tear collection is the better option.

4.4.4.A. Tear protein from capillary and Schirmer collected tear

Capillary and Schirmer extracted total tear protein was compared. The protein concentration in the tear extracted into the 8 M urea buffer was found to be 1.5 ± 0.76 mg /mL in the capillary collected tear and 1.65 ± 0.88 mg /mL in the Schirmer collected tear (Table 4.2). The extracted tear protein concentration did not show any significant difference.

Table 4.2: Tear protein in Capillary and Schirmer's extracted tear using urea buffer

S. No	Capillary Tear protein (mg/mL)	Schirmer Tear protein (mg/mL)
Mean ± SD	1.50 ± 0.76	1.650 ± 0.88

4.4.4.B. 1D PAGE of Capillary and Schirmer tear protein

The SDS-PAGE of the Schirmer and Capillary collected tear proteins showed only quantitative changes in the proteins as revealed by the densitometry (Figure 4.8). The quantity difference was observed by densitometry with respect to only two proteins at 25 kDa and 3 kDa regions which was not significant. These two tear proteins reduced quantitatively in the capillary method of collection. But this reduction was not significant (< 1-fold difference).

4.4.4.C. 2DE of capillary and Schirmer tear protein

The 2-DE of the tear proteins using 3–10 pH IPG strips of 17 cm showed a total spot number of 145 ± 7 in the Schirmer collected tear and a mean of 147 ± 8 in the capillary collected as analyzed by PD Quest analysis (Figure 4.9). All the 147 spots seen in capillary were seen in the Schirmer profile.

However, in order to see whether the two methods of collections were comparable, a statistical analysis was done.

Figure 4.8.: SDS-PAGE of capillary and Schirmer tear protein

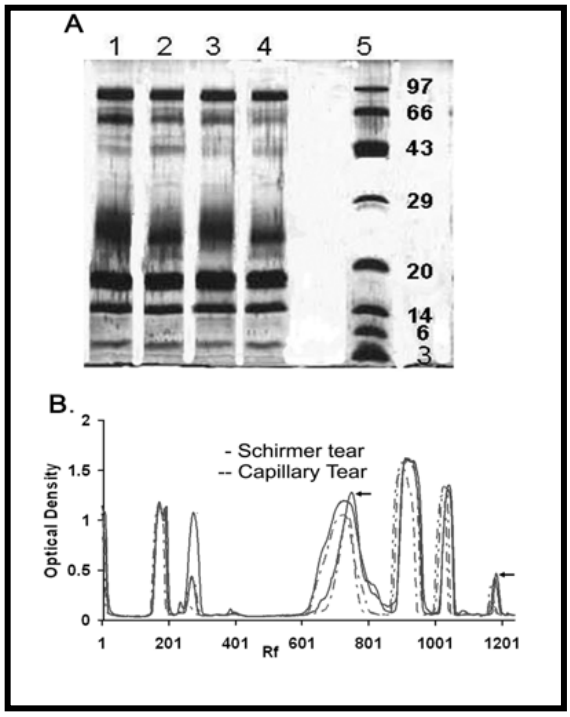


Fig 4.8: (A) Tear protein profiling by 13 % SDS-PAGE, (silver staining) Lane 1, 2: Schirmer tear protein (2 µg); Lane 3, 4 - Capillary tear proteins (2 µg), Lane 5- Molecular Weight marker (B) Densitogram of the SDS-PAGE

Figure 4.9: 2D profile of capillary and Schirmer extracted tear protein

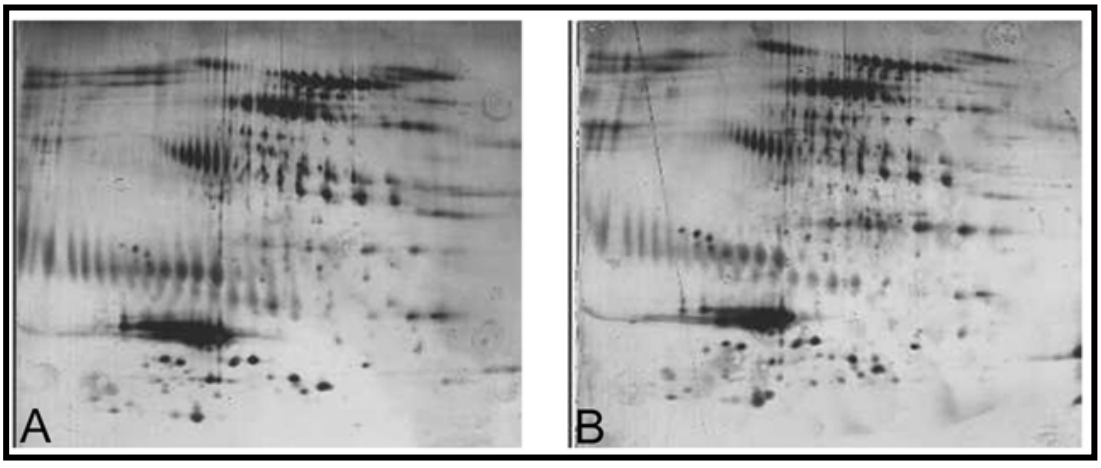


Fig 4.9: 2D Electrophoretic gel map of normal tear proteins. A: Schirmer tear and B: Capillary tear. Tear protein (30µg) were separated using 11 cm pH 3-10 IPG strip in the first dimension and 13% SDS-PAGE followed by silver staining in the second dimension.

4.4.4.D. Bland–Altman analysis of the 2-D profile by the two collection methods

All the protein spots detected by the PD Quest analysis based on their intensity, collected by the two methods were subjected to statistical analysis using the Bland–Altman tool. The agreement analysis showed that the Schirmer tear protein profile had good agreement with the capillary tear, which was based on the spot intensity values obtained from densitometry. The rate of agreement between the Schirmer and capillary tear collection methods was 95.6% and was statistically significant ($p < 0.001$) (Figure 4.10). The intra class correlation within the collection method was also calculated and the r value was found to be 0.93 ($p < 0.001$) for the Schirmer method and 0.91 ($p < 0.001$) for the capillary method. Thus, the Bland–Altman tool showed that both methods were in good agreement with each other and indicated that any type of tear collection could be used for further proteomic experiments.

Figure 4.10: Bland –Altman Analysis of Capillary and Schirmer extracted tear protein

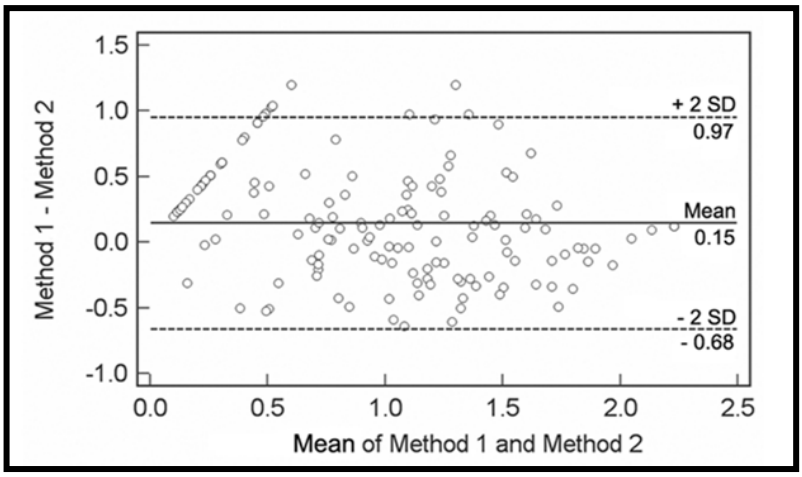


Fig 4.10: Bland Altman plot for protein spot of Capillary and Schirmer collection Methods. The x-axis indicates the mean of the spot densities of the two methods (Method 1: Schirmer, Method 2: Capillary). Y-axis indicates the difference of spot densities of the two methods. The Mean difference of both the methods is 0.15, with an Upper Specification Limit (USL) of 0.97 and a Lower Specification Limit (LSL) of -0.68. The two collection methods showed 95.2% confidence interval which is statistically significant ($p < 0.001$). Therefore both the methods are in agreement with each other.

4.5. Validation of Schirmer tear collection in Dry eye syndrome

Based on this study, Schirmer strip method was used for tear fluid collection both in controls as well as in Dry eye syndrome cases. Schirmer tear protein extraction procedure was used even for dry eye cases as discussed in this chapter section 3.3.3. Then 2DE was done with the tear protein to look for the differential expression. Dry eye tear protein

showed differential expression of proteins in dry eye associated with primary Sjogren's syndrome and Steven-Johnson syndrome as seen in figure 4.11 a and 4.12 a. This was repeated in 3 sets of tear samples from controls and DES which were un pooled.

Figure 4.11a: Differential expression of tear proteins in primary Sjogren's syndrome

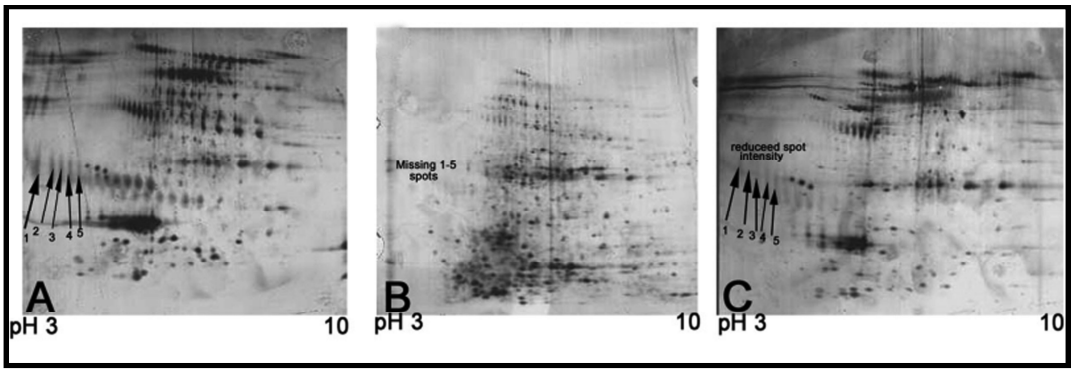


Fig 4.11 a: 2D gel map of tear proteins of A. Normal B and C. DES-Primary Sjogrens syndrome , severe and moderate respectively. Tear proteins (30 µg) separated in 17 cm pH 3-10 IPG strip in the first dimension and 13 % SDS-PAGE in the second dimension. The marked region shows the differential expression of tear proteins in the MW region of around 25- 30kDa absent in B gel and reduced in C gel.

Figure 4.11b: Zoomed images of Differential expression of tear proteins in primary Sjogren's syndrome

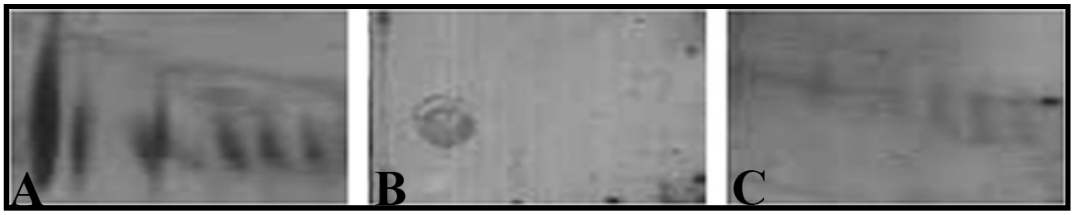


Figure 4.11b: Differential expression of tear proteins in DES. zoomed images of the 5 protein spots shown in fig 4.11 a.

Figure 4.12 a: Differential expression of tear proteins in Steven-John's syndrome

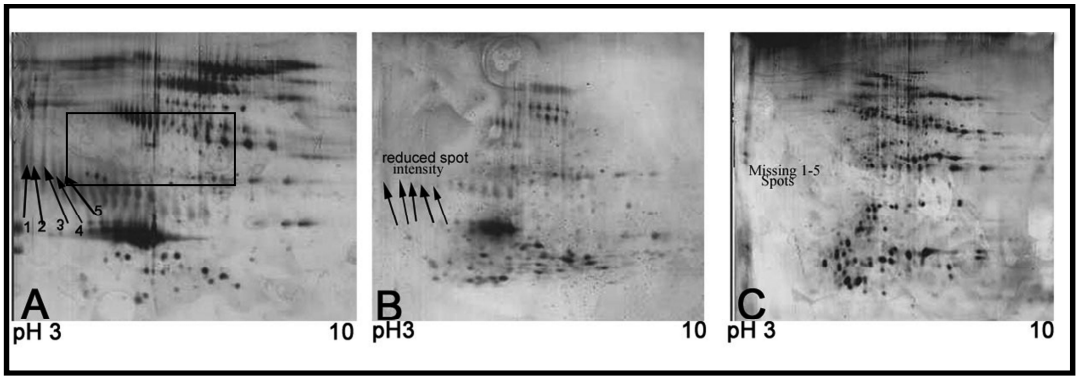


Fig 4.12a: 2D gel map of tear proteins of A. Normal. B & C. DES. Tear proteins (30 µg) separated in 17 cm pH 3-10 IPG strip in the first dimension and 13 % SDS-PAGE in the second dimension. The marked region shows the differential expression of tear proteins in the MW region of around 25- 30 kDa reduced in B gel and absent in C gel.

Figure 4.12 b: Zoomed images of differential expression of tear proteins in Steven-Johnson's syndrome

Figure 4.12b: Differential expression of tear proteins in DES with Steven-Johnson's syndrome. zoomed images of the 5 protein spots shown in fig 4.12 a.

4.6. SUMMARY

1. 8M Urea buffer showed better protein extraction from Schirmer strips compared to PBS and HEPES buffering system with maximum number of protein spots.
2. 17 cm IPG pH 3 – 10 is showed better resolution of tear protein and further profiling of tear protein can be used 17 cm IPG pH 3-10 compared to 7 and 11 cm IPG.
3. Capillary and Schirmer tear protein profile were comparable using 2DE, therefore Schirmer tear collection can be used in case of disease condition such as Dry eye syndrome for identification of biomarkers.

CHAPTER 5: 2D ELECTROPHORESIS PROFILING OF TEAR PROTEIN IN DRY EYE SYNDROME ASSOCIATED WITH NON SJOGRENS SYNDROME

5.1.1. Introduction

Dry eye is recognized as a disturbance of the Lacrimal Functional Unit (LFU). LFU controls the major components of the tear film, have the regulated controls and responds to stimulations includes environmental, endocrinological and cortical (Dartt 2009).

5.1.2. Classification of Dry eye

Dry eye is classified as aqueous tear deficient and evaporative dry eye as discussed in chapter 1.10. Further the aqueous tear deficient dry eye is classified into sub groups such as sjogrens with the involvement of auto antibodies and Non sjogrens which is associated with aqueous tear deficiency wherein no auto antibodies are involved. It is usually due to factors such as viral infections, various drugs such as antihistamines, beta blockers, diuretics and psychotropic drugs, apart from environmental factors such as humidity and wind. Stevens–Johnson syndrome (SJS) is a hypersensitivity complex disease affecting the skin and the mucous membranes, also leads to DES. SJS is caused by infections, usually due to infections such as herpes simplex virus, influenza, mumps, cat-scratch fever, histoplasmosis, Epstein-Barr virus and adverse effects of drugs mainly antibiotics and sulfa drugs such as allopurinol, diclofenac, etravirine, fluconazole, and also malignancies. Lid abnormalities can also cause non sjogrens DES. Evaporative dry eye classified as either intrinsic in which meibomian gland involved or extrinsic due to the vitamin A deficiency, allergy, and drug influence (2007).

5.1.3. Tear film changes in dry eye syndrome

Reports from the studies on DES associated with non sjogrens showed down regulated tear proteins like lysozyme, lipocalins, lactotransferrin, prolactin induced

tear protein. The protein changes may vary with the severity of the DES. Various groups are still working, conducting studies to look for specific proteins that contribute to disease mechanism. The current study focus to identify proteins characteristic of DES associated with non Sjogrens.

5.2. Objectives

1. Profiling of tear protein in Dry eye syndrome associated with non Sjogrens syndrome (DES-NSS) using 2D electrophoresis
2. To look at the differentially expressed tear proteins using PD quest software, validate the differentially expressed proteins using DIGE.
3. Identification of the differentially expressed proteins using mass spectrometry.
4. Correlation of the differentially expressed tear protein with the clinical condition

5.3.Methods

5.3.1. Diagnosis of dry eye cases from Ocular surface Clinic

Dry eye cases were diagnosed at ocular surface clinic for the recruitment. Total 4 groups were recruited for the study. Control subjects, Dry eye with Rheumatoid arthritis (DES-RA), Dry eye without Rheumatoid arthritis (RA alone), Rheumatoid arthritis without dry eye (DES) and primary Sjogren's (DES-pSS) cases were also recruited in the study. Diagnosis of DES was done based on the DEWS study as discussed in chapter 3. A detailed clinical proforma was used to document the clinical details of all the subjects. The non Sjogrens DES (DES-NSS) cases include DES with hormonal, Stevens-Johnson syndrome (DES-SJS), age related and post viral infection cases.

5.3.2. Subjects recruited for the study

39 healthy controls (mean age: 43 ± 12, 12M, 27F), 34 cases of DES-NSS (mean age: 39 ± 17 yrs, 16M, 18F) were recruited for the study. The cases with other systemic diseases, on any other medications were excluded during the recruitment of the study. Among the cases recruited for the study, the SJS were of the following

types. Of the 34 DES-NSS, 18 were SJS, 2 Neurotrophic DES, 3 MGD 1 post conjunctivitis (severe), 4 were due to other cause. Of the total, 4 were mild type, 6 moderate and 16 were severe.

5.3.3. Collection of tear fluid from dry eye and Controls

Tear fluid was collected from controls and dry eye cases using Schirmer strips as discussed in chapter 2. The sample was stored at -80 °C until processing.

5.3.4. Extraction of tear protein from Schirmer for 2DE and DIGE

Tear proteins from Schirmer strips were extracted using 300 µl of 8M urea buffer (pH: 7.0) containing DTT, CHAPS and with 30 µg protease inhibitor cocktail, incubated at 4°C for 3hrs with intermittent mixing. After 3 hrs, removed the strip, centrifuged the sample, desalted, and protein estimation was done by Bradford assay. **For DIGE** experiment tear protein was extracted using 30 mM Tris–HCl buffer containing with 8M urea, 3% CHAPS and 0.5 mM TCEP (pH: 8.5) and the extraction procedure was similar to the 2-DE extraction.

5.3.5. Processing of tear protein for 2DE and DIGE

2DE:17 cm IPG with pH 3-10 were used for tear protein separation in 1st dimension. IPG strips were passively rehydrated with 30 µg of tear protein for 16 hrs, then the strips were focussed for 8 hrs at 20 °C in IEF focussing unit, after the first dimension the strips were equilibrated using equilibration buffer I and II for 30 min each, separated the focussed proteins on 13% SDS-PAGE for 5 hrs using protein XI multigel system. Gel was stained using silver stain, scanned the gel using densitometer and analysis was done using PD quest software to look for the differentially expressed protein/peptide spots.

2D-DIGE: 30 µg of pooled tear protein from control and Non ss DES was labelled with 240 pmol of Cy dyes i.e Cy 3, Cy 5 and Cy 2 in dark, and then subjected to electrophoresis as described in chapter 2. Gels were scanned using typhoon scanner and spot analysis was done using Decyder 2DE 7 software for the quantitative changes of the differentially expressed spots.

5.4. Results

5.4.1. 2DE profiling of tear protein

Tear samples were profiled using 2DE from the non sjogrens cases. The amount of total tear protein was reduced significantly ($p < 0.001$) in all types of DES cases irrespective of the causative factor as seen in table 5.1.

Table 5.1: Tear protein in control vs DES-NonSS

	Control (µg/ml) tear protein	**DES-Non sjogrens (µg/ml)**
Mean ± SEM	**1726 ± 176**	**488 ± 111**
P value		**< 0.001**

5.4.2. 2DE of tear protein

Figure 5.1 shows the representative tear protein profile of DES-non Sjogrens tear protein comparing with control which shows changes in the total protein number as well as its spot intensity. Control and DES-non Sjogrens showed a total number of protein spots of 145 ± 7 and 100 ± 20.respectively as analyzed by PD Quest software.

Figure 5.1: Representative 2DE profile of tear protein in DES-Non SS

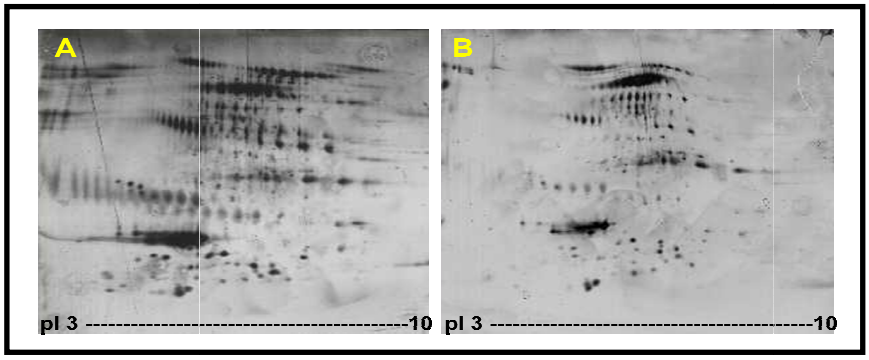


Fig 5.1: Tear protein profile A. Control B. DES-NSS. DES- NSS showed change in the tear proteins, most of the peptide spots were reduced compared to control.

The tear protein profile changed with the severity of the DES as seen in figure 5.2. The marked regions of 1-7 showed protein changes in DES condition. Peptide spots in regions 1,2,5,6, and 7 showed down regulation, and the proteins were observed to be more down regulated in severe compared to mild and moderate. Peptide spots at regions 3, 4 and 7 up were up regulated compared to control (figure 5.2).

Figure 5.2: DES-Non SS tear protein profile with DES severity

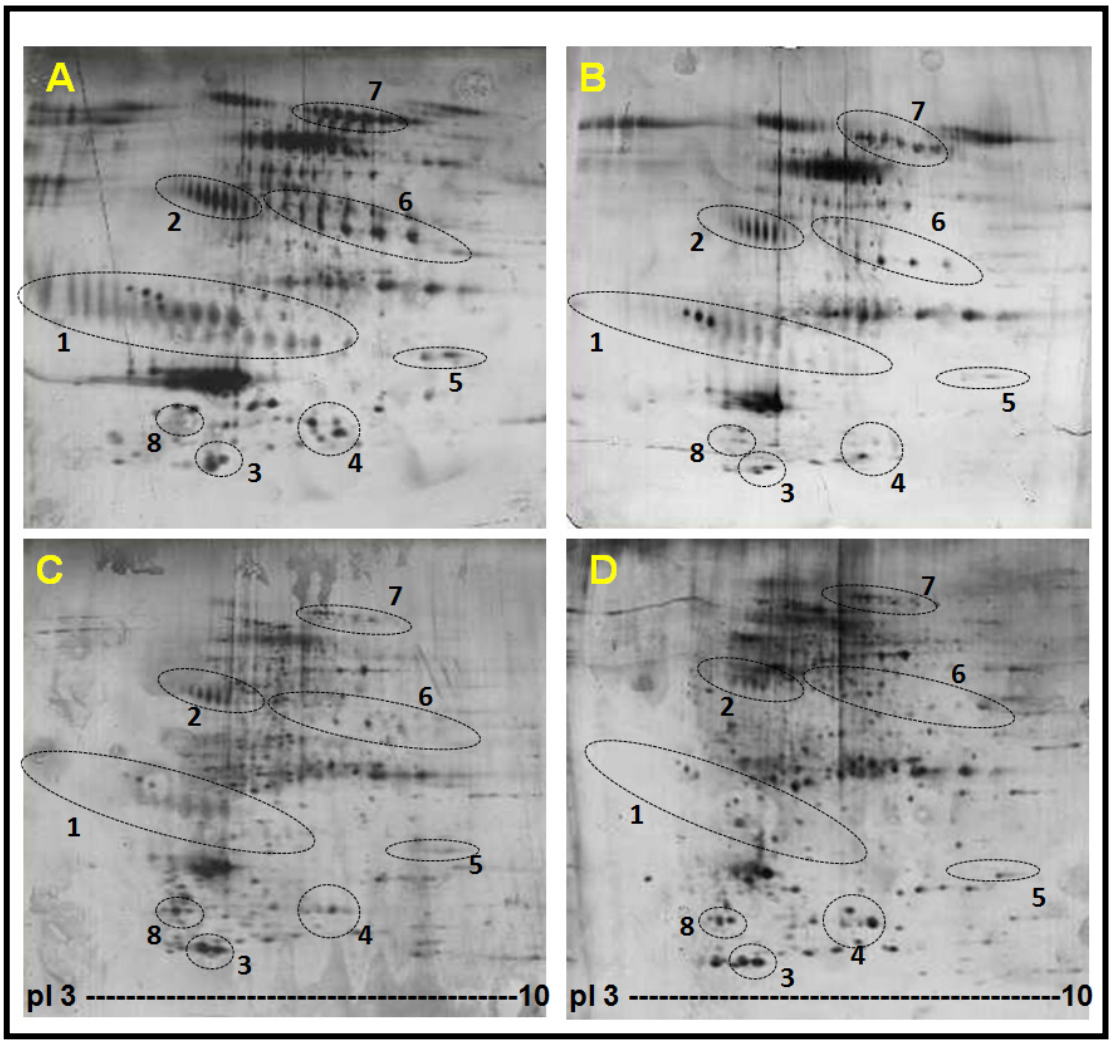


Fig 5.2: Tear protein profile A. Control B. Mild DES-NSS C. Moderate DES-NSS and D. Severe DES-NSS. As the severity increases quantitative changes in the proteins are observed seen as seen in circled region's 1-8 in DES-NSS compared to control.

Table 5.2 shows the differentially expressed spots in terms of change in % intensity and the number of cases showing the same as per PD quest analysis. Only 2 fold greater or lesser changes have been taken into consideration to see the up and down regulated peptide spots as given below.

- Down regulation of spot numbers 1-25, 56-72, was seen in 100% Severe DES, 60% of moderate DES and in 25 % of mild cases.

- Down regulation was seen in the spots 29-30 in 70 % of the severe DES, 50 % of the moderate DES and none in mild DES.
- Peptide spots 54-55, 63-66, 67-71 and 74 -76 showed down regulation in 70, 85, 89, and 90 % of severe DES respectively
- Spot 40-41 showed up regulation in 40% of severe DES, 25% of moderate DES and no change in mild DES
- Peptide spots 42-51 showed up regulation in 66 % severe DES,
- Peptide Spots A, B and C showed up regulation by 2.5 fold with an increase in > 75 % cases.

5.4.3. 2D-DIGE of Tear protein: To validate these differentially expressed tear proteins from 2DE, pooled tear protein was subjected to DIGE analysis and the differentially expressed protein spots were tabulated in table 5.3. DIGE also showed similar results as seen by 2DE. The representative DIGE Cy dye image of control vs Non SS DES is shown in figure 5.4. The red intensity shows the down regulation of the peptide spot in the test sample as the control sample is highly represented due to Cy5 (red). Up regulation is to be seen by intensity of the green spot while yellow (merged) indicated no change. This is analyzed by the Decyder software and is shown by the biological variance analysis (BVA) as given in table 5.3. This is given as spot volume ratio along with statistical significance. Figure 5.5 is the representative grey scale image of the DIGE that clearly shows the differential expression of proteins.

Table 5.3 shows the differentially expressed proteins in DES-Non SS as observed by DIGE analysis. The Biological variance analysis (BVA) of DIGE is done to look for the significant differential expression of peptide spots from DES-Non SS compared to controls as well as to primary and secondary Sjogrens DES. BVA was done during the spot analysis with Decyder software as discussed in chapter 2 methods section for DIGE analysis. One way ANOVA done by the decyder software and gives the significant peptide spots. p value < 0.05 is considered significant. The volume ratio of the peptide spot > or < 1.5 fold was considered as up regulated and down regulated respectively as mentioned in table 3. Accordingly, a total of 6 spots were down regulated and 4 spots up regulated in DES-Non SS.

Table 5.2: The differentially expressed proteins in DES-Non SS compared to normal tear by PD quest analysis.

Spot number	No of cases in Non SS DES			Change in spot intensity
	Mild DES (n= 4)	Moderate DES (n= 8)	Severe DES (n= 22)	Spot intensity (fold change)
Spot 1-8	25% ↓	70 % ↓	100 % ↓	3.5
Spot 9-20	25 % ↓	65 % ↓	100 % ↓	4.0
Spot 21-22	No change	50 % ↓	100% ↓	2.0
Spot 23	No change	60 % ↓	100 % ↓	3.5
Spot 24	No change	60 % ↓	100 % ↓	3.5
Spot 25	No change	60 % ↓	100 % ↓	3.5
Spot 29-30	No change	50% ↓	70 % ↓	3.0
Spot 40-41	No change	25 % ↑	38 % ↑	2.1
Spot 42-51	20% ↑	50% ↑	66% ↑	2.5
Spot 54-55	No change	60 % ↓	70% ↓	2.0
Spot 56-62	No change	65% ↓	100% ↓	3.5
Spot 63 -66	20 % ↓	50% ↓	85 % ↓	2.0
Spot 67-71	25 % ↓	67 % ↓	89 % ↓	2.0
Spot 72-76	No change ↓	63 % ↓	90 % ↓	2.0
Spot 35-39	60% ↑	65% ↑	75 % ↑	3.5
Spot A	ND	40% ↑	70% ↑	2.5
Spot B	ND	50% ↑	100% ↑	2.5
Spot C	ND	60% ↑	74% ↑	2.5

These spots can be considered as the proteins that are characteristically altered in Non SS DES. The 2D data had revealed the differentially expressed proteins in Non SS DES with respect to control. (63 peptide spots) However this has been short listed (10 peptide spots) based on the DIGE analysis. Further identification of these spots, followed by validation can qualify them as biomarkers. Figure 5.6 shows these 10 differentially expressed peptide spots based on DIGE.

The differentially expressed spots from DES–Non SS that were up regulated and down regulated as given by the DIGE Decyder was shown in Figure 5.5. The spot view represents the position of spot, (figure 5.6A) the graphical view indicates the up or down regulation of the spot in respect to all the groups under comparison, (figure 5.6B) and 3D view of the same spot from which spot volume is calculated (figure 5.6C).

Figure 5.3: Representative DIGE tear profile of DES-Non SS

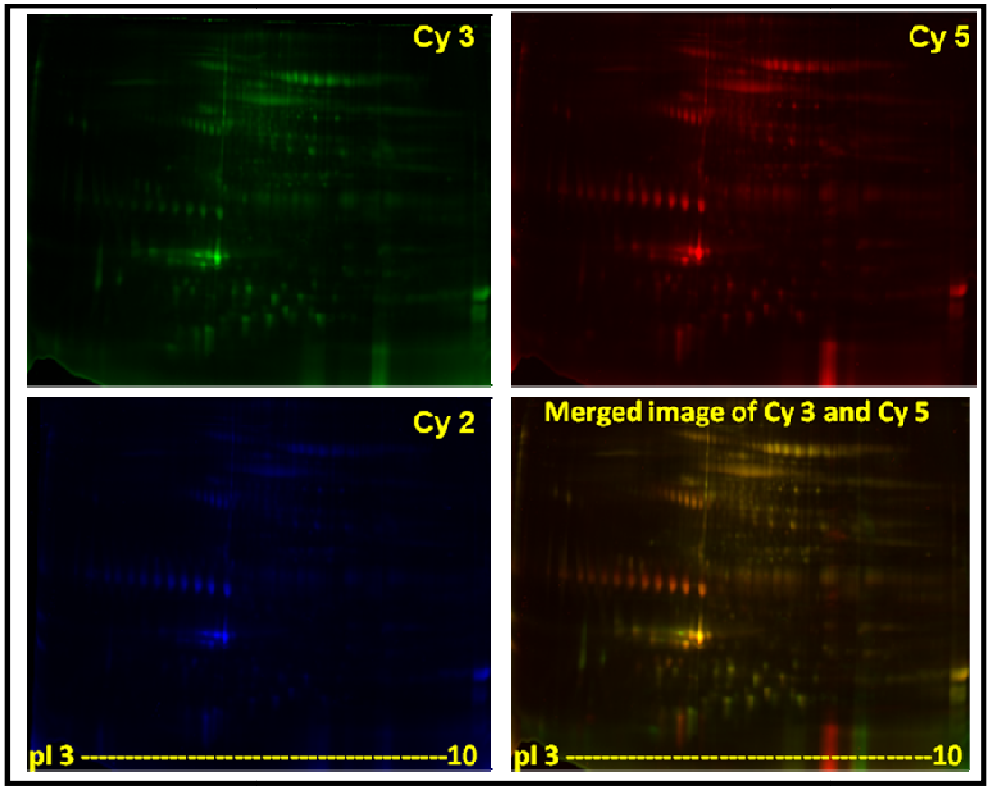


Fig 5.3.: The tear protein (30 µg) of control(red) and DES (green) labelled with the Cy dye Cy 5 and Cy3, and pool of control and DES protein (15 µg) labelled with Cy 2 as an internal standard (blue) during the DIGE spot analysis. The merged image of Cy 3 and Cy 5 image to look for the differential expression. The more the colour it shows indicated the up regulation of that peptide spot.

Table 5.3: Differentially expressed tear peptide spots in DES-Non SS based on DIGE BVA analysis.

S. No.	Down regulated Peptide DIGE spots	Corresponding 2D spot No.	Spot Volume ratio	P value
1	270	43	2.1	0.011
2	293	24 a	3.5	0.006
3	328	20	4.8	0.04
4	430	40 a	2.2	0.02
5	432	40 b	5.8	0.00016
6	351	**20 a**	**3.4**	0.03
	Up regulated Peptide spots			
7	**412**	**38**	**2.5**	**0.02**
8	258	80	2.0	0.02
9	240	75	1.9	0.045
10	129	60	1.9	0.01

Figure 5.4.: The representative tear protein profile grey scale image of 2D-DIGE

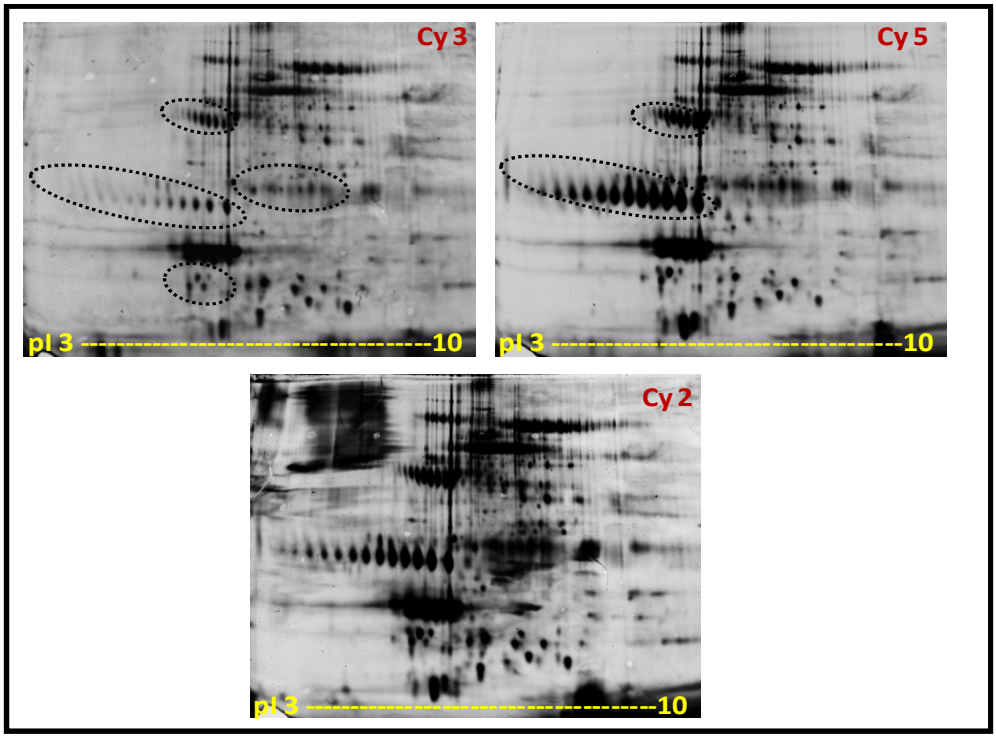


Fig 5.4.: The representative grey scale image of DIGE, 30 µg of control and DES tear was labelled with Cy 5 and Cy3, 15 µg of each control and DES was mixed and labelled with Cy 2 as an internal control. Initially the proteins were focussed on IPG strips with pI 3-10, and then the focussed proteins were resolved on 13% SDS-PAGE. DES-Non SS showed changes in the proteins compared to control.

Figure 5.5: Differentially expressed proteins of DES-Non SS based on DIGE

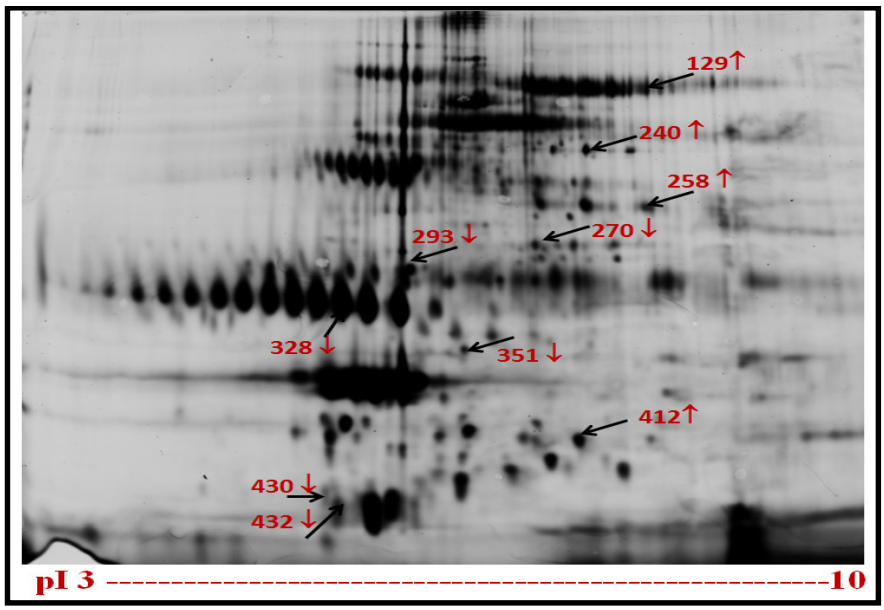


Figure 5.6: Representative gel image from BVA analysis of tear proteins in DES-NonSS

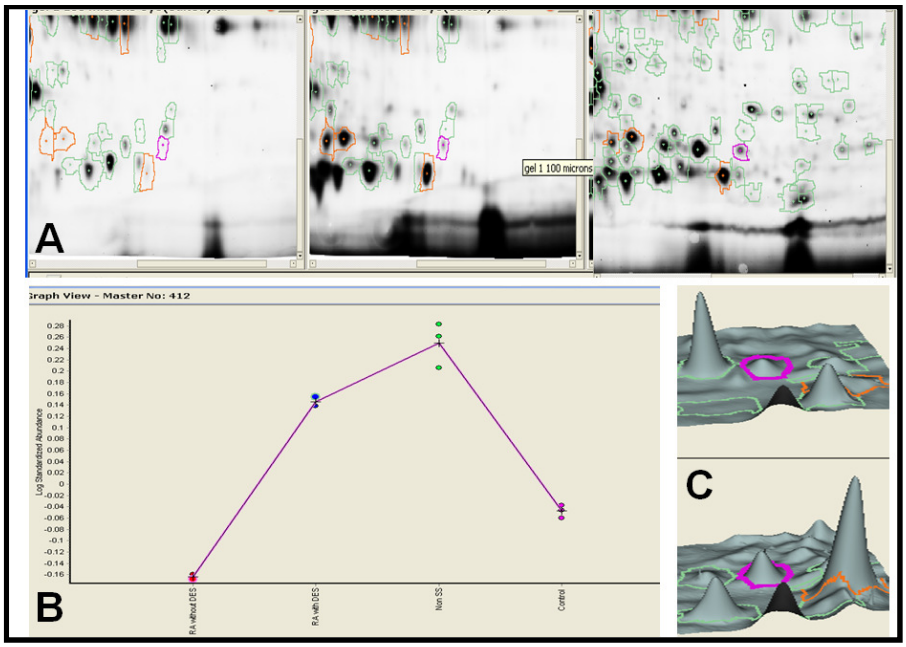


Figure 5.6: BVA spot view. A. pink coloured marking – selected spot in the gel B. Graphical representation of the selected spot across the groups. C. 3D view of the selected spot.

5.4.4. *Ingel* tryptic digestion of differentially expressed proteins

The *ingel* tryptic digested proteins observed from both 2DE and DIGE were identified by mass spectrometry nano LC/MS/MS as shown in table 5.4. The major proteins that were down regulated are lacrimal proline rich protein (LPRR4), lacritin precursor (spot 1-8,15 &16), extracellular glycoprotein lacritin precursor(spot 18,19), immunoglobulin J (spot 23-25), Znic – alpha-glycoprotein(spot 56-32), cystatin (spot 29,30), lactotransferrin isoform 1 precursor and isoform 2(spot 72-74), dermicidin (spot 54-55), lipocalin (spot 35-37) and the proteins that were up regulated are mammaglobin B (spot 40), Protein S100A10 (spot A) Methenyltetrahydrofolate synthase domain containing protein (spot C), as shown in table 5.4. Two proteins namely zinc alpha glycoprotein and cystatin are reported to be reduced in dry eye condition already known from the literature.

Out of the significantly altered proteins spot number 412 was identified as protein S100 A8 and neutrophil defensin 3 by 2D nano-LC/MS/MS. The sequence evidence spectrum of each identified protein with 59 % and 65 % coverage, 3 peptide sequences LLETECPQYIR, LTELEK, MLTELEK, with m/z value 1421.7, 732.4 and 863.4 and with +2 charge are mentioned in the figure 5.7.

Figure 5.7. MS/MS Spectrum of Spot 412 identification as S100 A8 with 3 peptide sequence

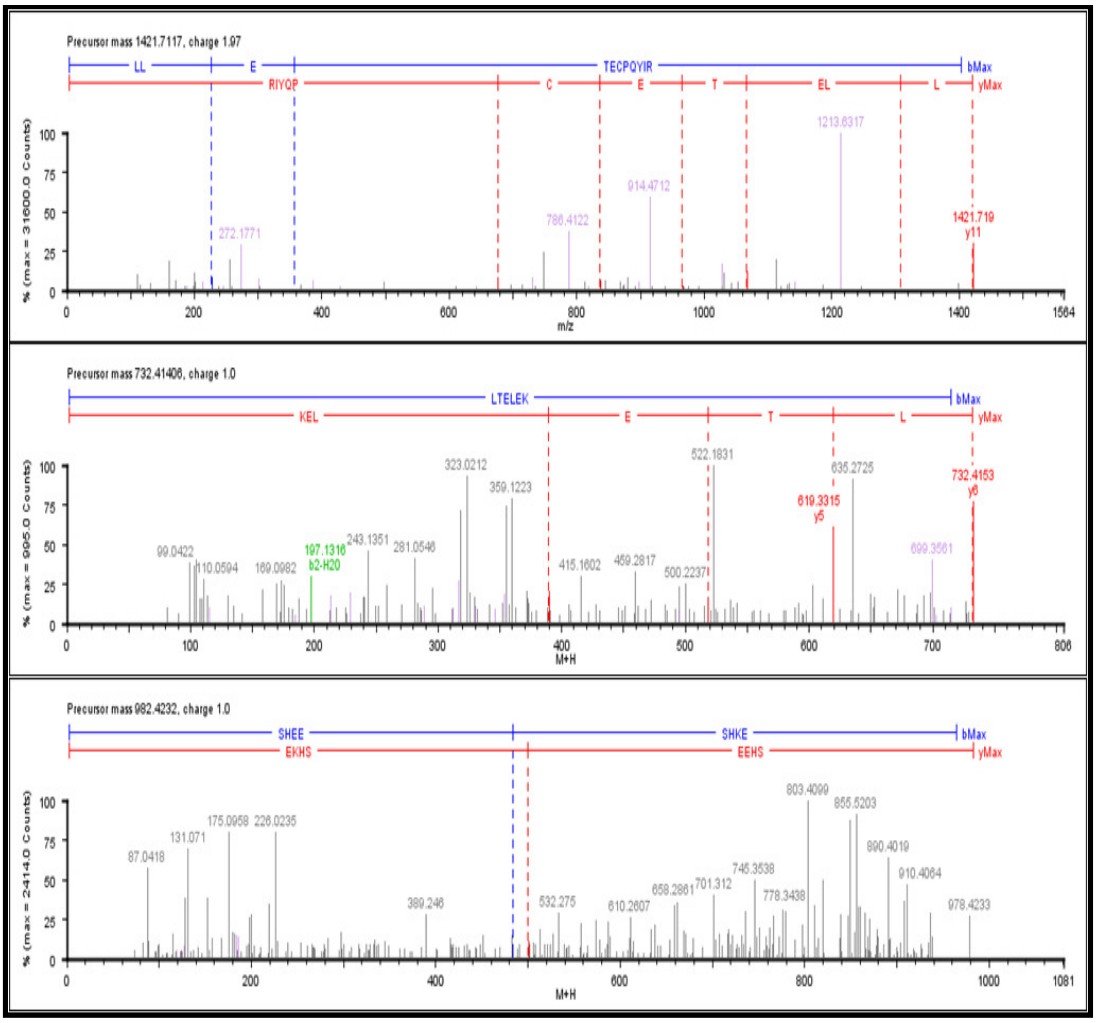


Figure 5.7: Protein S100 A8 with 3 peptides LLETECPQYIR, LTELEK, MLTELEK, with m/z value 1421.7, 732.4 and 863.4 respectively.

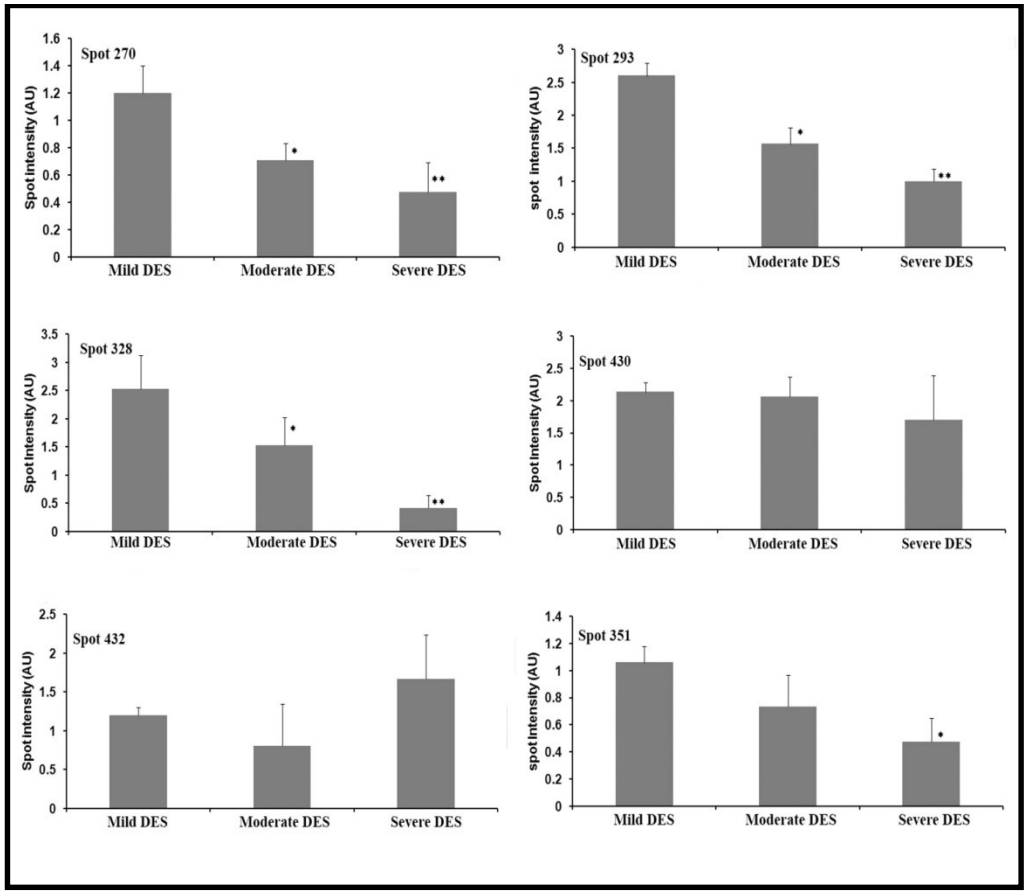
Table 5.4.: List of identified peptides differentially expressed in DES

Spot No. 2D	Volume ratio (DIGE-spot no)	P value	Identified protein	Accession no.	Peptide sequence	% covera ge
Spot 1-8, 15,16	2.6 ↓ spot no: 315,316,317, 318,319, 320,321,322, ↓	< 0.01	LPRR4	gil154448886	FPSVSLQEASSFFQR (1peptide)	37%
Spot 21-22	2.4 ↓ 334,335 ↓	< 0.03	Lacritin precursor	gil54607120	DGAGDVAFIR LADFALLCLK LRPVAAEVYGTER (3 peptides)	25%
Spot 18,19	2.1 ↓	< 0.03	extracellular glycoprotein lacritin precursor	gil15187164	SILLTEQALAK (1 peptide)	26%
Spot 23 - 25	1.8 ↓ 301,302, 303	< 0.05	Immunoglobu lin J	gil21489959	SSEDPNEDIVER CYTAVVPLVYGGEK (2 peptides)	31%
Spot 42-51	2.0 ↑	< 0.04	Yet to identify	--	---	--
Spot 54-55	1.9 ↓	<0.05	Dermicidin	P81605	DAVEDLESVGK	20 %
Spot 29-30			Cystatin (From literature)	---	---	---
Spot 40-41	2.3 ↑	< 0.035	Mamma globulin B precursor	gil4505171	ELLQEFIDSDAAAEA MGK TINSDISIPEYK QCFLNQSHR (3 peptides)	43%
Spot 56 - 62	2.5 ↓	< 0.03	From literature Zn-alpha-glyco protein	---	---	---
Spot 67 – 71	1.9 ↓	< 0.03	Yet to identify	----	----	----
Spot 72 – 74	2.1 ↓ 20,21 ↓	< 0.04	Lacto transferrin isoform 1 precursor and isoform 2	gil54607120 gil312433998	DGAGDVAFIR (1 peptide) DGAGDVAFIR (1 peptide)	17%
Spot 75, 76	2.5 ↓	< 0.04	yet to identify	---	---	---
Spot A	2.5 ↑		S100A10	P60903	VGFQSFFSLIAGLTIA CNDYFVVHMK	26.8
Spot C	2.5 ↑		Methenyltetra hydrofolate synthase domain containing protein	Q2M296	ELGSVPLR NYSVPIGLDSR SVPLR	19.5
Spot 38	2.5 ↑ 412		S100A8	P05109	LLETECPQYIR, LTELEK, MLTELEK	59 %
81	2.0 191 ↑		Lipocalin precursor	gil4504963	NNLEALEDFEK	19.9 %

5.4.5. Disease correlation of spots variation with DES grading

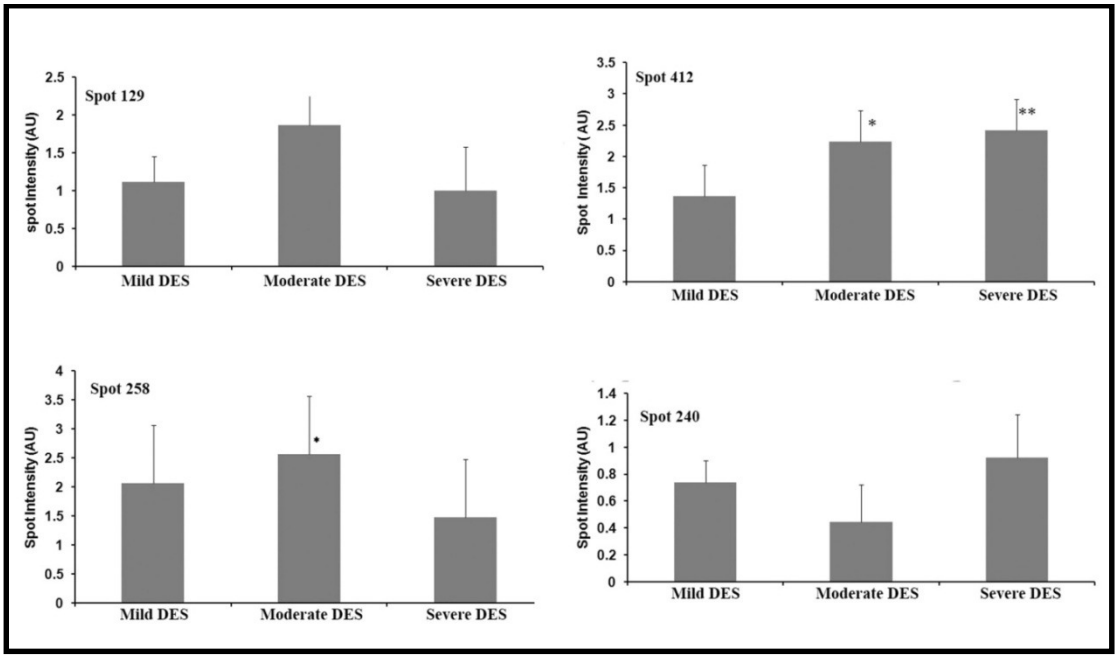
When looked for the correlation of differentially expressed peptide spots in un pooled 2DE with spot intensity, spot 270,293, 328 showed significantly down regulated in moderate and severe DES. Spot 430, 432 did not show any change with the severity. Spot 351 was down regulated significantly in severe DES than Moderate DES as seen in figure 5.8.

Figure 5.8.: Disease correlation of down regulated spots of DES-Non SS



Spot 129,240 showed up regulation in DES – Non SS. But it did not show significant increase with DES disease correlation. Spot 412 showed significant up regulation in moderate and severe DES. Spot 258 showed significant up regulation in moderate DES, no change in severe DES as seen in figure 5.9.

Figure 5.9: Disease correlation of Up regulated spots of DES-Non SS



5.4.6. *Insol* tryptic digested tear protein identification:

The whole tear protein after extracted into the buffer was also subjected to *insol* tryptic digestion and then the proteins were identified by nano LC/MS/MS. The tear proteins identified from the insol tryptic digested tear in all types of DES are tabulated in table 5.5 and table 5.6 gives proteins characteristic of DES-Non Sjogrens.

For *in sol* MS analysis no pooling of the tear protein was done. A total of 38 tear proteins were identified from control tear sample, among which 13 proteins were also detected in DES-Non SS. DES-non SS also showed 19 characteristic proteins specific to the disease as seen in table 5.5 and 5.6 in which the major proteins identified are calcium binding proteins such as S100A9, S100A6, P,A7, enolase variant, peroxidise precursor, Histone H2B type 2 protein, plastin 2, cytokeratin 13, BRD 1 protein, Zn finger protein, actin alpha cardiac muscle 1 proprotein as listed in table 5.6.

Table 5.5. The proteins identified from the *in sol* tryptic digested tear protein by nano LC/MS/MS

Accession no / peptide / sequence coverage	Control (n=9)	DES-Non SS (n=11)
spt|P12273	Prolactin induced protein(n=7)	Prolactin-inducible protein (n=2)
pdb|1YAN	Lysozyme (n=8)	Lysozyme precursor(n=1)
trm|Q8N4N0	Zinc Alpha 2 glycoprotein(n=8)	Alpha-2-glycoprotein 1, zinc (n=1)
spt|Q9GZZ8	Lacritin precursor (n=5)	lacritin precursor (n=1)
cra|hCP1734353.1	Proline rich 1 protein (n = 5)	Not detected
cra|hCP1915488	Proline rich 4 protein (n=7)	Proline-rich protein 4 lacrimal(n=2)
Gi|4505171	Secretoglobin 2A(n=1)	secretoglobin family 2A member 1(n=1)
spt|P01036	Cystatin S precursor(n-1)	Cystatin S precursor (n=1)
Gi|55666285	Lipocalin like protein(n=5)	lipocalin 1 like protein(n=2)
pdb|1LFH	Lactoferrin(n=6)	Lactoferrin(n=1)
Gi|25058739	Albumin(n=5)	serum albumin(n=4)
spt|P01833	Polymeric Ig receptor 9(n=2)	polymeric immunoglobulin receptor(n=1)
Gi|55667085	Similar to pHL E1F1 isoform 2 (n=2)	Not detected (ND)
Gi|3954893	Ig kappa light chain(n=3)	immunoglobulin kappa light chain VLJ region (n=1)
Gi|21669677	Ig lambda light chain(n=1)	immunoglobulin variable region (n=1)
trm|Q96F97	Chitinase 3 like 2 protein(n=4)	ND
Gi|52426735	Ankyrin 2 isoform 1(n=1)	ND
trm|Q8IZY7	hepatocellular carcinoma associated TB6 protein (n=4)	ND
Gi|854182	SOX(n=1)	ND
Gi|15822535	cyclin dependent kinase protein(n=1)	ND
spt|P06703	Calcyclin(n=1)	ND
spt|O75556	mammaglobin B precursor (n=4)	ND
rf|NP_001053.2	trans cobalamin I precursor(N=1)	ND
Gi|19574296	hCG1999003 (n=1)	ND
Gi|119608459	hCG201503 (n=1)	ND
Gi|19882251	Cystatin SN precursor (n=5)	ND
Gi|124248531	LOC441476 (n=1)	ND
P02786	human transferrin receptor(n=1)	human transferrin receptor (n=1)
Spt|p01130	LDL receptor precursor (n=1)	ND
Trm|Q86X06	ERD 1 protein(n=1)	ND
Gi|7657532	protein S 100 A6 (n=1)	protein S 100 A6 (n=2)
Gi|40385873	probable G protein coupled receptor protein 150 (n=2)	ND
Gi|188528616	testis-specific gene 10 protein(n=2)	
Q8NEM0		Microcephalin (n=1)

Table 5.6: Proteins characteristically seen in DES- Non SS by *Insol* nano LC/MS/MS.

Accession No.	DES-Non SS (n=11)
Trm|Q68DN5	Hypothetical protein DKFZp779N1935 (n = 4)
gi|4506773	protein S100 A9 (n =2)
gi|4503571	enolase 1 variant (n= 2)
Trm|Q9Y2F3	KIAA0676 protein ((n= 2)
gi|7657532	protein S100 A6 ((n= 2)
gi|5174663	protein S100 P (n= 2)
gi|115298657	protein S100 A7 (n= 2)

5.4.7. Comparision tear protein Changes in DES-Non SS and DES-SJS

Dry eye is due to various etiologies, in which DES-Non SS is one. SJS due to infection, drug allergy is one of the DES-NonSS types. Current study observed differential expression of proteins between the DES-SJS and other DES-NonSS. Though DES showed differential expression of tear proteins, the % difference in the spot intensity vary between DES-NSS and SJS cases as shown in table 5.7. SJS cases showed more severe DES than NSS as seen in the changes of the proteins Fig 5.10. LPPP4, immunoglobulin J, lacritin, cystatin, Zn-alpha glycoprotein, dermicidin were down regulation and cytoskeletal protein 10, S100 protein, mammaglobulin, lipocalin and lipocalin precursor showed up regulation in both SJS and Non SS, but SJS showed > 90% cases than other DES-Non SS. Table 5.8 shows the clinical details of the Non SS cases where most of them were severe in terms of clinical parameters Schirmer's value, TBUT, TMH and disease grade Correlating with the expression of proteins.

Table 5.7: Differential tear proteins of DES-non SS and DES-SJS using 2DE.

Spot No.	Volume ratio	Significance	Name of the Identified protein	Non SS % cases	SJS % cases
Spot 1-8, 15,16	2.6 ↓	< 0.01	LPRR4	80%	100 %
Spot 21-22	2.4 ↓	< 0.03	Lacritin precursor	80%	100 %
Spot 18,19	2.1 ↓	< 0.03	extracellular glycoprotein lacritin precursor	75%	100 %
Spot 23 - 25	1.8 ↓	< 0.05	Immunoglobulin J	60%	90 %
Spot 42-51	2.0 ↑	< 0.04	Yet to identify		
Spot 54-55	1.9 ↓	<0.05	Dermicidin	70 %	100 %
Spot 29-30			Cystatin (From literature)	80%	100 %
Spot 40-41	2.3 ↑	< 0.035	Mammaglobulin B precursor	80 %	80 %
Spot 56 - 62	2.5 ↓	< 0.03	From literature Zn-alpha-glycoprotein	50 %	95 %
Spot 67 – 71	1.9 ↓	< 0.03	Yet to identify		
Spot 72 – 74	2.1 ↓	< 0.04	lactotransferrin isoform 1 precursor and isoform 2	80%	95 %
Spot 75, 76	2.5 ↓	< 0.04	yet to identify		
Spot 35,36, 37	1.8 ↑	0.03	Lipocalin	70 %	90 %
Spot 81	2.0 ↑	0.02	Lipocalin precursor	70 %	95 %

Figure 5.10: Tear proteins expression comparison of DES non Sjogren's and SJS

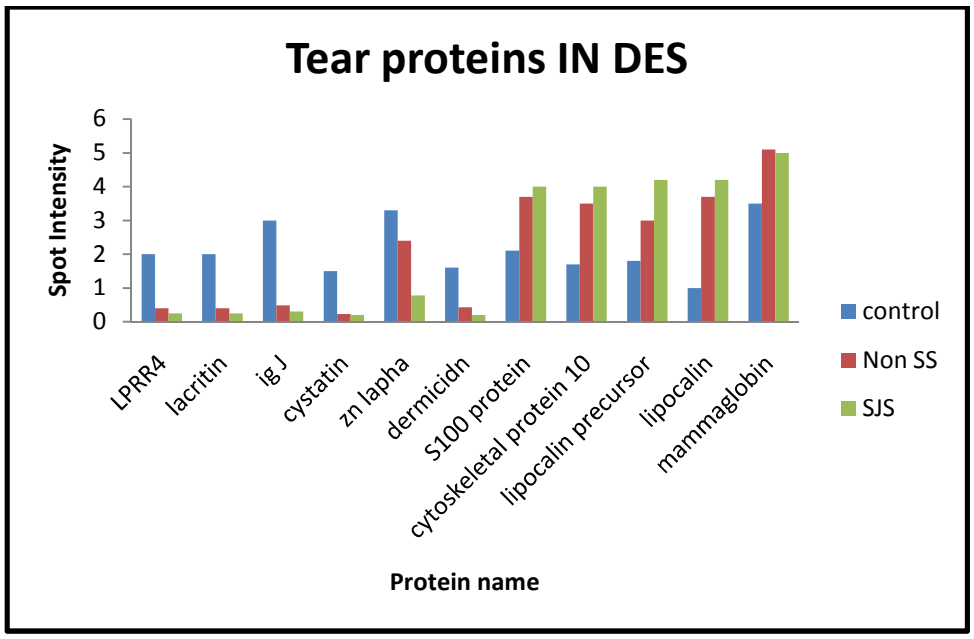


Table 5.8: Functions of the proteins identified in DES based on *in gel* and *insol* tryptic digestion

S.No.	Name of the identified protein	Function of the protein	Major expression tissue/cell
1	Lacrimal proline rich 4 protein(LPRR4)	Exact mechanism is not known yet. Protective role could be as antimicrobial.	Lcarimal gland acinar cells, secreted protein
2	Extracellular glycoprotein lacritin precursor	Promotes tear secretion	Lacrimal and salivary acinar cells
3.	Immunoglobulin J	Linking immunoglobulin monomers (IgM to pentamers, IgA to dimers) and binding these immunoglobulins to secretory component	All cell types
4.	lactotransferrin isoform 1 precursor and isoform 2	Anti-inflammatory and anti-microbial	All tisues
5	Cytoskeletal protein 1	regulate the activity of kinases such as PKC and SRC	All types of cells
6	Cytoskeletal 10 protein	Keratinocyte differentiation, epidermis development	All types of cells suprabasal cell layers
7	Lipocalin 1 precursor	non-immunological defence	Lacrimal and salivary gland
8	Zn-alpha 2- glycoprotein	Regulates lipid metabolism,	Serum, saliva,
9	Cystatin	Cysteine proteinases	Saliva, tears
10	Mammaglobin B	Transcriptional reguloation of steroid hormones	Uterus, breast, testis, salivary gland
11	S 100 A6, A9,P,A7, S100A8	Antimicrobial activity, Calcium binding protein. regulation of protein phosphorylation, transcription factors, Ca++ homeostasis, the dynamics of cytoskeleton constituents, enzyme activities, cell growth and differentiation, and the inflammatory response	All cell types

Tear proteins from DES-Non SS that were differentially expressed and identified in the current study are mostly involved in ocular defence with its anti microbial activity, calcium binding, protease function, anti microbial activity. Further validation these proteins has to be done.

5.5. Summary

1. The tear protein form Dry eye of non sjogrens was profiled using 2D electrophoresis. And then further validated using DIGE analysis.

2. Proteins that showed down regulation in DES were LPRR4, Immunoglobulin J, Cystatin, Zinc alpha glycol protein, dermcidin. Up regulated proteins were mammaglobulin, S100 A8 protein, lipocalin, lipocalin precursor more than 70 % of cases with 2 fold changes.

3. DIGE showed 10 differentially expressed spots among which 6 were down regulated and 4 were up regulated. 3 of the down regulated protein spots 270, 293 and 328 showed significant down regulation with disease severity. 1 of the up regulated proteins spot 412 showed significant up regulation that correlated with disease severity. Spot 412 was identified as protein S100 A8 by nano-LC/MS/MS. Further validation should be done for this protein.

4. DES with Non SS and SJS were compared, changes in the proteins either up regulation or down regulation were more severe in SJS compared to other non SS DES cases. Zinc alpha glycoprotein showed significant down regulation in DES-SJS than DES-NSS. This has to be validated further.

CHAPTER 6 : 2D ELECTROPHORESIS PROFILING OF TEAR PROTEIN FOR BIOMARKER IDENTIFICATION IN SECONDARY SJOGREN'S DRY EYE SYNDROME ASSOCIATEDWITHRHEUMATOID ARTHRITIS

6.1. INTRODUCTION

6.1.1. Dry Eye Syndrome (DES): Dry eye (DES) is a multifactorial disease that affects tear film and ocular surface. Increased osmolarity and inflammation is associated with DES (Ubels 2005). Dry eye is associated with auto immune disease such as Sjogren's syndrome. Sjogrens syndrome is a chronic inflammatory disease that affects exocrine glands, including salivary glands and lacrimal glands resulting in dry mouth and dry eyes (Haga, Naderi et al. 2012). Sjogren's syndrome is termed secondary Sjogren's when it is present with connective tissue disease like Rheumatoid arthritis (RA) and Systemic lupus erythematosis (SLE) (Ham, Jacob et al. 2007). This part of the study deals with DES associated with RA and the tear protein changes associated.

6.1.2. Rheumatoid arthritis (RA): RA is a chronic disease characterized by painful inflammation of the joints and the surrounding tissues leading to long term disability (Agarwal 2011). Chronic painful inflammation of synovial membrane and destruction of articular cartilage leads to muscle cachexia, joint destruction and permanent deformity (Mahto, Dave et al. 2011). Rheumatoid Arthritis can begin at any age (Huizinga and van der Helm-van Mil 2011) but its more prevalent between 35-55 years of age (Deal, Meenan et al. 1985). It is also known as rheumatoid disease (systemic illness) as it can affect multiple organs of the body such as lungs, heart, eyes, blood vessels etc., in addition to the tissues around the joints such as tendons, ligaments and muscles (Cojocaru, Cojocaru et al. 2010).

6.1.3. Prevalence of RA

(i) International: Globally 1.3 million adults suffer from RA. RA predominantly occurs in females. The prevalence of RA is around 1% worldwide (Sheehy, Murphy et al. 2006), with women suffering 3-5 times more than men (Agarwal 2011; Bergsten, Bergman et al. 2011; Huizinga and van der Helm-van Mil 2011).

(ii) India: RA is a common disease in India affecting elderly women. The Indian prevalence is 0.9% almost equal to the world prevalence (Malaviya, Kapoor et al. 1993).

(iii) RA is more prevalent in smokers than non smokers but not enough evidence which supports this correlation (Huizinga and van der Helm-van Mil 2011). Persons having positive Rheumatoid factor are more susceptible to develop RA.

6.1.4. Causes for RA: Specific cause to RA is not known sofar, but infection due to immune response against the hosts own body leads to RA. Organisms suspected to trigger for RA are mycoplasm, erysipelothrix, parvo virus, Rubella, Epstein - Barr virus and human herpes virus (Masuko-Hongo, Kato et al. 2003; Lossius, Johansen et al. 2012) Hormonal changes also be linked to the disease, particularly in women (Pikwer, Nilsson et al. 2012). RA may be genetically inherited (Huizinga and van der Helm-van Mil 2011).

6.1.5. Diagnosis of RA: Multiple tests are required to diagnose RA, includes x-ray, ultra sonography, blood investigations such as rheumatoid factor (RF) (Aho, Heliovaara et al. 1991), anti-citrullinated peptide antibodies (anti-CCP) (Agarwal 2011), antinuclear antibody (ANA), ESR, C-reactive protein, liver function tests etc.

6.1.6. Pathophysiology of RA: RA is an autoimmune disease, driven primarily by activated T-cells (TCD4 cells, T helper cells and activated T helper cells) giving rise to T cell derived cytokines such as IL-1 and TNF alpha found in the rheumatoid synovium (Parameswaran and Patial 2010; Baria, Joshi et al. 2011). Activated T cells also activate macrophages and B cells which intern triggers cytokine production as well as chemokine production (Szekanecz and Koch 2007), (McInnes and Schett 2007). These cytokines triggers osteoclast (Zhao, Guo et al. 2011), fibroblasts and further influx of inflammatory cells, release of lysosomal enzymes at synovium which leads to inflammation of synovium, progress to the damage of cartilage, bone erosion (Forsblad-d'Elia and Carlsten 2011) leading to RA.

6.1.7. Treatment for RA: Treatment is generally based on the severity of RA. Initially treatment for RA is to reduce the joint inflammation and pain with the use of analgesics and anti-inflammatory agents, then the joint function will be restored by administering disease modifying anti-rheumatic drugs (DMARDs) to preventing joint deformity (Uhlig, Kvien et al. 1999).

6.1.8. RA association with Dry Eye Syndrome: RA is a syndrome which affects most of the body organs including the eye. The common eye problem associated with RA is "dry eye", also associates with other eye pathologies like scleritis, uveitis, glaucoma, and cataract. Since RA is an autoimmune disease, it mainly affects extracellular glands like lacrimal, salivary and therefore dry eye symptoms are more prevalent in RA. More than 90 % of RA patients shows dry eye, out of which 50 % show moderate to severe dry eye (Haga, Naderi et al. 2012).

6.1.9. Proteomics changes reported in RA: Inflammation is the key mechanism involved in both RA and DES. Serum and synovial fluid protein profile in RA using proteomic approach (Ortea, Roschitzki et al. 2012). T-Cell mediated inflammatory molecules, cytokines, chemokines showed changes in RA serum, synovial fluid. Immune mediated molecules transthyretin, aquaporins, actin molecules, calcium depending proteins showed variations in RA plasma (Gibson, Blelock et al. 2009). Tear protein changes in DES associated with NSS and pSS showed down regulation of major proteins such as lysozyme, lactoferrin, IgA, zn-α-glycoprotein (Hassan, Waheed et al. 2008). Pro-inflammatory cytokine were reported to be increased along with significant reduction in anti-inflammatory cytokines in non SS and SS dry eye syndrome. (Brignole, Pisella et al. 2000).

Specific proteins such as SAA (serum amyloid A), SOD (superoxide dismutase) and TPI (triose phosphate isomerase) were elevated in plasma and synovial tissue of RA as seen by proteomic studies. Since the lacrimal and salivary gland involvement seen in RA, tear specific proteins were not identified so far. Therefore tear proteomics plays an important role to understand DES with RA. Therefore this work aims to profile the tear protein to understand the protein changes in DES associated with RA which is not reported so far.

6.2. OBJECTIVES

1. Profiling of tear protein in Dry eye syndrome associated with Rheumatoid Arthritis using 2-DE approach and to identify the differentially expressed ones in tear proteins using PD quest software.
2. To validate the differentially expressed proteins using statistics based on DIGE analysis.
3. Identification of the differentially expressed proteins using mass spectrometry.
4. Correlation of the differentially expressed tear protein with the clinical condition

6.3. METHODS

6.3.1. Diagnosis of Dry Eye cases and rheumatoid cases for the study.

A. Diagnostic Criteria for DES as in chapter 3 section 3.1.2A
B. Diagnostic criteria for RA as in chapter 3, section 3. 1.2B

6.3.2. Subjects recruited for the study : As a prospective age and sex matched case- control study, 39 healthy controls (mean age: 43 ± 12, 12M, 27F), 26 cases of dry eye secondary to Rheumatoid arthritis (RA), (mean age: 48 ± 10 y, M, 20 F), 4 RA without DES (mean age: 50 ± 10 yrs, 4 F) were recruited for the study.

DIGE was done in another set of 18 controls (mean age: 43 ± 12 y, 8M, 10F), 16 cases of dry eye secondary to Rheumatoid arthritis (RA), (mean age: 49 ± 8 y, 2 M, 14 F) and 2 RA without DES (mean age: 50 ± 10 yrs, 2 F).

6.3.3. Blood investigations: All the RA parameters were done using ELISA method as detailed in chapter 3 in section 3.19.

6.3.4. Collection of tear fluid from dry eye and Controls: Tear fluid was collected from controls and dry eye cases using Schirmer strips as discussed in chapter 2. The samples were stored at -80 °C until processing.

6.3.5. Extraction of tear protein from Schirmer for 2DE and DIGE as in chapter 3, section 3.4

6.3.6. A. Processing of tear protein for 2DE as in chapter 3, section 3.9.

B. 2D-DIGE: 30 µg of pooled tear protein from samples were used as described in table 1. While Cy 2 was used was used for internal standard labelling which is a mixture of the specimen used for the corresponding Cy3 and Cy5 samples as in the table 6.1. Labelling was done in dark, and then subjected to electrophoresis as described in chapter 3 section 3.10. The combinations (pool of 3 specimen each) processed are mentioned in table 6.1. Gels were scanned using typhoon scanner, spot analysis was done using Decyder 2 DE 7 software for the quantitative changes of the differentially expressed spots.

Table 6.1: Details of the pooled tear specimen and the corresponding Cy dye labelling used for DIGE analysis for each of the gel

Gel No	30 µg Tear protein labelling for DIGE		
	240 pmol Cy 3	240 pmol Cy 5	240 pmol Cy 2
1	DES alone (NSS)	RA-DES	DES + RA-DES
2	DES alone (NSS)	RA-DES	DES + RA-DES
3	DES- RA	RA alone	RA-DES + RA
4	DES -RA	RA alone	RA-DES + RA alone
5	RA alone	RA-DES	RA + RA-DES
6	DES- RA	Control	RA-DES + control
7	DES- Primary SS (pSS)	RA-DES	DES-pSS+ RA-DES

6.4. RESULTS

6.4.1. RA blood investigations

To diagnose RA, blood investigations were done apart from clinical examination, by the rheumatologist in the study while DES was assessed by the ophthalmologist. The details of blood investigations are shown in table 6.2 with the diagnosis of RA and DES including the grading of RA and DES. The diagnosed RA cases with the presence of RF is considered as sero positive RA, while the RF negative considered as sero negative RA as seen in table 6.2.

Table 6.2: RA investigations done for the patients recruited in the study

S.No	Age	Sex	RF	CRP	ANA	SSA	SSB	DsDNA	CCP	ESR	RA Diagnosis + Grade	DES Diagnosis + Grade
1	55	F	400	2.1	0.18	0.96	0.21	0.47	8.46	ND	SP RA Class I	DES Grade 2
2	60	F	300	1.0	0.13	0.77	0.18	0.25	4.15	45	SP RA Class I	DES Grade 3
3	48	F	240	0.8	0.16	0.51	0.11	0.16	7.19	31	SP RA Class I	DES Grade 3
4	56	M	640	0.5	0.22	1.4	0.16	0.32	4.73	43	SP RA Class I	DES Grade 3
5	36	F	130	3.5	0.09	0.64	0.15	0.21	10.14	54	SP RA Class 1	DES Grade 3
6	60	M	10	0.01	0.21	0.83	0.47	0.84	4.63	ND	SN RA Class I	DES Grade 1
7	55	F	17.5	0.007	0.04	0.22	0.20	0.22	0.29	33	SN RA Class I	DES Grade1
8	54	F	395	0.000	1.54	2.03	7.39	0.46	0.13	52	SP RA Class 1	DES Grade 3
9	45	F	150	2.7	0.38	1.03	0.74	0.59	6.12	ND	SP RA Class 1	DES Grade 3
10	42	F	100	0.0012	0.361	0.91	0.28	1.643	6.19	20	SP RA Class I	DES Grade 4
11	45	M	120	0.014	0.42	2.26	1.43	0.8	3.64	75	SP RA Class I	DES Grade 4
12	55	F	ND	0.23	0.53	1.87	1.12	1.93	11.1	105	SP RA Class I	DES Grade 2
13	66	F	ND	0.65	0.36	0.82	0.42	1.39	10.7	56	SP RA Class I	DES Grade 1
14	M	ND	0.95	0.22	0.63	0.56	3.5	1.7	ND		SN RA Class I	DES Grade 3
15	56	M	ND	0.47	0.35	0.48	0.87	0.45	8.5	ND	SP RA Class I	DES Grade 2
16	62	F	8	90	0.7	9.5	8.2	0.55	5	131	SN RA Class I	DES Grade 3
17	45	F	7.5	3	5.2	11	9.3	17	2.3	40	SN RA Class I	DES Grade 2
18	54	F	8	190	8.5	8	7	26	3.2	145	SN RA Class I	DES Grade 2
19	66	F	37	90	0.75	5	7	0.8	40		SP RA Class I	DES Grade 2
20	42	M	35	50	0.6	9	8.1	0.45	13	24	SP RA Class I	DES Grade 2
21	56	F	180	0.3	0.69	2.37	2.5	0.23	0.13	98	SP RA Class II	Grade 1
22	56	F	200	0.1	0.45	1.71	0.16	0.25	0.33	35	SP RA Class II	DES Grade 1
23	51	M	15	0.0015	0.342	0.665	0.21	0.243	5.03	4	SN RA Class II	DES Grade 2
24	53	F	200	0.004	0.4	1.3	1.03	0.34	7.88	ND	SP RA Class II	DES Grade 3
25	61	F	255	190	0.82	45	40	0.7	8.9	80	SP RA Class II	DES Grade 4
26	44	M	536	3.5	0.24	0.67	0.10	0.3	5.26	40	SP RA Class III	DES Grade 1
27	70	M	ND	1.03	1.28	0.6	0.64	0.73	11.67	ND	SN RA Class III	DES Grade 3
28	45	F	70	30	0.7	8.6	6	0.45	50	ND	SP RA Class III	DES Grade 2

RF – Rheumatoid factor
CRP – C- reactive protein
ANA- Anti nuclear antibody
SSA,SSB - Anti Ro, anti La- nuclear antigens
Anti CCP – Anti Cyclic Citrulinated peptide
DES – Dry Eye Syndrome
SP RA- Seropositive Rheumatoid arthritis
SN RA- Seronegative Rheumatoid arthritis
ND – Not done
ESR – Erythrocyte sedimentation Rat

6.4.2. Grading of RA and DES cases

Based on the clinical details of patients and the clinical grading of RA with DES, table 6.3 revealed that 90 % RA cases had Dry eye. 60% of RA class I showed severe DES thus degree of severity of RA does not correlated with DES severity.

Table 6.3: Grading of DES and total number of RA cases.

DES grading	RA without DES	RA severity grading		
		Class I	Class II	Class III
Mild-DES : **Grade 1**	All were Class I	n = 6 (24 %)	n = 3(25%)	n = 1(33%)
Moderate-DES: **Grade 2**		n = 4 (16 %)	n = 3(25%)	n = 1(33%)
Severe –DES : **Grade 3 & 4**		n = 15 (60%)	n = 6(50%)	n = 1(33%)
Total No. of patients	**4**	**25**	**12**	**3**

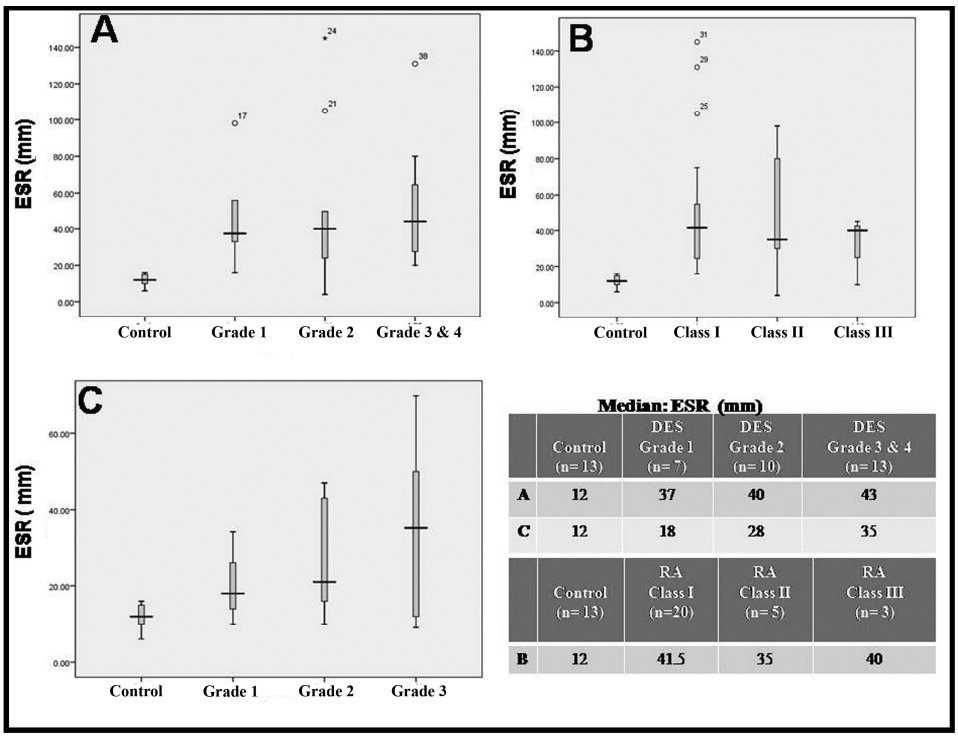
6.4.3. RA blood parameter analysis in DES-RA:

6.4.3.a. An increase in ESR is seen in both primary and secondary Sjogrens (RA) compared to control. The ESR level is 2 fold higher in secondary Sjogrens (RA) than in primary Sjogrens (pSS) in the mild to moderate severity of DES. All RA cases irrespective of the class of severity and grade of DES showed higher levels of ESR by at least 3 fold increase compared to control (figure 6.1). Normal reference range for ESR is 0- 30 mm/hr. The RF factor was found to be increased in all cases of Secondary Sjogrens due to RA except in severe cases of primary. Severe DES grade (grade 3 and 4) was found to have higher levels of RF by 1.7 fold over that of mild to moderate i.e DES grade 1 and 2. < 16 units/ml is considered normal level of RF in serum (figure 6.2). 0- 0.8 mg/dL is the normal reference range of CRP and the median levels did not indicate any significant alterations in primary and secondary Sjogrens (figure 6.3). No increase above the reference range of ANA was observed in the primary and secondary Sjogrens cases (figure 6.4). An ab index < 0.9 units is considered normal for ANA. An ab index < 0.9 units is considered as normal levels of anti SSA and anti SSB ab. However both in primary and in secondary Sjogrens in RA there was an increase in the mean levels by 3 fold and 6 fold increase in secondary RA. In moderate cases of DES in RA a maximal increase was observed. Anti-SSB in Secondary Sjogrens to RA and primary SS showed an increase by 2.5

fold and 3.8 fold in the mean levels with RA grade. In moderate cases of DES of RA there was maximal increase (Figure 6.5 and 6.6).

An ab index of < 0.95 units is considered as normal levels of anti CCP ab. Increase in anti-CCP levels was found to be associated with increase in DES grade as well as RA severity as seen by the shift in the median. Severe DES associated with Primary SS showed increase in mean levels anti-CCP levels by 14 fold (Fig 6.7). This suggests primary SS with increased anti-CCP levels may be indicative of developing RA.

Figure 6.1: ESR levels in DES –RA and primary SS



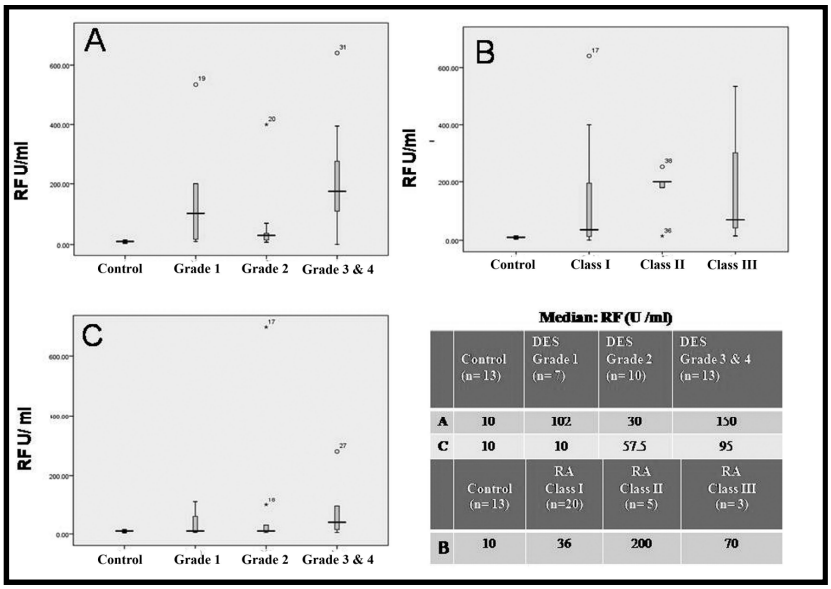
Median: ESR (mm)

	Control (n=13)	DES Grade 1 (n=7)	DES Grade 2 (n=10)	DES Grade 3 & 4 (n=13)
A	12	37	40	43
C	12	18	28	35

	Control (n=13)	RA Class I (n=20)	RA Class II (n=5)	RA Class III (n=3)
B	12	41.5	35	40

Figure 6.1: ESR levels (A). RA-DES with grade (B). RA-DES with RA class (C).DES- pSS. DES severity in Grade 1 (mild) - 2 (moderate) and 3,4 (severe) is represented in the x-axis in A and C. RA severity shown as Class I, II and III in the x-axis of B**.** Median of DES-RA and DES-pSS shown in the table.

Figure 6.2: Serum Anti RF levels in DES –RA and primary SS



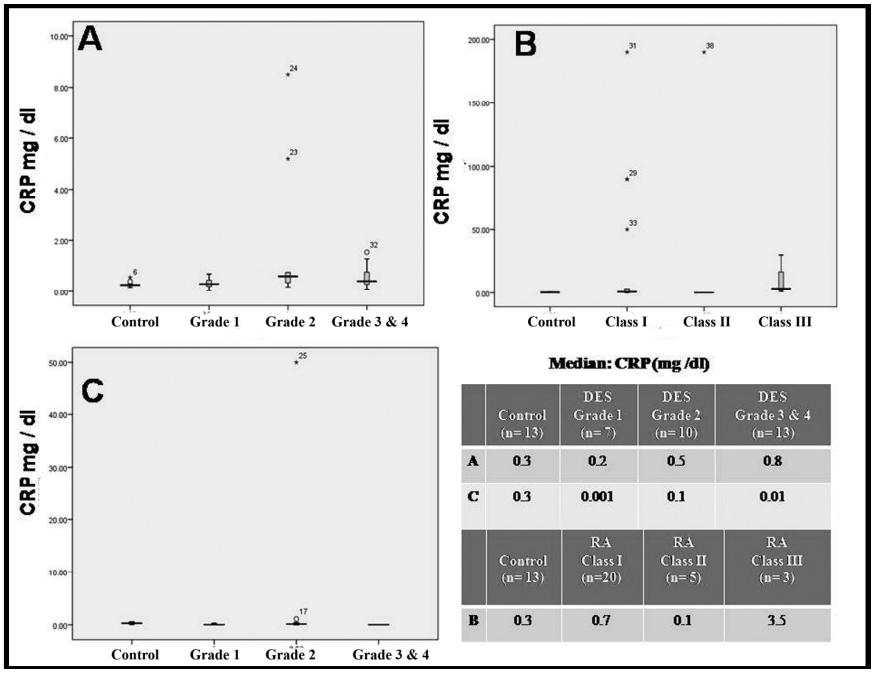
Median: RF (U /ml)

	Control (n= 13)	DES Grade 1 (n= 7)	DES Grade 2 (n= 10)	DES Grade 3 & 4 (n= 13)
A	10	102	30	150
C	10	10	57.5	95

	Control (n= 13)	RA Class I (n=20)	RA Class II (n= 5)	RA Class III (n= 3)
B	10	36	200	70

Figure 6.2: RF levels in (A). RA-DES with grade (B). RA-DES with RA class (C). DES- pSS. DES severity in Grade 1 (mild) - 2 (moderate) and 3,4 (severe) is represented in the x-axis in A and C. RA severity shown as Class I, II and III in the x-axis of B**.** Median of DES-RA and DES-pSS shown in the table.

Figure 6.3: Serum CRP levels In DES-RA and In DES-pSS



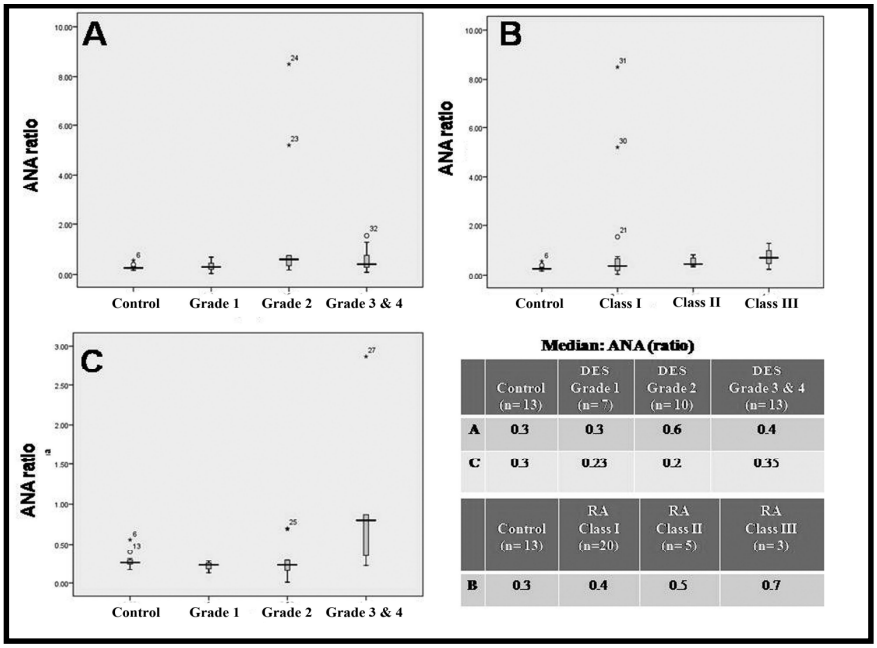
Median: CRP (mg /dl)

	Control (n= 13)	DES Grade 1 (n= 7)	DES Grade 2 (n= 10)	DES Grade 3 & 4 (n= 13)
A	0.3	0.2	0.5	0.8
C	0.3	0.001	0.1	0.01

	Control (n= 13)	RA Class I (n=20)	RA Class II (n= 5)	RA Class III (n= 3)
B	0.3	0.7	0.1	3.5

Figure 6.3.: Serum CRP levels (A). RA-DES with grade (B). RA-DES with RA class (C).DES- pSS. DES severity in Grade 1 (mild) - 2 (moderate) and 3,4 (severe) is represented in the x-axis in A and C. RA severity shown as Class I, II and III in the x-axis of B**.** Median of DES-RA and DES-pSS shown in the table. No change observed in all the groups.

Figure 6.4: Serum ANA levels in DES-RA and DES-pSS



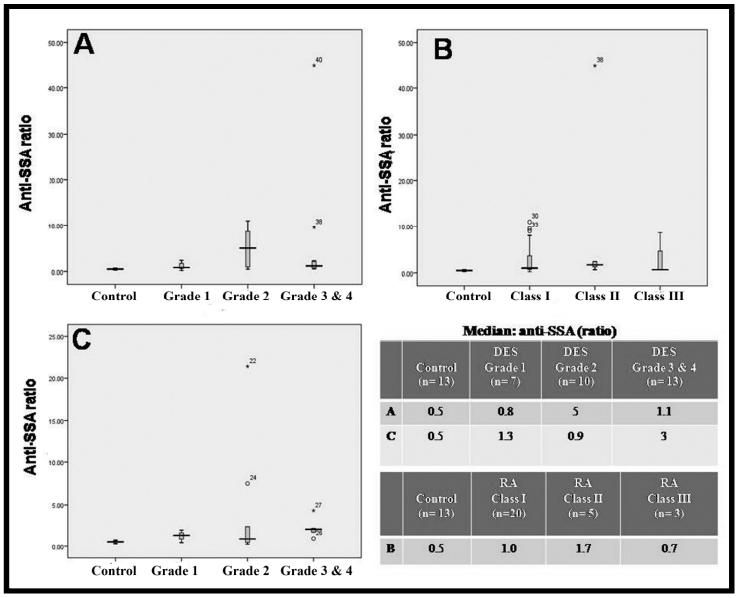
Median: ANA (ratio)

	Control (n= 13)	DES Grade 1 (n= 7)	DES Grade 2 (n= 10)	DES Grade 3 & 4 (n= 13)
A	0.3	0.3	0.6	0.4
C	0.3	0.23	0.2	0.35

	Control (n= 13)	RA Class I (n=20)	RA Class II (n= 5)	RA Class III (n= 3)
B	0.3	0.4	0.5	0.7

Figure 6.4: Serum ANA levels (A). RA-DES with grade (B). RA-DES with RA class (C).DES- pSS. DES severity in Grade 1 (mild) - 2 (moderate) and 3,4 (severe) is represented in the x-axis in A and C. RA severity shown as Class I, II and III in the x-axis of B**.** Median of DES-RA and DES-pSS shown in the table.

Figure 6.5: Serum anti-SSA levels in DES-RA and DES-pSS.



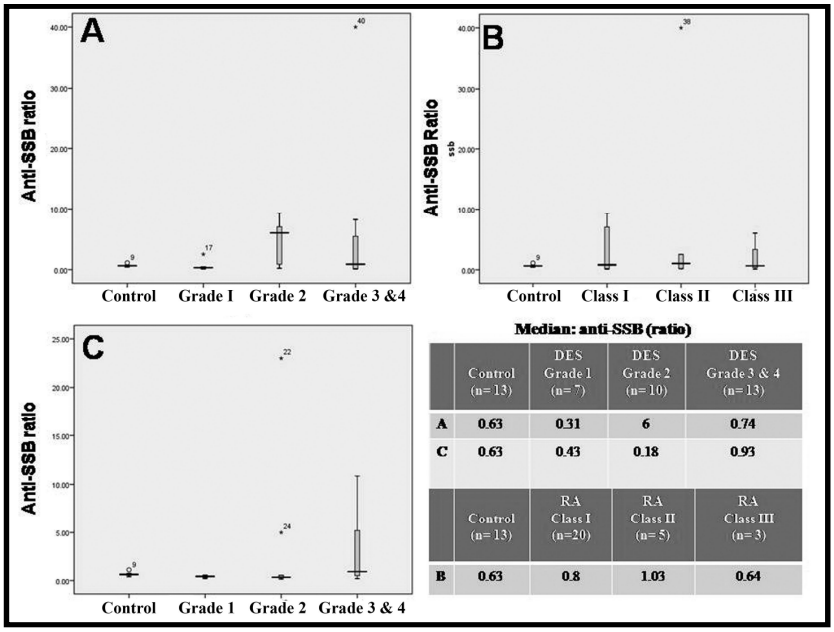
Median: anti-SSA (ratio)

	Control (n= 13)	DES Grade 1 (n= 7)	DES Grade 2 (n= 10)	DES Grade 3 & 4 (n= 13)
A	0.5	0.8	5	1.1
C	0.5	1.3	0.9	3

	Control (n= 13)	RA Class I (n=20)	RA Class II (n= 5)	RA Class III (n= 3)
B	0.5	1.0	1.7	0.7

Figure 6.5. Serum anti-SSA levels (A). RA-DES with grade (B). RA-DES with RA class (C).DES- pSS. DES severity in Grade 1 (mild) - 2 (moderate) and 3,4 (severe) is represented in the x-axis in A and C. RA severity shown as Class I, II and III in the x-axis of B**.** Median of DES-RA and DES-pSS shown in the table.

Figure 6.6: Serum anti- SSB levels in DES-RA and DES –pSS



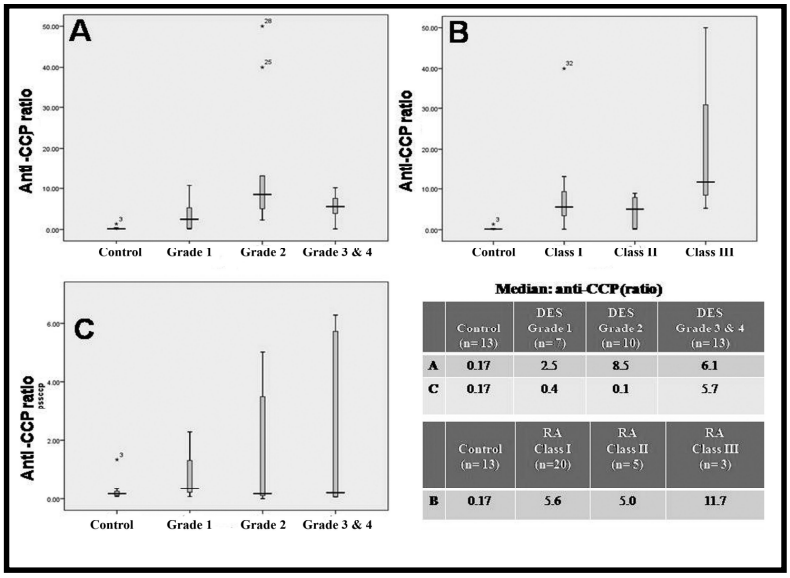
Median: anti-SSB (ratio)

	Control (n=13)	DES Grade 1 (n=7)	DES Grade 2 (n=10)	DES Grade 3 & 4 (n=13)
A	0.63	0.31	6	0.74
C	0.63	0.43	0.18	0.93

	Control (n=13)	RA Class I (n=20)	RA Class II (n=5)	RA Class III (n=3)
B	0.63	0.8	1.03	0.64

Figure 6.6. Serum anti-SSB levels (A). RA-DES with grade (B). RA-DES with RA class (C).DES- pSS. DES severity in Grade 1 (mild) - 2 (moderate) and 3,4 (severe) is represented in the x-axis in A and C. RA severity shown as Class I, II and III in the x-axis of B**.** Median of DES-RA and DES-pSS shown in the table.

Figure 6.7: Serum Anti CCP levels in DES-RA and DES-pSS



Median: anti-CCP (ratio)

	Control (n=13)	DES Grade 1 (n=7)	DES Grade 2 (n=10)	DES Grade 3 & 4 (n=13)
A	0.17	2.5	8.5	6.1
C	0.17	0.4	0.1	5.7

	Control (n=13)	RA Class I (n=20)	RA Class II (n=5)	RA Class III (n=3)
B	0.17	5.6	5.0	11.7

Figure 6.7. Serum anti-CCP levels (A). RA-DES with grade (B). RA-DES with RA class (C).DES- pSS. DES severity in Grade 1 (mild) - 2 (moderate) and 3,4 (severe) is represented in the x-axis in A and C. RA severity shown as Class I, II and III in the x-axis of B. Median of DES-RA and DES-pSS shown in the table. Increased Anti-CCP with the DES grade increases. And also with RA class increases. No change in DES-pSS.

6.3.4.b. Summary of all the serum clinical parameters, shows increase in anti-RF, ESR and anti-CCP levels with increase in DES grade and RA class and also in DES-pSS. ANA, CRP showed no change in DES-RA and DES-pSS. Anti-SSA and anti-SSB increased.

6.3.4.c. Serum parameters change in sero positive and sero negative DES-RA

Sero positive showed increase in RF, CRP, SSA, ESR, anti-CCP, ANA by 95%, 13%, 45%, 29% 36%, and 69% than sero negative cases respectively (figure 6.8). Based on more than 50% shift in the median RF, SSA, SSB, ANA and CCP Seems to differentiate sero positive from negative While ESR and CRP does not (fig 6.8).

Figure 6.8: Distribution of serum parameters in Sero positive and Sero negative DES-RA.

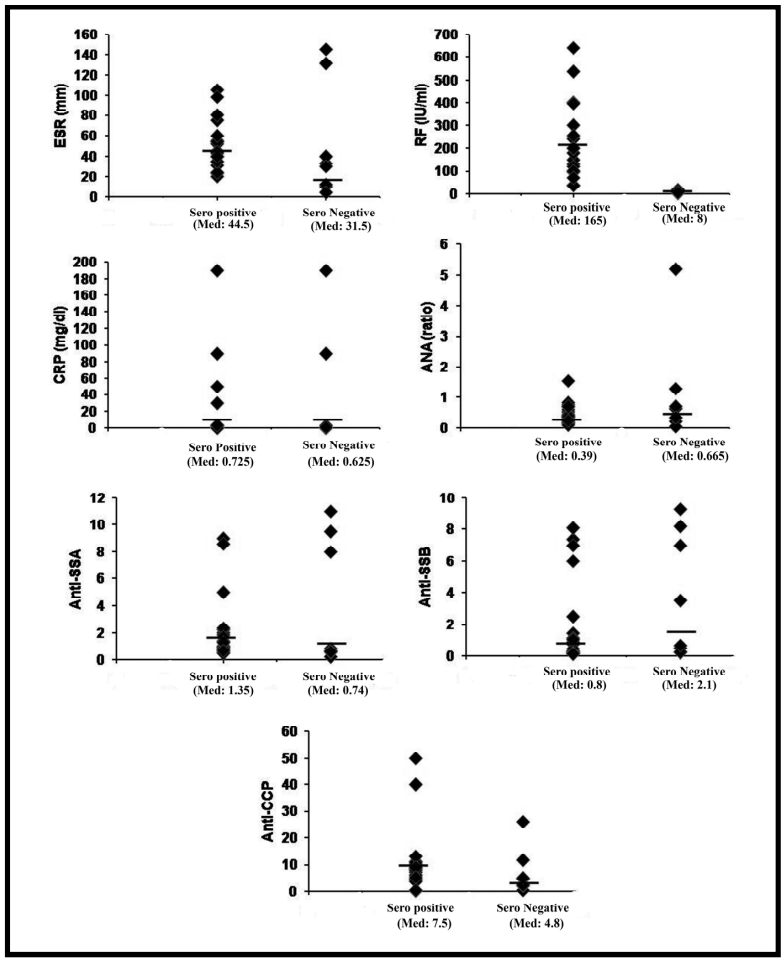


Fig 6.8: Median shift in parameters ESR, Anti RF, Anti-CCP seen in Seropositive DES-RA (N = 19) compared to Sero negative DES-RA (N= 7).

6.4.4. 2DE profiling of tear protein

(i) Total Protein

The amount of total tear protein was reduced significantly in RA with DES ($p < 0.00$) cases than RA without DES as seen in table 6.4. All the DES cases irrespective of the type, showed a significant reduction in total protein as seen in chapter 5 table 2.

Table 6.4: Total protein in tear samples of control and RA cases

	Control (µg/ml)	DES-RA (µg/ml)	RA without DES (µg/ml)
Mean ± SD	1726 ±176	757 ± 112	1523 ± 174
p value		< 0.001	0.63

(ii) Differential expression of Proteins based on 2D

The differentially expressed spots of DES-RA compared to RA alone and control was assessed based on the spot density by PD Quest software as shown in figure 6.9. Accordingly spots grouped as 1 -25, 32, 33, 35-39, 42-51, 54,55, 56-62, 63-76. The zoomed images of the differentially expressed spots are shown in figure 6.9.

The proteins differentially expressed in RA alone (figure 6.10.B) was not significantly altered compared to the control (Figure 6.10.A) but changes were seen in RA-DES (figure 6.10 C) compared to the control. However most of the changes were characteristic of DES rather than RA, because there was no significant difference in spot intensity changes between DES alone and RA-DES (table 6.5).

Figure 6.9.: Differentially expressed tear proteins in DES-RA

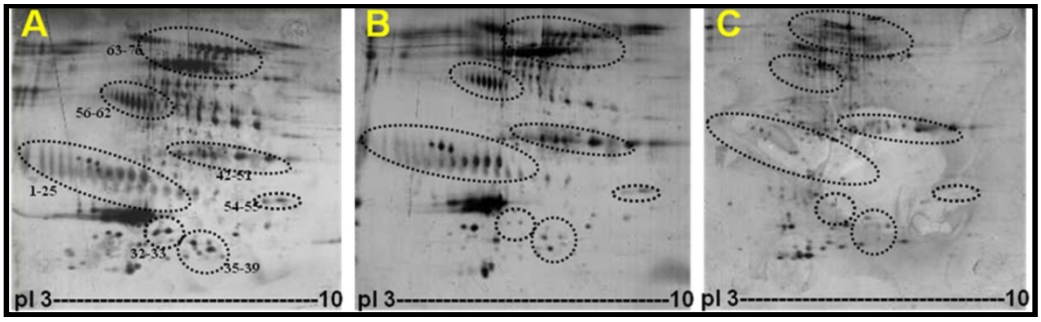


Fig 6.9: Tear protein separation of control and DES using 17 cm, pH 3-10 IPG strip in 1st dimension and on 1% SDS-PAGE in 2nd dimension. DES-RA A. Control B. RA alone C. DES-RA showed most of down regulated peptide spots as seen in the figure compared to control and RA alone.

Figure 6.10: The zoomed images of differentially expressed spots in DES-RA.

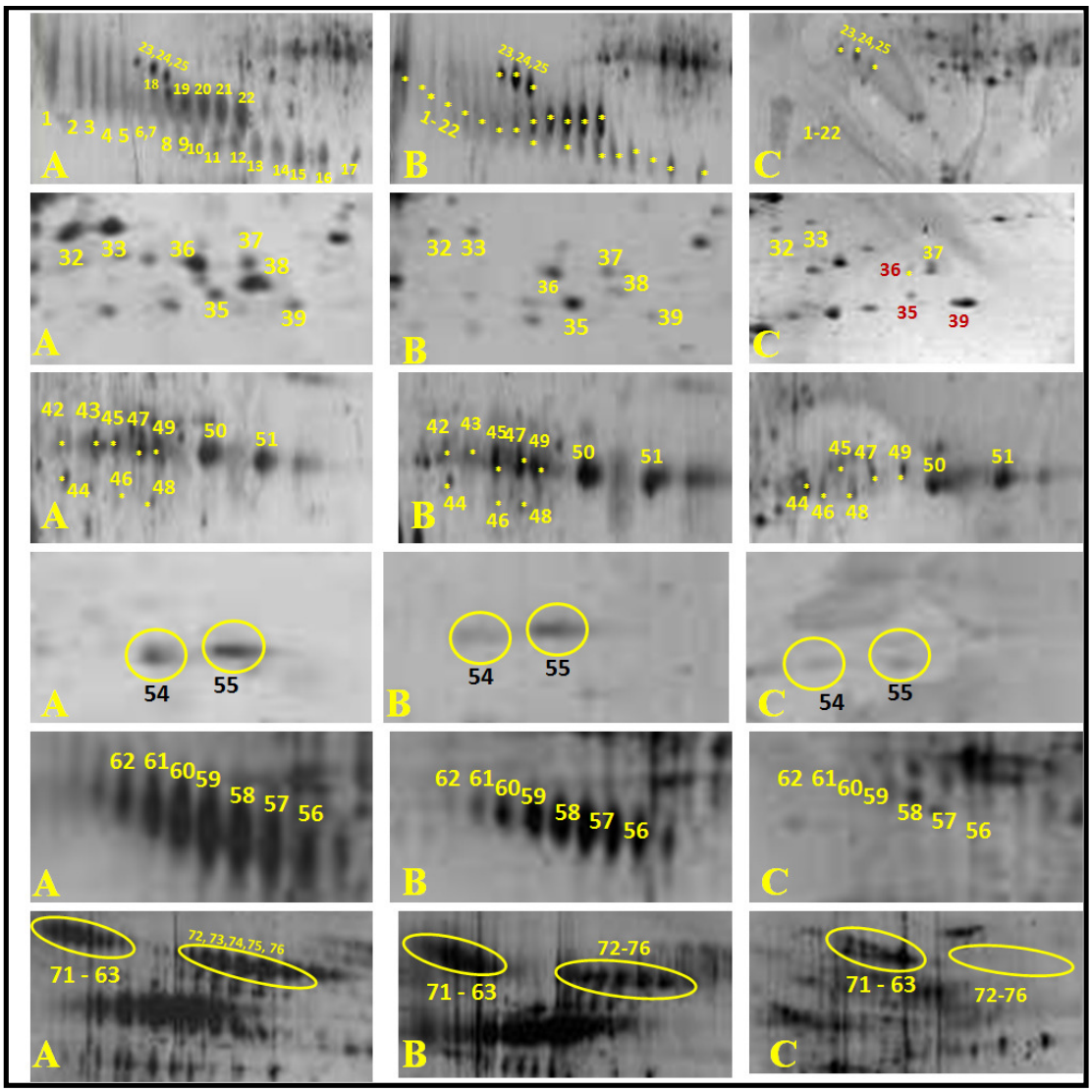


Figure 6.10: the zoomed images of 2DE images of control and DES tear proteins separated using 17cm pH 3-10 IPG strips in firs dimension and in 2nd dimension using13% SDS-PAGE. A. Control B. RA alone and C. DES with RA**.** DES-RA showed down regulation of peptide spots 1-25, 32, 33, 35-39, 54, 55, 56-62 and 63-76 as seen in the figure 6.10. RA alone tear protein 2DE did not reveal down regulation of the same, but the % change in protein observed. It is similar to control tear protein profile.

Table 6.5: Differentially expressed proteins in DES (Non DES) and RA-DES based on spot intensity as per PD Quest analysis following 2D electrophoresis.

Spot number	DES Groups					
	DES alone n = 30		RA alone n = 4		RA-DES N = 26	
	% cases	Fold intensity	% cases	Fold intensity	% cases	Fold intensity
Spot 1-8 ↓	100 %	1.5	No change	-	87 %	1.5
Spot 9 -20 ↓	100 %	2. 3	No change	-	88 %	2.0
Spot 21-22 ↓	100 %	2.4	No change	-	90 %	1.6
Spot 23↓	100 %	3.0	No change	-	80 %	2.8
Spot 24 ↓	100 %	3.5	No change	-	80 %	2.5
Spot 25 ↓	100 %	3.50	No change	-	80 %	2.8
Spot 29-30 ↓	60 %	4.5	No change	-	38 %	2.5
Spot 32-33 ↓	80 %	3.5	90 % ↓	1.7	**77 %**	**1.75**
Spot 40-41	**38 % ↑**	**1.5**	No change	-	**25 %↓**	**2.0**
Spot 42-51	60% ↓	1.7	30 % ↑	1.4	**72 %**	**1.4**
Spot 54-55	70% ↓	2.2	No change	-	67 %	2.2
Spot 56-62 ↓	100%	2.5	No change	-	100 %	2.8
Spot 63 -66 ↓	85 %	1.5	No change	-	71 %	1.5
Spot 67-71 ↓	89 %	1.8	No change	-	69 %	2.2
Spot 72-76 ↓	90 %	2.4	No change	-	70 %	3.7
Spot 35-39	70 % ↑	1.6	35 % ↓	1.5	**67 % ↓**	**1.4**
Spot A ↑	70 %	2.5	No change	-	76 %	2.5
Spot B ↑	100 %	1.8	No change	-	65 %	1.5
Spot C ↑	74 %	1.5	absent	-	55 %	**1.5**
Spot D	absent		**absent**		68 %	**0.15**

6.4.5. 2D-DIGE Analysis: Tear protein in DES with and without RA was profiled using DIGE. 30 µg tear protein of each of the condition i.e. RA alone was labelled with 240 pmol of Cy 3(green), RA-DES tear protein was labelled with 240 pmol Cy 5 (red). Mixture of RA (15 µg) and RA-DES (15 µg) labelled with 240 pmol of Cy 2 (blue) (Figure 6.11A). Labelling with Cy 2, serves as internal standard during the analysis for calculating the spot volume which helps to look for the differentially expressed proteins during BVA analysis of DIGE. Figure 6.11B is the grey scale image of the RA with and without DES samples as seen in Figure 6.11 A. The reduced red fluorescence showed the down regulation of that particular spot.From

DIGE BVA analysis a total of 9 proteins were down regulated, 2 proteins up regulated in DES-RA alone and 4 proteins down regulated, 2 proteins up regulated in RA alone from BVA statistical analysis using De cyder software as seen in table 6.6, figure 6.12.

The differentially expressed proteins in DES-RA, RA alone from BVA analysis were further subjected to differential spot analysis to look for its overall expression in the form of Heat map (Figure 6.13). Heat map showed the total 34 proteins are showed differential expression. On x-axis pink dot represents the control group and the blue dot represents DES-RA. Green colour indicates down regulation of the protein, red colour indicates up regulation of the protein and black colour indicates no change. From this analysis, majority of the proteins in DES-RA showed green colour, indicating down regulation.

From the discriminate analysis using EDA tool, proteins with > 95 % accuracy were denoted as disease biomarker. From this data a total of 4 proteins that can serve as biomarker for DES-RA, 2 protein for RA alone and 7 peptide spots for DES-Non-Sjogrens were identified (Figure 6.14, table 6.7).

Figure 6.11A: Tear protein from RA-DES and RA separation using DIGE.

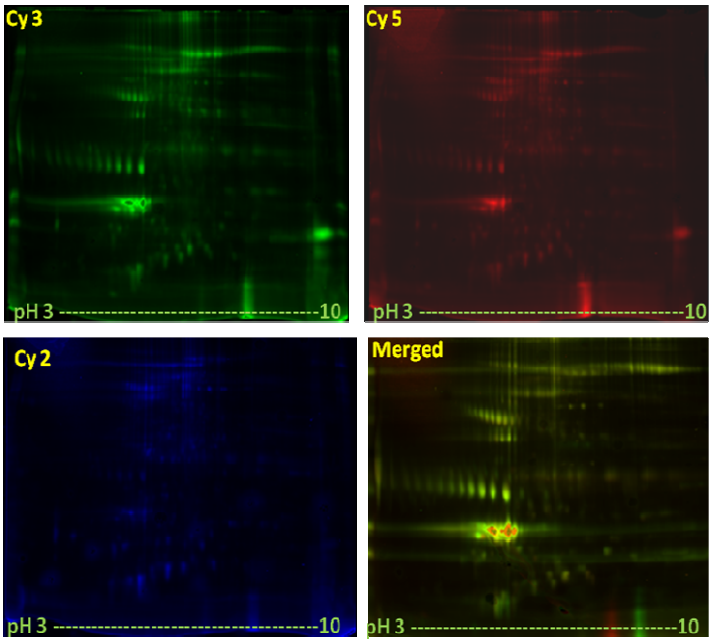


Fig 6.11A: Representative tear protein separation using DIGE. 30 µg of RA tear protein labelled with Cy 3 (green), 30 µg RA-DES labelled with Cy 5 (red) and 15 µg of RA-DES + 15 µg of RA labelled with Cy 2 (blue). The merged image is the combination of Cy3 and Cy 5 indicates the tear protein expression. The more the green colour indicates that particular spot is up regulated in Cy 3 labelled sample in RA alone.

Figure 6.11B: Representative grey scale image of tear protein using DIGE.

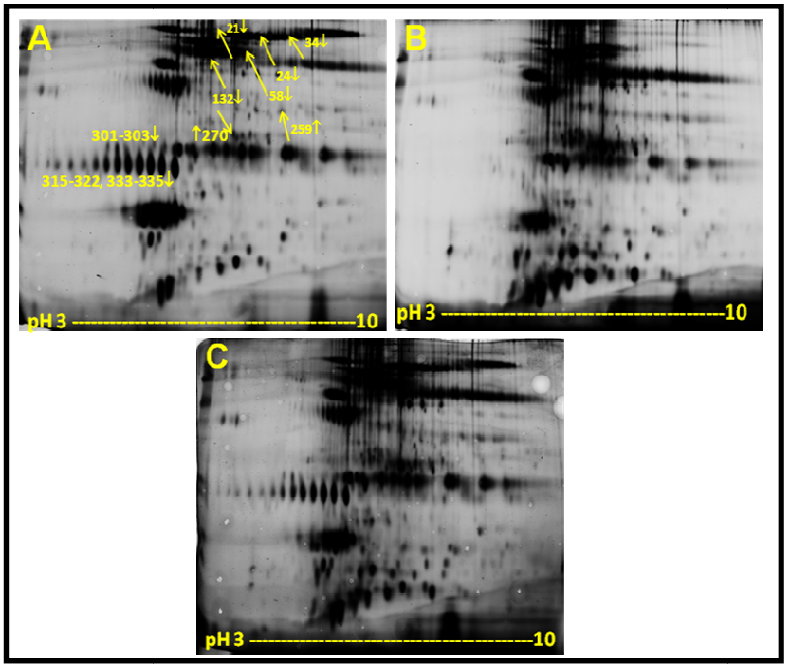


Fig 6.11B: Representative grey scale image of above DIGE image 6.12A. Tear protein separation using DIGE. 30 µg of RA tear protein labelled with Cy 3 (A), 30 µg RA-DES labelled with Cy 5 (B) and 15 µg of RA-DES + 15 µg of RA labelled with Cy 2 (C). DES-RA showed differentially expressed proteins compared to RA alone**.** Spot 132, 21, 24, 34, and Spot 270,259 were up regulated.

Figure 6.12: BVA analysis view differentially expressed protein in RA

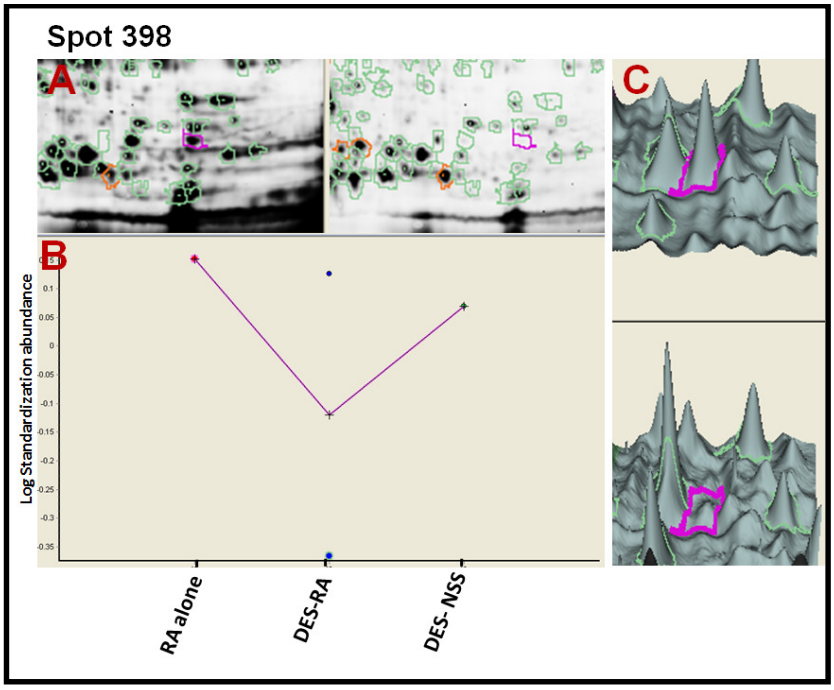


Fig 6.12: BVA analysis of DIGE gels, gives the differentially expressed proteins. Spot 398, one of the up regulated protein in RA, compared to DES-RA and DES-Non SS. A. Spot position in the gel B. Graphical view of the spot across the gels and C. 3D view of spot 398, that shows up regulation of the spot (C. upper panel – spot in RA gel, lower panel – spot in other DES gel).

Based on the BVA analysis, the significant peptide spots that showed differential expression in DES-RA, RA alone DES-Non SS compared to controls were listed in table 6.7.

Table 6.6: The differentially expressed spots based on 2D-DIGE

condition	Differential expression	Spot No. from DIGE analysis	Spot Volume ratio and *p*- value
DES - RA	Down regulated	**24**, 26, **34**,39,**58,21, 132**, 272,214. 301,302,303,315.316,319,320,321, 322,333,334,335	-2.13 to – 3.25 (0.0004 – 0.03)
	Up regulated	**259,270**	1.5 to 2.3 (0.03-0.01)
RA alone	Down regulated	192,260,411,156	- 2.0 to 2.5 (0.028- 0.031)
	Up regulated	419,293	1.5 to 2 (p = 0.013, 0.006)

Figure 6.13: Heat map view of differentially expressed proteins from Extended Data Analysis (EDA) in Control vs RA-DES.

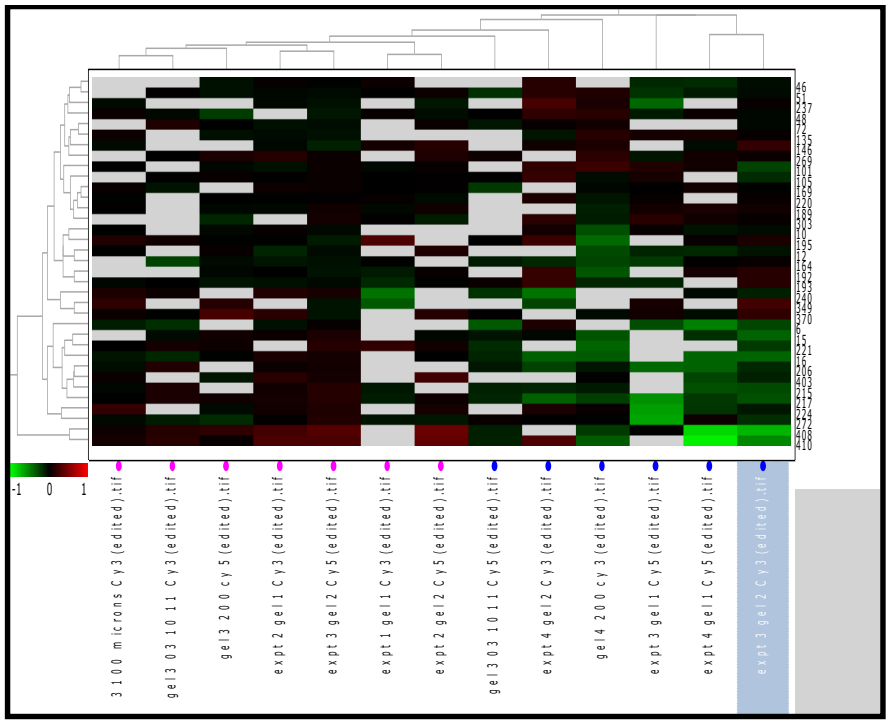


Figure 6.13.: Heat map view of tear proteins of DES-RA (samples with blue dots) compared to controls (pink colour dots). As indicated in the figure more proteins showed down regulation as indicated by green colour compared to control.

Figure 6.14: Discriminate analysis of differentially expressed proteins using EDA for the biomarker selection.

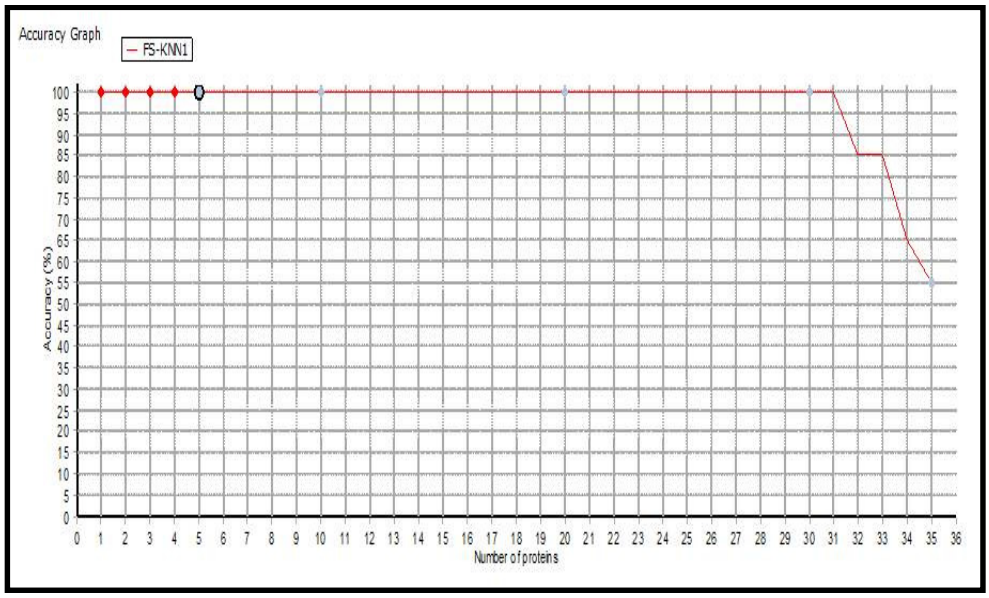


Figure 6.14: Based on the > 95% accuracy, potential biomarker protein was selected using discriminate analysis as shown above. X-axis is the number of proteins that were potentialbiomarkers. Spot numbers were mentioned in table 6.7.

Table 6.7: The peptide spots from DIGE discriminate analysis that is potential biomarkers based on heat map analysis

Peptide spot number as biomarker	DES condition	Name of the protein
24 ↓	DES-RA	Ribonuclease p protein subunit 20 and Protocadherin
34 ↓	DES-RA	Heterogeneous nuclear ribonucleoprotein Q isoform 6
132 ↓	DES-RA	SHC transforming 1 isoform
270 ↑	DES-RA	Ecto-ADP ribosyltranferase -5-precursor and Rho-related GTP-binding protein RhoQ precursor
192 ↓	RA alone	Yet to identify
411 ↓	RA alone	Yet to identify
10 ↓	DES-Non SS	Yet to identify
105 ↓	DES-Non SS	Yet to identify
408 ↓	DES-Non SS	Yet to identify
410 ↓	DES-Non SS	Yet to identify
101 ↓	DES-Non SS	Yet to identify
15 ↓	DES-Non SS	Yet to identify
403 ↑	DES-Non SS	Yet to identify

6.4.6. Identification of differentially expressed spots of DES-RA and RA

From 2D and DIGE the differentially expressed specific DES-RA peptide spots were short listed based on heat map analysis and were then identified by nano-LC/MS/MS and they were identified as listed in table 8.

Five of the down regulated proteins in DES-RA are identified as

1. Spot No. 132: SHC transforming 1 isoform,
2. Spot No. 24: Ribonuclease protein subunit 20 and Protocadherin
3. Spot No. 58: Cystatin SN precursor
4. Spot No. 21: Lactotransferrin isoform 1 precursor,
5. Spot No. 34: Heterogeneous nuclear ribonucleoprotein Q isoform 6.

Three proteins that showed up regulation in DES-RA are identified as

1. Spot No.259: Keratin type II cytoskeletal protein
2. Spot No. 270: Ecto-ADP ribosyltranferase -5-precursor and Rho-related GTP-binding protein RhoQ precursor, RhoJ precursor.

The proteins identified by MS are showed in figures 6.15 - 6.17. They showed peptide sequence > 60 % confidence to 99 % confidence as seen in table 6.9. Based on the presence of continuous 5 y or b ions during cleavage gives good confidence for the identification.

Figure 6.15: Mass spectrogram of cystatin SN (gi|19882251) precursor with peptide sequence IIPGGIYNADLNDEWVQR, mass 1055, charge + 2.

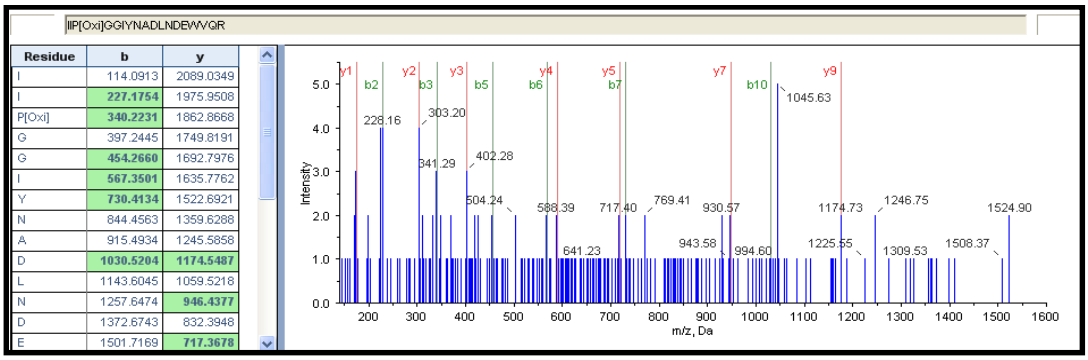


Figure 6.16: Mass spectrogram of down regulated peptide Ribonuclease p protein subunit 20 in DES-RA with its sequence LPSRLPR mass of 458 and charge +2

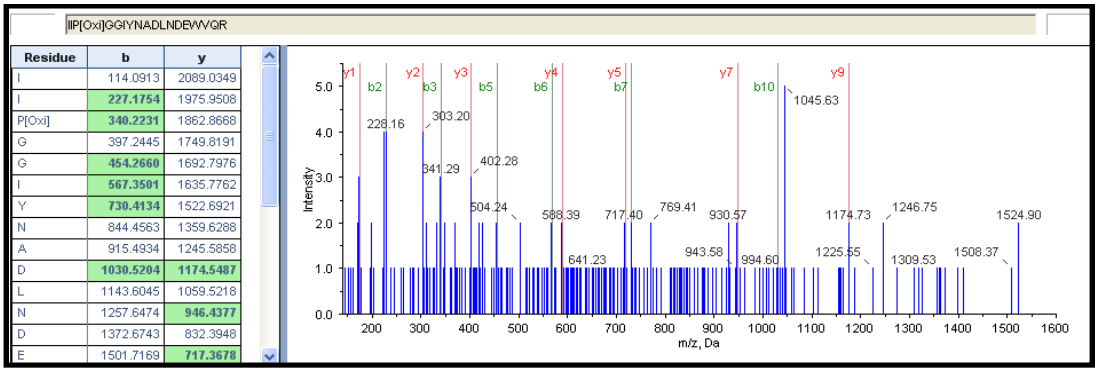
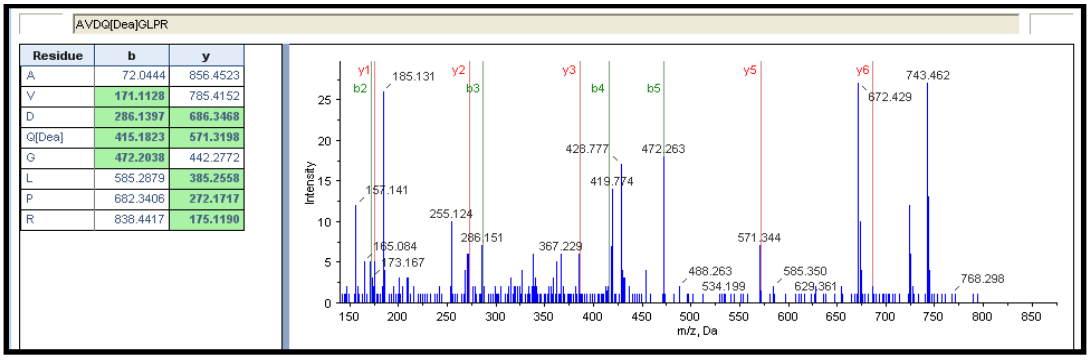


Figure 6.17: Mass spectrum of spot 24 as Protocadherin with sequence AVDQGLPR, m/z is 428 with a charge +2.



4 proteins namely Ribonuclease protein subunit 20, Protocadherin, cystatin SN precursor, Heterogeneous nuclear ribonucleoprotein Q isoform 6, lactotransferrin isoform 1 precursor showed down regulation with > 2.5 fold change down regulation in 80 % cases of DES-RA whereas protein SHC transforming 1 isoform showed down regulation in 60 % cases. There was also upreguation observed with respect to 4 proteins. 2 proteins namely Ecto-ADP ribosyltranferase -5-precursor and rho-related GTP-binding protein RhoQ precursor, RhoJ precursor showed > 80 % of cases upregulation and Keratin type II cytoskeletal protein showed up regulation in 60 % DES-RA cases by > 2 fold (Table 6.8).

Table 6.8: RA-DES specific proteins identified from DIGE using nano LC/MS/MS

Spot No. from Gel	Name of the protein	Accession Number	Sequence and No. peptides	confidence	% coverage	% of cases showed Change
132 ↓	SHC transforming 1 isoform	gil32261324	LSGGGGR (1 peptide)	86 %	4.6 %	63%
24 ↓	Ribonuclease p protein subunit 20 Protocadherin	gil153791431 gil66346693	LPSRLPR (1 peptide) AVDQGLPR (1 peptide)	99% 99%	6.4% 0.5%	82% 82 %
58 ↓	cystatin SN precursor	gil19882251	IIPGGIYNADLNDEWVQR (1 peptide)	99%	44.7%	80%
34 ↓	Heterogeneous nuclear ribonucleoprotein Q isoform 6	gil228008400	GVRGARGGAQQQR (1 peptide)	60%	2.3%	80%
259 ↑	Keratin type II cytoskeletal	gil47132620	FLEQQNQVLQTK (1 peptide)	95.5%	3.8%	60%
270 ↑	Ecto-ADP ribosyltranferase -5-precursor	gil30089992	ESWEAAQETWEDK 1 peptide	79%	5.5%	80%
270 ↑	Rho-related GTP-binding protein RhoQ precursor, RhoJ precursor	gil50263042 gil16903164	DDPKTLAR (1 peptide)	50%	3.9%	80% 80%
21 ↓	lactotransferrin isoform 1 precursor	gil54607120	DGAGDVAFIR (1 peptide)	99%	16.5%	100%

The differential proteins of DES-RA in % cases and its change in terms of spot intensity showed in Figure 6.18 and figure 6.19 showed the differential proteins of DES-RA in % respectively.

Figure 6.18: Graphical representation of differentially expressed tear proteins and its % cases as analysed by DIGE

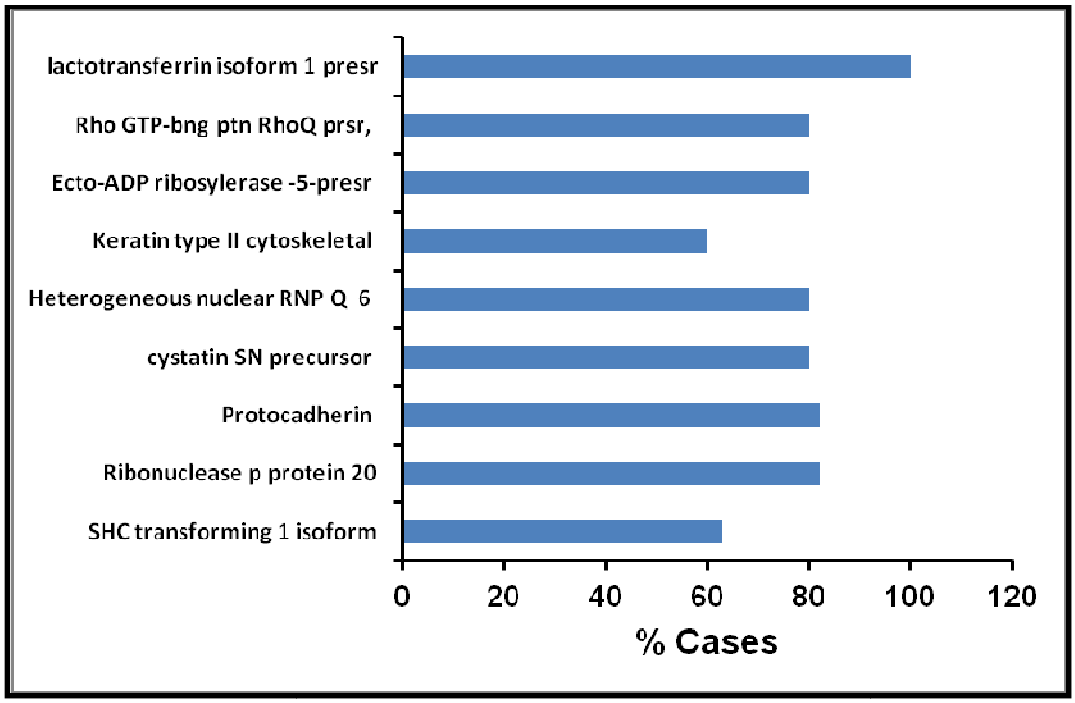
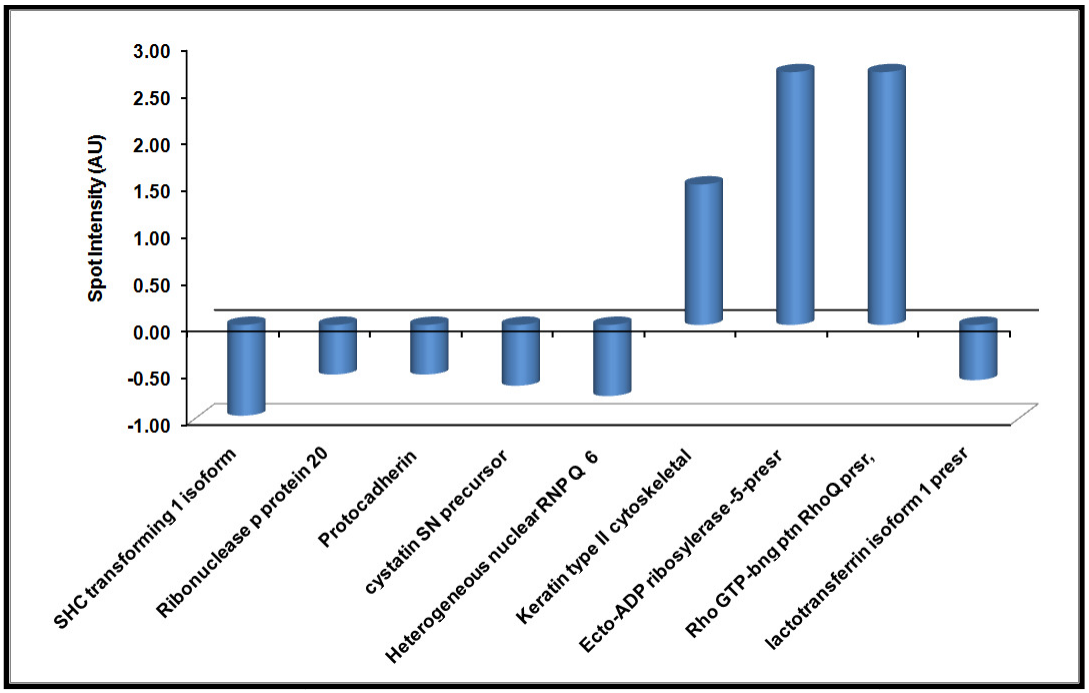


Figure 6.19. Graphical representation of differentially expressed tear proteins and its spot intensity as analysed by DIGE



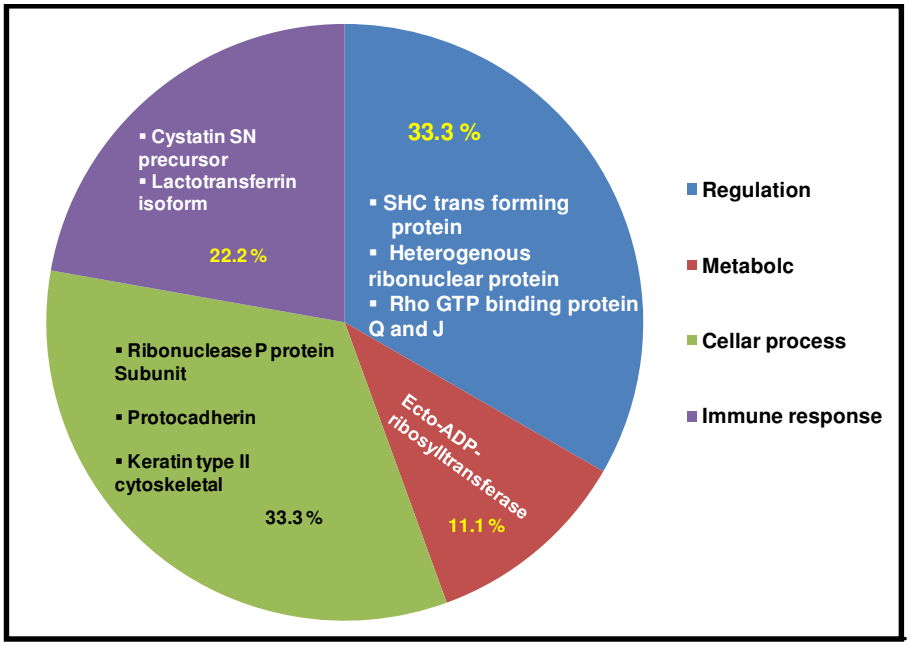
6.4.7: DES-RA proteins identified using nano LC/MS/MS and their possible functions

Table 6.9: Functions of the proteins identified in DES-RA condition by nano-LC/MS/MS

Spot No from gel	Name of the protein	Functional role
132 ↓	SHC transforming 1 isoform (Uniport ID: **P29353)**	Regulatory at signaling level Signaling adapter that couples activated growth factor receptors to signaling pathway molecules like Isoform p46Shc and isoform p52Shc, once phosphorylated, couple activated receptor tyrosine kinases to Ras via the recruitment of the GRB2/SOS complex and are implicated in the cytoplasmic propagation of mitogenic signals. Also interacts with EGFR, which phosphorylates MUC1. In androgen deficiency associated DES MUC1 expression is reduced in the conjunctival cells (Mantelli, Moretti et al. 2007). Sjogrens syndrome is reportedly associated with androgen deficiency(Porola, Laine et al. 2007). In this current study , out of 36 RA cases, 27 numbers are females in which 25 cases are in post menopausal state.
24 ↓	Ribonuclease p protein subunit 20 Uniport No: **O75817**	Regulatory role: (transcriptional) Rpp2 is a protein subunit of nuclear RNase P that is functionally conserved in eukaryotes from yeast to humans. Rpp1 protein is an orthologue of the human scleroderma autoimmune antigen, Rpp30 (Kikovska, Svard et al. 2007; Martinez-Azorin, Remacha et al. 2008).
58 ↓	cystatin SN precursor (Uniport ID: **P01037)**	Antimicrobial activity: Is a cysteine proteinase inhibitor. Expressed in submandibular and sublingual saliva but not in parotid saliva. Expressed in saliva, **tears,** urine and seminal fluid (Molloy, Bolis et al. 1997; Messana, Cabras et al. 2008). This protein play role in Immune response and response to stimulus
34 ↓	Heterogeneous nuclear ribonucleoprotein Q isoform 6	Regulatory role- transcriptional: mRNA processing mechanisms. hnRNP Q is a splicing modulator of *SMN* gene transcript (associated with spinal muscular atrophy SMA)- a neuro muscular disorder) augmenting the expression level of hnRNP Q1 may be a therapeutic strategy for SMA(Chen, Chang et al. 2008).
270 ↑	Ecto-ADP ribosyltranferase -5- precursor (UniportID: **Q96L15)**	Catalytic activity as Arg-specific ADP-ribosyltransferase family. Arginine metabolic processes are involved in cell trafficking, inflammation and apoptosis. Activation of ecto ADP ribosyltransferase leads to cell death (Koch-Nolte, Reyelt et al. 2007). This study showed this protein increases in 80% RA cases.
21 ↓	lactotransferrin isoform 1 precursor (Uniport ID: **P02788)**	Antimicrobial activity: A serine protease of the peptidase S60 family that cuts arginine rich regions which contributes to the antimicrobial activity. Lactotransferrin has antimicrobial activity and this protein is also identified in the synovial fluid of arthritis(Wong, Francis et al. 2009). Other proteins identified as RA specific proteins are not completely studied, but their general functions are mentioned in table 11.

Based on the DAVID annotation tool all the 10 proteins identified in DES-RA condition are classified to 6 functional categories a distribution as follows figure 6. 20. 25% biological regulation, 23% Cellular organisation, 13% cellular process, 13% Immune response/ response to stimuli, 17 % developmental, 12 % locomotion, 17 % metabolic process, 3% organisamal process, and 13 % localization process as seen in figure 6.20.

Figure 6.20: The Gene ontology functional classification of the 9 proteins identified by MS in DES-RA



Based on the functions of the 7 DES-RA specific proteins identifie, most of them involves biological regulation both at transcriptional and signalling level, immune response, localization, metabolic regulation and other cellular process. Further validation of these proteins give clue to understand functional role and then can understand the disease mechanism.

SUMMARY

1. RA clinical parameters namely ESR, RF and Anti-CCP increased with increasing DES severity and RA severity. ANA, CRP did not show any change with disease severity. Anti-SSA and anti-SSB increased in moderate DES-RA.

2. Tear proteins from DES-RA were profiled using 2DE and then confirmed by 2D-DIGE. 7 proteins were identified that are characteristically altered in DES RA, among which 5 were down regulated and 2 were up regulated.

3. Five of the down regulated proteins were identified as SHC transforming isoform 1 protein, Ribonuclease p protein subunit 20, protocadherin, cystatin SN precursor and lactotransferrin and 2 of the up regulated proteins identified as Rho-related GTP-binding protein RhoQ precursor, Ecto-ADP ribosyltranferase -5-precursor. Other proteins are yet to be identified.

4. Using DAVID annotation tool, these proteins were grouped to be as 25 % in biological regulation, 13 % in Cellular process, followed by 13 % in immune response, 17 % metabolic process, and a minor extent in locomotion as seen by DAVID annotation tool.

CHAPTER 7: 2D ELECTROPHORESIS PROFILING OF TEAR PROTEIN IN DRY EYE SYNDROME ASSOCIATED WITH PRIMARY SJOGRENS SYNDROME

7.1 INTRODUCTION

Sjogrens syndrome is a progressive, inflammatory autoimmune disease affecting primarily the exocrine glands(Ramos-Casals, Tzioufas et al. 2005). Diagnostic hallmarks are diminished tear production, xerostomia and presence of auto antibodies, especially Ro (SS-A) and La (SS-B) antibodies (Moutsopoulos 1994).

SS can occur at all ages, but it affects primarily females during the fourth and fifth decades of life. SS frequency appears to increase with age, with a prevalence of about 3% in people above an age of 50 years. The female to male ratio is about 9:1 and is 3.59 % corresponds to the combined definite/probable prevalence of primary SS (Taiym, Haghighat et al. 2004).

Sjögren's syndrome is considered an autoimmune disease characterized by chronic inflammation of exocrine glands namely lacrimal and salivary glands. Various environmental factors such as viral infections lead to epithelial cell activation and a protracted inflammatory response with features of systemic autoimmunity(Nikolov and Illei 2009). In the absence of an associated systemic autoimmune disease, patients with this condition are classified as having primary SS (Ramos-Casals, Tzioufas et al. 2010). The histological hallmark is focal lymphocytic infiltration of the exocrine glands, and the spectrum of the disease extends from an organ-specific autoimmune disease (autoimmune exocrinopathy) to a systemic disease with extra glandular manifestation (Moutsopoulos 1994). The diagnosis of pSS is based upon the combination of several clinical, serological, histological and instrumental elements suggestive both of excocrine gland involvement and typical laboratory abnormalities mostly with Ro/SSA and La/SSB (Seror, Ravaud et al. 2010).

The peculiar involvement of salivary and lacrimal glands during the pSS course, led to the research in biochemical studies to look for any enzyme or protein that might

be altered owing to the disease process (Giusti, Baldini et al. 2007). In pSS decreased expression of lactoferrin, lysozyme, salivary IgA, beta 2 microglobulin in both salivary and lacrimal fluid was observed (Avisar, Menache et al. 1979; Janssen and van Bijsterveld 1986; Markusse, Otten et al. 1992; Bjerrum 1997; Zoukhri, Rawe et al. 2012). Very limited number of studies on the proteomic studies using tear in pSS reported so far. Characteristic protein changes in tear fluid not identified yet. Table 7.1 gives the various proteomic studies using different specimens to see protein changes in primary Sjogren's syndrome.

Table 7.1: Proteomic studies in primary Sjogren's syndrome

S.No	Specimen	Altered protein	References
1	Tear fluid, lacrimal gland	Lysozyme, lactoferrin, pre albumin, glycoprteins, Ig A, beta 2 microglobulin, alpha 1 antitrypsin, peroxidise, lipocalin	(Giusti, Baldini et al. 2007)
2	Parotid saliva	beta-2-microglobulin, lactoferrin, immunoglobulin (Ig) kappa light chain, polymeric Ig receptor, lysozyme C and cystatin C	(Ryu, Atkinson et al. 2006)
3	Whole saliva	Lactoferrin, lysozyme, IgA, Ig G1,2,3 and IgM, kalikerin, Beta 2 microglobulin, calmodulin binding protein, albumin	(Giusti, Baldini et al. 2007)
4	Parotid glands	La/SSB autoantigen PTM changes	(Stea, Routsias et al. 2007)
6	Minor salivary glands	Heat shock proteins, mucins, carbonic anhydrases, enolase, vimentin and cyclophilin B	(Hjelmervik, Jonsson et al. 2009)

Since dry eye is one of the main component in primary Sjogrens syndrome, finding the specific altered proteins in tear of DES with pSS give clues for the disease mechanism and in diagnosis. Therefore the current study aims at a 2D based proteomic approach using tear fluid to search for the relevant disease biomarkers that can be informative for DES-pSS detection and its management.

7.2. OBJECTIVES

1. Profiling of tear protein in Dry eye syndrome associated with primary Sjogrens syndrome using 2D electrophoresis
2. To look at the differentially expressed tear proteins using PD quest soft ware, validate the differentially expressed proteins using DIGE.
3. Identification of the differentially expressed proteins using mass spectrometry.
4. Correlation of the differentially expressed tear protein with the clinical condition

7.3. MATERIALS AND METHODS

7.3.1. Diagnosis of Dry Eye cases and primary Sjogren's syndrome cases for the study.

Diagnostic Criteria, Inclusion and exclusion criteria for Dry Eye Syndrome: as described in chapter 3.1.2.

7.3.2. Diagnostic criteria for primary Sjogren's syndrome as discussed in 3.1.3 and in chapter 6.2. A detailed clinical proforma was used to document the clinical details of all the subjects both DES and RA. DES- pSS cases were recruited for the study. Blood investigation for the RA and pSS was done.

Subjects recruited for the study : As a prospective age and sex matched case-control study, 39 healthy controls (mean age: 43 ± 12 years, 12M, 27F), total of 26 cases of primary Sjogrens, 15 cases of primary Sjogrens (PSS) (mean age: 48 ± 11years, 5M, 10F), were recruited and was used for 2DE. DIGE was done in 18 controls (mean age: 43 ± 12 years, 8M, 10F), 8 cases of primary Sjogrens (PSS) (mean age: 46 ± 11 years, 4M, 4F), were recruited for the study.

7.3.3.Blood investigations : For the diagnosis, Blood parameters namely ESR, anti-RF, CRP, ANA, anti-SSA, Anti-SSB, Anti-dsDNA and anti-CCP as stated in section 7.3.1.were done using ELISA method as detailed in chapter 3.19.

7.3.4.Collection of tear fluid from dry eye and Controls: Tear fluid was collected from controls and dry eye cases using Schirmer strips as discussed in chapter 3. The sample was stored at -80°C until processing.

7.3.5. Extraction of tear protein from Schirmer for 2DE and DIGE : Tear proteins from Schirmer strips were extracted using 300 µl of 8M urea buffer (pH: 7.0) containing DTT, CHAPS and with 30 µg protease inhibitor cocktail, incubated at 4°C for 3hrs with intermittent mixing. After 3 hrs, centrifuged the sample, desalted, and estimated the protein using Bradford assay. For DIGE experiment tear protein was extracted using 30 mM Tris–HCl buffer containing 8M urea, 3% CHAPS and 0.5 mM TCEP (pH: 8.5) and the extraction procedure was similar to the above 2DE extraction protocol. Samples were then desalted and protein estimated as discussed in chapter 3.4.

7.3.6. Processing of tear protein for 2DE and DIGE as discussed in chapter 3.9 and 3.10

7.3.7. Identification of differentially expressed peptide/protein spots by mass spectrometry

Ingel tryptic digestion of spots was performed as discussed in chapter 3.11

Insol digestion tryptic digestion of tear protein for Mass spectrometry was performed as discussed in chapter 3.12

7.4. RESULTS

7.4.1.Blood parameters

The Blood investigations for the diagnosis of primary sjogrens done. Based on the clinical proforma data 3 cases were mild, 12 cases were moderate and 11 cases were with severe DES. The results are given in chapter 6 sections 6.4.1 wherein the tests were done to diagnose RA and to differentiate primary from secondary Sjogrens. Briefly, an increase in ESR is seen in both pSS and DES-RA compared to control. The ESR level is 2 fold higher in DES-RA than in pSS. The RF factor was found to be increased in severe cases of DES- pSS. CRP and ANA showed no significant

alterations in primary Sjogrens. Anti-SSA showed increase in the mean levels by 3 fold in primary Sjogrens cases. Anti-SSB in primary SS showed an increase by 2.5 fold. Severe DES associated with Primary SS showed increase in mean anti-CCP levels by 14 fold. This suggests primary SS with increased ant-CCP levels may be indicative of developing RA.

7.4.2. **2DE profiling of tear protein**: Tear protein samples of control and DES-pSS were profiled using 2DE.The amount of total tear protein was reduced significantly in DES-pSS cases compared to controls ($p < 0.001$) as seen in table 7.2.

Table 7.2: Tear total protein in control vs DES-pSS

Parameter	Control (µg/ml) tear protein	DES-primary SS (µg/ml)
Mean ±SEM	1726 ± 176	694 ± 57
P value		$P < 0.001$

The differential expression of proteins in DES-pSS is shown in figure 7.1. Spot E, G and H are seen only in DES-pSS but not in controls. These are specific to DES-pSS as seen in figure 7.1 and table 7.3. The Changes in the other spots marked are not specific to pSS as they are also seen in other types of DES like Non SS and DES-RA. However the level of expression seems to vary with the types of DES.

The differentially expressed proteins in DES- pSS case based on PD quest analysis are listed in table 7.3. The proteins differentially expressed in DES are also showed differential expression in pSS. But the percentage of reduction in terms of spot intensity, change in the number of cases varied compared to non Sjogrens and DES-RA condition as seen in table 7.3.

Figure 7.1: The differential expression of proteins in DES-pSS

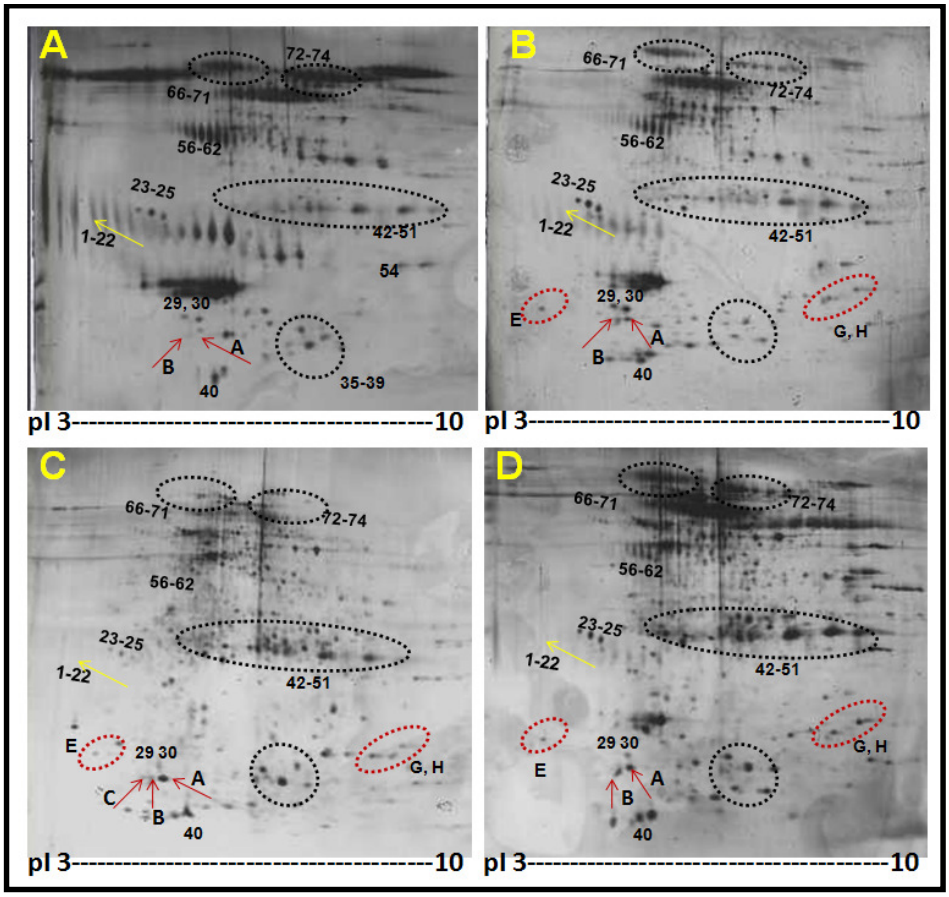


Figure 7.1: 30 µg tear protein was separated using 17 cm IPG strip with pH 3-10. A. Control B. mild DES-pSS, C: moderate DES-pSS and D: Severe DES-pSS. Differentialy expressed of spots compared to control are marked.

Table 7.3: Differentially expressed proteins in DES-NonSS, DES-RA and DES-pSS based on PD Quest analysis

Spot Number	DES – pSS (severe)		DES- RA (severe)		DES –non SS (Severe)	
	% cases	% Spot intensity	% cases	% Spot intensity	% cases	% Spot intensity
Spot 1-8	**95% ↓**	2.0	87 %	1.5	100 %	1.5
Spot 9 -20	**90 % ↓**	2.0	88 %	2.0	100 %	2. 3
Spot 21-22	90 % ↓	2.0	90 %	1.6	100 %	2.4
Spot 23	**60 % ↓**	1.5	80 %	2.8	100 %	3.0
Spot 24	**60 % ↓**	2.0	80 %	2.5	100 %	3.5
Spot 25	**60 % ↓**	2.5	80 %	2.8	100 %	3.50
Spot 29-30	**60 % ↓**	2.5	38 %	2.5	60 %	4.5
Spot 32-33	90 % ↓	3.0	**77 %**	**1.75**	80 %	3.5
Spot 40-41	**40 % ↓**	3.5	**25 %↓**	**2.0**	**38 % ↑**	**1.5**
Spot 42-51	60 % ↑	3.2	**72 %**	**1.4**	60% ↓	1.7
Spot 54-55	30 % ↓	2.0	67 %	2.2	70% ↓	2.2
Spot 56-62	90 % ↓	3.5	100 %	2.8	100%	2.5
Spot 63 -66	80 % ↓	2.0	71 %	1.5	85 %	1.5
Spot 67-71	**85 % ↓**	2.0	69 %	2.2	89 %	1.8
Spot 72-76	80 % ↓	2.0	70 %	3.7	90 %	2.4
Spot 35-39	40 % ↓	3.5	**67 % ↓**	**1.4**	70 % ↑	1.6
Spot A	60 % ↑	2.5	76 % ↑	2.5	70 % ↑	2.5
Spot B	62% ↑	2.5	65 % ↑	1.5	100 % ↑	1.8
Spot C	30% ↑	2.5	55 % ↑	**1.5**	74 % ↑	1.5
Spot D	---	2.5	68 % ↑	**1.5**	---	
Spot E	**80 % ↑**	1.5	ND	ND	----	
Spot G	**90 % ↑**	1.6	ND	ND	----	
Spot H	**90 % ↑**	1.5	ND	ND	-----	

7.4.3. 2D-DIGE of tear protein of DES-pSS: Normal 2DE revealed differential expression of tear proteins in DES -pSS as seen figure 7.1, which is complied in table 7.3. Further to validate the same and look for proteins specific to DES-pSS, DIGE was done with pooled tear samples. Representative tear protein profile using DIGE – tear protein separation of DES (DES-pSS) vs (control). 30 μg tear protein from DES-pSS alone was labelled with Cy 3 (green), control tear protein labelled with Cy 5 (red) and mixture (15 μg) of Control and DES-pSS labelled with Cy 2 (blue) which served as internal standard as showed in Figure 7.2. From DIGE analysis a total of 16 proteins showed down regulation and 2 proteins up regulated in the DES-pSS as per statistical analysis BVA using Decyder software as seen in table

7.4. Among this 5 proteins are also shown to be down regulated by normal 2DE. The differentially expressed peptide spots were seen in the figure 7.3. Proteins that were also seen in 2DE are marked in yellow. The representative down and up regulated protein BVA view of DES-pSS condition are shown in figure 7.4 and 7.5.

The discriminate analysis done to conclude the differentially expressed proteins that can serve as disease biomarkers having > 95 % accuracy using EDA analysis with decyder software showed a total of 6 peptide spots 46,101,109,163,137 and 192 are potential markers for DES-pSS (Table 7.4).

Figure7.2. Representative DIGE image of DES-pSS.

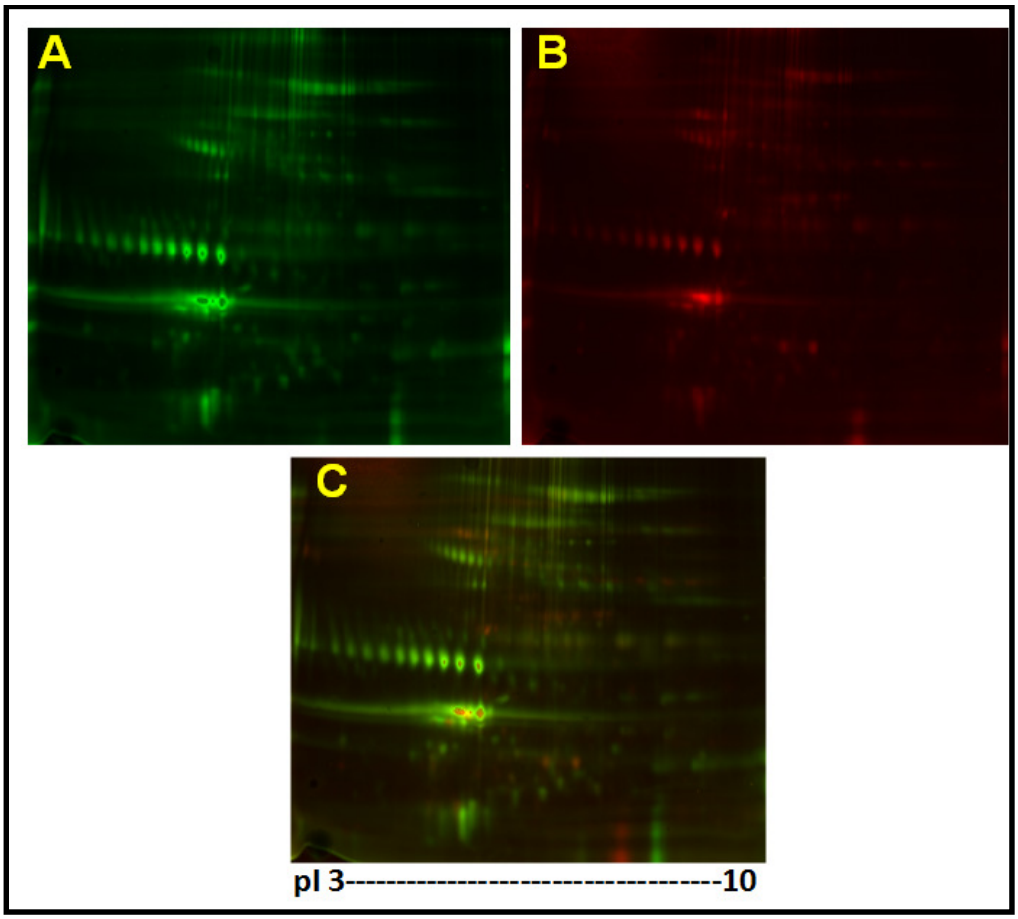


Figure 7.2: Representative tear protein (30 µg) DIGE image of A. Cy 3 labelled DES-pSS B. Cy 5 lebelled control and C. Merged image of DES-pSS and Control.

Table 7.4: The differentially expressed spots in DES-pSS using DIGE

DES - Condition	Differential expression	Spot No. by DIGE	Volume ratio and significance
DES - primary SS	Down regulated	**46**,75,77,82,83,86,,98,**101**,**109**, 131,**163**,180,188,172,177,308,328 ,	-1.97 to - 7.3 *p value:* > 0.001
	Up regulated	**137**,**192**	2.9, 3.33 *p value*: 0.01- 0.04

Figure 7.3.: The differentially expressed spots of DES-pSS by DIGE BVA analysis.

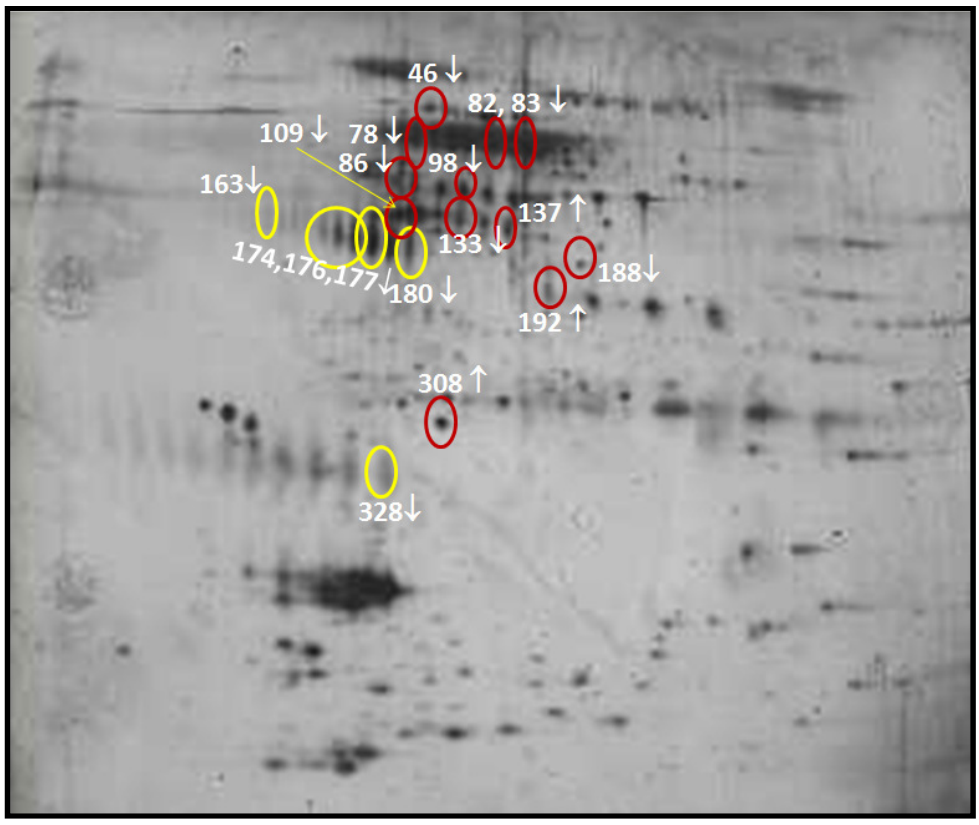


Figure 7.3: the differentially expressed spots of DES-pSS by DIGE. Among this yellow coloured circled spots also seen in other types of DES. Spot 163, 46 (down regulated), Spot 137, 192 (up regulated) are suggested potential biomarkers by DIGE discriminate analysis.

Figure 7.4: Representative BVA analysis of Spot 188 in DES-pSS

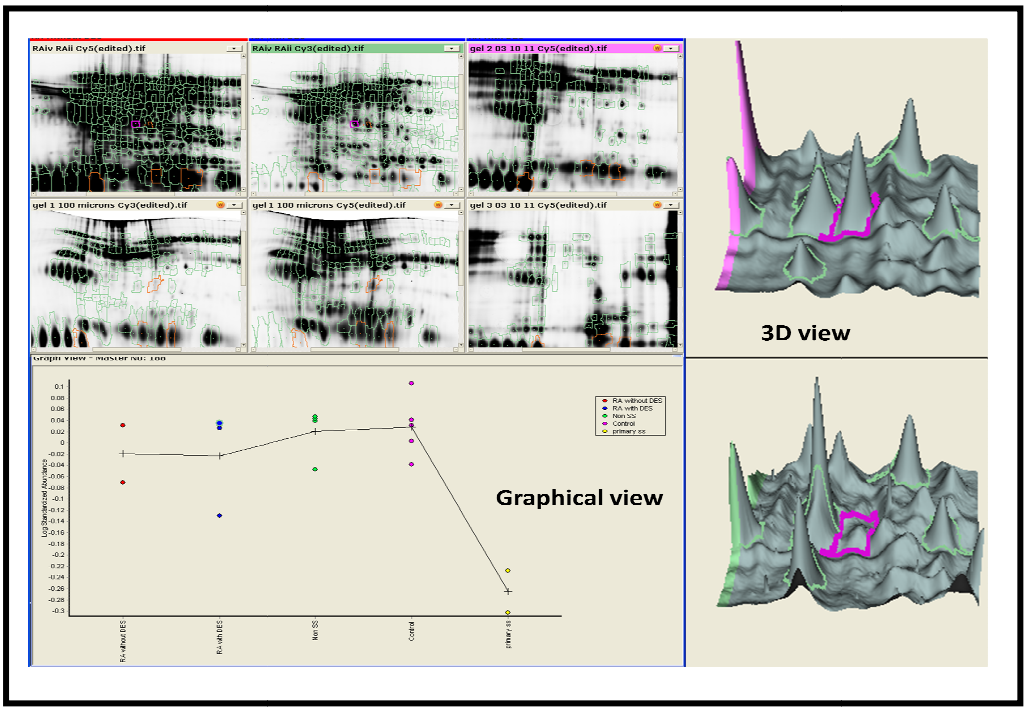


Fig7.4: BVA view of DIGE gel in DES-pSS. Shows the spot position in the gel, graphical view, 3D view to show it's down regulation.

Figure 7.5: Representative BVA view of Spot 137 in DES-pSS

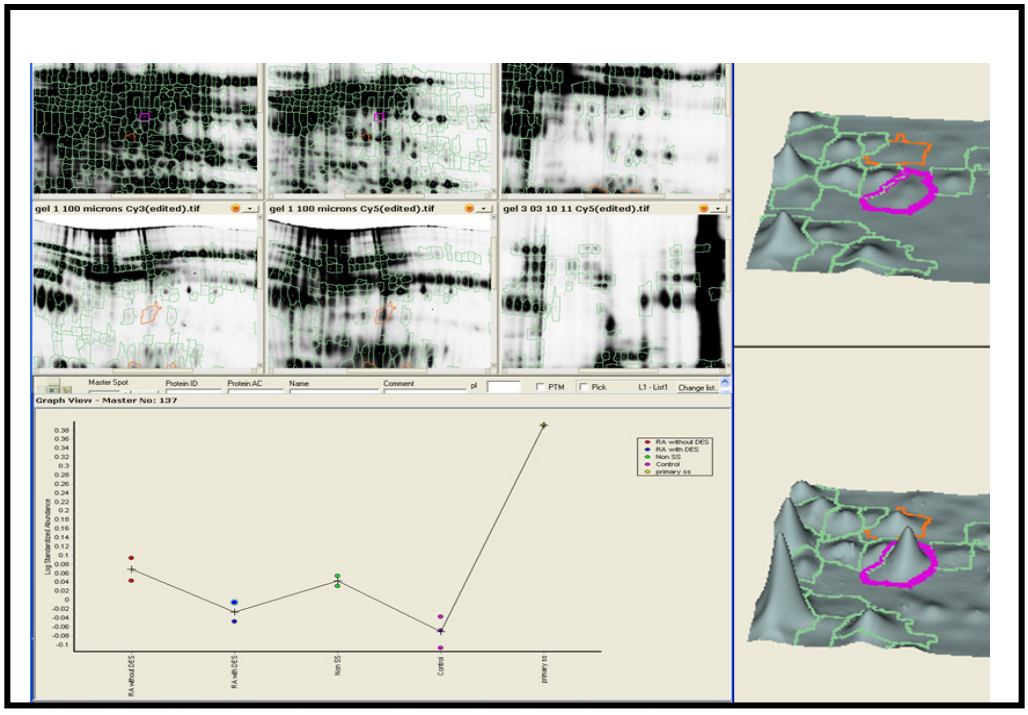


Table 7.5.: The peptide spots from DIGE discriminate analysis as biomarkers

Peptide spot number as potential biomarker	DES condition	Identified protein
46 ↓	DES - pSS	Yet to identify
163 ↓	DES - pSS	Znic-alpha glycoprotein
101 ↓	DES - pSS	Yet to identify
109 ↓	DES - pSS	Transcription cofactor HES-6 and keratin cytoskeletal protein
192 ↑	DES - pSS	Yet to identify
137 ↑	DES – pSS	Yet to identify

7.4.4. Identification of differentially expressed spots of DES-pSS and DES-RA: The peptide/protein from DIGE was ingel tryptic digested and subjected to nano LC/MS/MS. One of the down regulated spot in DES-pSS spot 109 was identified as Keratin type 1 cytoskeletal 13 with 3 peptides of 99 % confidence (figure 7.6). Another protein of the same spot was Transcription cofactor HES-6 with 1 peptide of 85% confidence was detected, in 70 % of DES-pSS cases showed down regulation with 2.5 fold decrease. This protein involves in developmental process. Spot 163 showed down regulation, and also down regulated in other DES types, and this protein from the literature identified as Zn-alpha- glycol protein. Spot 328 is identified as lacritin precursor in this study also down regulated in other DES types.

Table 7.6: Ingel tryptic digestion of differentially expressed spots in DES – pSS

Spot No. from Gel	Name of the protein	Accession Number	Sequence	No of peptide/ Confidence	% coverage	% of DES-cases showed Change
109 ↓ Primary Sjogren's-DES	**1. Keratin type I cytoskeletal 13**	**gi|131412228**	**AGLENTV AETECR ALEEANA DLEVK**	4 peptides 99%	15.1%	70%
	2.Transcription cofactor HES-6	**gi|218751874**	**LEQEIAT YR**	1 peptide 85%	9.9%	

7.4.5. Tear protein identification in DES-pSS by *in sol* tryptic digestion

The total tear protein of control and DES-pSS, DES-non SS and DES-RA tear was subjected to *Insol* tryptic digestion and then the proteins were identified by nano LC/MS/MS along with the proteins of (table 7.7). A total of 37 proteins were detected in control tear. DES- Non SS showed15 proteins, DES-RA showed 16 proteins, DES-pSS showed 7 proteins that were present in control tear. 19 proteins characteristic to DES-RA, 19 proteins characteristic to DES-Non SS and 9 proteins characteristic of primary Sjogrens were seen in the insol digestion of tear protein. Among which 2 proteins Hypothetical protein DKFZp779N1935, and alpha enolase was found in all types of DES condition. 3 proteins actin variant IGHA and S100 A9 present in DES-RA and DES-Non SS. 2 proteins, Heat shock protein 27 and ORM 2 present in DES-RA and DES-pSS. One protein KIAAO676 present in both DES-pSS and DES-Non SS.

Table 7.8 shows proteins that were characteristically seen in DES but not in controls. **10 proteins specific to DES-RA** are serine protease inhibitor clade A, apolipoprotein preproprotein, N-acetyl transferase 10, Annexin A1, alpha 1 acid glycoprotein precursor, GALGT protein, similar to filaggrin, PRO0684, hypothetical protein LOC1244220, actin cytoplasmic 2, chromosone 14 OPF 153, alpha 1 antitrypsin precursor. **10 proteins specific to DES-Non SS** are core histone macro H2A1 isoform 2, histone H2B type 2 F a, Peroxidase precursor, predicted belch BTB containing domain, SET and MYND domain containing protein, plastin 2, cytokeartin 13, BRD 1 protein, zinc finger protein 169, actin alpha cardiac muscle 1 proprotein, protein S100 A6, S100 P, S100 A7 and 4 proteins specific to DES-pSS are immunoglobulin heavy chain variable region, similar to USP6 N-terminal like isoform, insulin activator factor, similar to ribosaomal protein L18. A venn diagram shows the tear proteins identified in DES by In sol tryptic digestion (Figure 7.6).

Table 7.7: Tear proteins identified in all DES types compared to control using nano-LC/MS/MS

Accession No.	Control (n=9)	DES -RA (n=6)	DES-Non SS (n=11)	Primary SS (n=3)
spt|P12273	Prolactin induced protein	prolactin induced protein	Prolactin-inducible protein	
pdb|1YAN	Lysozyme	lysozyme precursor	Lysozyme precursor	
trm|Q8N4N0	Zinc Alpha 2 glycoprotein	Zinc alpha 2 glycoprotein	Alpha-2-glycoprotein 1, zinc	Zinc alpha 2 glycoprotein
spt|Q9GZZ8	Lacritin precursor	extracellular glycoprotein lacritin precursor	lacritin precursor	lacritin precursor
cra|hCP1734353.1	Proline rich 1 protein	Proline-rich protein 1 precursor		
cra|hCP1915488	Proline rich 4 protein	proline rich 4 (lacrimal)	Proline-rich protein 4 lacrimal	proline rich 4 (lacrimal)
Gi|4505171	Secretoglobin 2A		secretoglobin family 2A member 1	
spt|P01036	Cystatin S precursor	cystatin S precursor	Cystatin S precursor	
Gi|55666285	Lipocalin like protein	lipocalin 1 precursor	lipocalin 1 like protein	lipocalin 1 precursor
pdb|1LFH	Lactoferrin	Lactoferrin	Lactoferrin	Lactotransferrin
Gi|25058739	Albumin	serum albumin	serum albumin	serum albumin precursor
spt|P01833	Polymeric Ig receptor		polymeric immunoglobulin receptor	
Gi|55667085	Similar to pHL E1F1 isoform 2	similar to pHL E1F1 isoform 2		
Gi|3954893	Ig kappa light chain	immunoglobulin kappa light chain VLJ region	immunoglobulin kappa light chain VLJ region	
Gi|21669677	Ig lambda light chain	immunoglobulin labda like polypeptide 5	immunoglobulin variable region	
trm|Q96F97	Chitinase 3 like 2 protein			
Gi|52426735	Ankyrin 2 isoform 1			

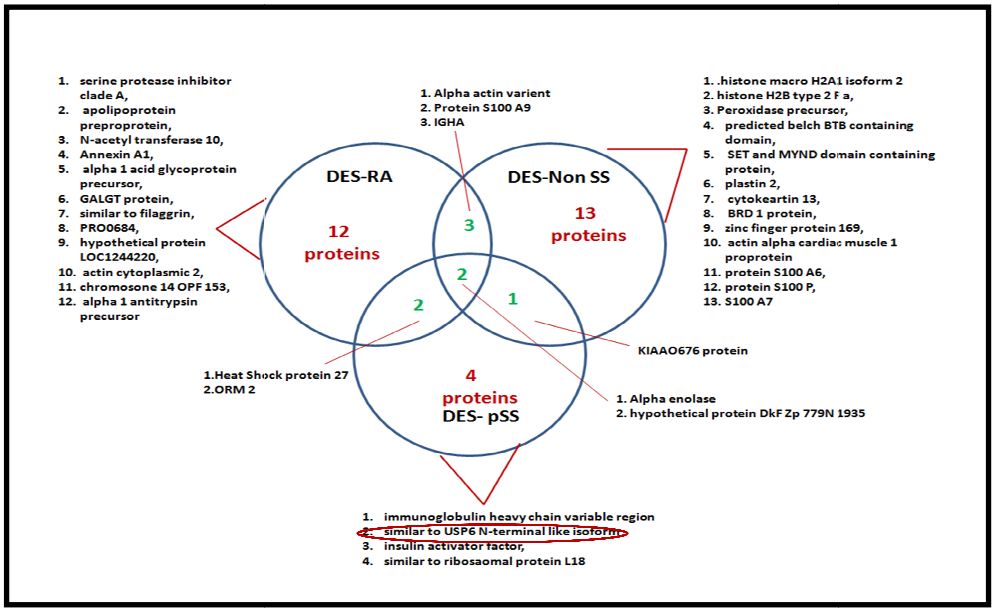
Accession No.	Control (n=9)	DES -RA (n=6)	DES-Non SS (n=11)	Primary SS (n=3)
trm|Q8IZY7	hepatocellular carcinoma associated TB6 protein			
Gi|854182	SOX			
Gi|15822535	cyclin dependent kinase protein			
spt|P06703	Calcyclin			
spt|O75556	mammaglobin B precursor	mammaglobin B precursor		
rf|NP_001053.2	trans cobalamin I precursor			
Gi|19574296	hCG1999003			
Gi|119608459	hCG201503	hCG201503		hCG201503
Gi|19882251	Cystatin SN precursor			
Gi|124248531	LOC441476			
P02786	human transferrin receptor		human transferrin receptor	
Spt|p01130	LDL receptor precursor			
Trm|Q86X06	ERD 1 protein			
Gi|7657532	protein S 100 A6			
Gi|40385873	probable G protein coupled receptor protein 150			
Q8NEM0			microcephalin	
spt|P31025		Von Ebner's gland protein precursor		

Table 7.8: Proteins characteristic of DES from *insol* tryptic digestion by nano-LC/MS/MS

Accession No.	DES -RA (n=6)	DES-Non SS (n=11)	Primary SS (n=3)
gi|50363217	serine protease inhibitor clade A		
gi|662841	heat shock protein 27		heat shock protein 27
	beat actin variant	alpha actin 1 isoform b	
gi|48145977	ORM2		ORM 2
gi|21362641	N-acetyl transferase 10		
gi|4502101	Annexin A1		
gi|167857790	alpha 1 acid glycoprotein precursor		
trm|Q8N636	GALGT protein		
gi|49258100	IGHA protein	IGHA2 protein	
gi|113413655	**similar to filaggrin**		
gi|6855601	PRO0684		
gi|94536866	hypothetical protein LOC1244220		
Trm|Q68DN5	hypothetical protein DkF Zp 779N 1935	Hypothetical protein DKFZp779N1935	hypothetical protein DkF Zp 779N 1935
gi|4506773	protein S100 A9	protein S100 A9	
gi|4503571	alpha enolase	enolase 1 varient	enolase 1 varient
gi|4501887	actin cytoplasmic 2		
gi|190341102	chromosone 14 OPF 153		
gi|50363221	alpha 1 antitrypsin precursor		
gi|4557321	apolipoprotein preproprotein		
gi|93141020		core histone macro H2A1 isoform 2	
gi|66912162		histone H2B type 2 F a	
		Peroxidase precursor	
		predicted belch BTB containing domain	

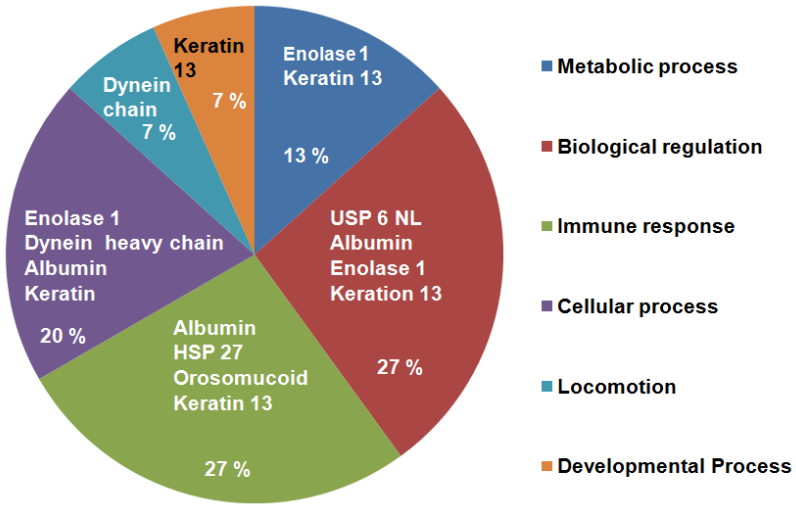
Accession No.	DES -RA (n=6)	DES-Non SS (n=11)	Primary SS (n=3)
gi|90101756		SET and MYND domain containing protein	
Trm|Q9Y2F3		KIAA0676 protein	KIAA0944 protein
gi|67614506		plastin 2	
gi|7657532		protein S100 A6	
gi|5174663		protein S100 P	
gi|115298657		protein S100 A7	
gi|131412228		cytokeartin 13	
Trm|Q86X06		BRD 1 protein	
Cra|hcp|805194		zinc finger protein 169	
gi|4885049		actin alpha cardiac muscle 1 proprotein	
Cra|hcp1744655 1			similar to ribosaomal protein L18
gi|685120			insulin activator factor
gi|113426782			similar to USP6 nterminal like isoform
gi|112700686			immunoglobulin heavy chain variable region

Figure 7.6: Venn diagram of proteins identified in all types of DES by *Insol* tryptic digestion



7.4.6. Functional classification of identified proteins: The characteristic proteins of DES-pSS are subjected to DAVID annotation tool and found 27 % of proteins belongs to biological regulation, cellular process and immune response, 20 % cellular process, 13 % metabolic process, locomotion and 7% of proteins belongs to locomotion developmental process as seen in figure 7.7.

Figure 7.7: Functional classification of DES-pSS proteins using DAVID annotation tool



7.5. SUMMARY

1. The Clinical parameters such as serum anti-SSA, anti-SSB levels were increased in DES-pSS. Serum anti-CCP also increased in severe DES-pSS compared to others. ESR, RF levels also showed increase with increase in DES severity.
2. Tear proteins of DES- primary SS were profiled using 2DE and DIGE. 4 new proteins, Total 16 peptide spots were down regulated and 4 peptides were up regulated. Among which 5 peptides were also down regulated in other DES conditions and were identified as Zn-alpha-glycoprotein and lacritin precursor.
3. 1 of the down regulated protein from ingel tryptic digestion in DES – pSS is identified as keratin cytoskeletal protein 13. Other specific potential biomarker spots are yet to identify.
4. *Insol* tryptic digestion of tear protein from all DES types were identified. DES-pSS showed 4 proteins specific to the disease, and are namely USP 6 NL, insulin activator factor, similar to ribosaomal protein, immunoglobulin heavy chain variable.
5. DAVID annotation tool showed 36 % of proteins play role in cellular process, 30 % in cell organization, 25 % proteins involved in biological regulation, 17 % in immune response, 13 % of proteins localization and 3 % in locomotion.

CHAPTER 8: VALIDATION OF BIOMARKER LACRIMAL PROLINE RICH 4 PROTEIN (LPRR4)

8.1.1. INTRODUCTION

Dry eye syndrome (DES) is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface(2007). DES is observed with increased age. Apart from age, various other reasons to develop DES mainly includes, other systemic diseases such as Sjogren's syndrome both primary and secondary to RA, SLE, Diabetes and also use of contact lens, computer usage, other eye pathologies such as glaucoma, cataract surgery.

Tear fluid proteins play an important role in the ocular surface integrity and to maintain healthy environment. Changes in the tear protein and its role would help to understand the disease mechanism of DES. Therefore, this study aimed to look for potential biomarkers of DES, DES associated with pSS and RA to RA using proteomics approach. As discussed in chapter 4,5 and 6 one of the differentially expressed tear protein that showed 100% down regulation in all types of DES was identified as Lacrimal proline rich 4 protein (LPRR4). This chapter deals with the validation of LPRR4 protein in tear fluid of DES condition by ELISA and also LPRR4 expression in lacrimal gland tissue at protein level using IHC as well as at mRNA level using RT-PCR.

Lacrimal proline rich protein is a member of the proline-rich protein family. It is similar to saliavary PRP functionally and has a protective role. The PRPs is reported as a family of highly abundant salivary proteins produced by the submandibular and parotid glands of a wide phylogenetic range of mammals. This protein is encoded by a single gene present on the Chromosome 12 p13 region. They are characterized by a typical amino acid composition of 25-45% proline, and they can be classified into acidic, basic, and which can be in glycosylated /phosphorylated form(Dickinson and Thiesse 1995).

In situ Hybridization analysis revealed the expression of PRP gene in the human lacrimal gland in the acinar cells but not in the intra lobular ducts (Dickinson and Thiesse 1995)

Despite their overall similarity, the actual protein sequence of lacrimal PRR is significantly different from the salivary acidic PRR. The salivary PRRs involved in protecting the epithelial surfaces, with specific function of binding to hydroxy apatite, tannins in agglutination of bacteria (Hay, Bennick et al. 1988). Similar functions are speculated for LPRR4 protein. The other forms of salivary proline rich proteins are mentioned below based on sequence based bioinformatics approach.

8.1.2. Memebers of the PRR family

The Lacrimal PRR includes,

a. PROL1- Proline rich protein 1

b. PROL4 (2) - Proline rich protein 4 isoform 2

c. PROL4 (1) - Proline rich protein 4 isoform 1

Non lacrimal PRR proteins (salivary)

a. PRP1- Basic salivary proline rich protein 1

b. PRB2-- Basic salivary proline rich protein 2

c. PRB3-- Basic salivary proline rich protein 3

d. PRB4-- Basic salivary proline rich protein 4

e. PRPC – Salivary acid proline rich phosphoprotein1/2 - Acidic

The amino acid composion, mRNA sequence length, molecular weight comparisons of salivary PRPs and lacrimal PRR are mentioned in table 8.1. LPRR4 is small molecular weight protein among all the PRRs apart from the basic salivary proline rich protein as seen in table 8.1.

Table 8.1: Comparison of salivary proline rich proteins with lacrimal proline rich protein.

NAME	UNIPORT ID	Amino acid length	Molecular weight (kDa)	Theoretical pI	m-RNA sequence length
Proline-rich protein 1	Q99935	248	27.216 5	10.42	947
Proline rich protein 4 isoform 2	Q16378	134	15.124 8	6.97	507
Basic salivary proline-rich protein 1	P04280	392	38.545 8	11.22	1173
Basic salivary proline-rich protein 2	P02812	416	40.799 2	11.63	502
Basic salivary proline-rich protein 3	Q04118	309	30.980 1	10.80	1058
Salivary acidic proline-rich phosphoprotein 1/2	P02810	166	17.016 4	4.63	659

8.1.3. Sequence Similarity

The species variation and its sequence similarity with other species PRRs mentions in table 8.2. LPRR4 showed 100% identity with Proline rich 4 (PRR4) of Homo sapiens. PRRs also showed 100% identity with LPRP4 of *Pan troglodytes* as seen in table 8.2.

Table 8. 2: Sequence similarity of LPRR4 with other species

Protein Name	Organisms	Accession.NO	Sequence coverage %
proline rich protein 2	Rattus norvegicus	NP_001013229.1	49
proline-rich protein HaeIII subfamily 1 precursor	Mus musculus	NP_035304.3	44
proline-rich salivary protein	Mus musculus	AAA40003.1	34.3
proline rich protein MP4	Mus musculus	NP_444481.1	29
proline-rich proteoglycan 1, isoform CRA_a	Rattus norvegicus	EDM01685.1	28

Secreted form of LPRR4 contains 134 amino acids with amino acids 1-16 contributing to the signal peptide. All members of this protein family have a characteristic architecture. Following a conserved secretory peptide leader sequence the secreted proteins have an acidic N-terminal domain. This domain can be subdivided into the Nl region, carried by both the acidic and the basic subfamilies, and an N2 extension, carried only by the acidic PRPs. This domain is followed by a number of tandem repeats of an oligopeptide comprised mainly of proline, glycine, and glutamine, leading to the characteristic amino acid composition of the proteins.

8.1.4. Tissue specificity of LPRR4:

Abundantly expressed in lacrimal gland where it is found in the acinar cells but not in the intralobular ducts. Also found in the submandibular gland, the parotid and sublingual glands. The distribution LPRR is 25.8 % in eye. Trachea showed 53 % of LPRR4, 9.8 % in salivary gland with minor % of other tissues in human tissues using Unigene &EST Expression information.

8.1.5. Homology of LPRR4: The homology of LPRR4 with other members of PRR family tabulated in table 7. It has 49 % homology with Salivary acid proline rich phosphoprotein1/2 – Acidic (PRPC) protein, other PRR proteins had only around 11 – 16 % homology as seen in Table 8.3.

Table 8.3: LPRR4 homology with PRR family members using UNIPROT search

Lacrimal			**Salivary**		
Name	**Uniprot ID**	**% similarity**	**Name**	**Uniprot ID**	**% Similarity**
PROL1	Q99935	11.9	PRP1	P04280	12.6
PROL4(2)	Q16378	100	PRB2	P02812	15.1
PROL4(1)	Q16378	100 **(R->Q)**	PRB3	Q0411	16.2
			PRB4	P10163	15.9
			PRPC	P02810	**45**

The NetPhos result shows that LPRR4 can undergo post translational modification such as phosphorylation. Table 8.4 explains the possible amino acid residues at specified position that can undergo phosphorylation.

Table 8.4: The NetPhos results for the possible phosphorylation sites of LPRR4.

Residue	Position	Sequence	Score [0-1.0]
S	103	HRQLSLPRF	0.970
S	39	VEDSSQRPD	0.968
Y	25	NDVNYEDFT	0.949
S	38	DVEDSSQRP	0.914
S	18	SSAQSTDND	0.901

All these information on species specificity and homology with the other salivary PRRs gives information that LPRR4 is a protein specific to lacrimal gland tissue, doesn't have 100 % similarity with other PRRS. This helps to further validate the protein for its functional and structural aspects using *insilico* approach.

Despite their overall similarity, the actual protein sequence of lacrimal PRP is significantly different from the salivary acidic PRPs, and it lacks conservation in the repetitive domain. The pattern of tissue-specific expression of the lacrimal PRP mRNA is distinct from that of the salivary PRPs, which are not expressed in the human lacrimal gland.

Lacrimal expression is widely distributed in human anterior exocrine glands, whereas the acidic PRPs are expressed mainly in the submandibular and parotid glands and the basic and glycosylated PRPs in the parotid (Bennick and Connell 1971). However, even though these PRP genes show different tissue-specific patterns of expression, they are expressed in the same type of cells in each tissue, the serous acini and demilunes, and are not expressed in ductal cells. The high levels of LPRP expression in the lacrimal acinar cells could be a phenotypic marker of differentiation and can be used in the *in vitro* studies of lacrimal gland function.

8.1.6. Functional aspects of PRP: Three functions have been proposed for these proteins. Namely binding to hydroxyapatite presumably, to stabilize the mineralization on the surfaces of the teeth as well as binding to bacteria and to clear potential pathogens by agglutination and probably clearance of even benign species on the tooth surface. Thirdly, they also bind to tannins, a highly toxic, carcinogenic component of certain major foods such as fruits and grains. Hence, the salivary PRPs may be involved in protecting the epithelial surfaces from these substances (Mehansho, Hagerman et al. 1983; Carlson 1993)

8.1.7. Lacrimal PRP: The function of LPRR4 is not yet known. However, the lacrimal PRP is expected to provide protective functions in the eye similar to those proposed for the salivary acidic PRPs in the oral cavity. Modulation of the microflora at the ocular surfaces appears to be the more obvious function of lacrimal PRP. This could be by promoting agglutination and clearance of bacteria by the flow of the tear film over the eye, or by promoting adherence of benign species to the epithelial surfaces, thus providing a competitive advantage over non adherent species. However, binding to minerals or tannins by LPRP also may be important for the protection of the ocular surfaces (Dickinson and Thiesse 1995).

8.1.8. LPRP4 in DES report from literature: LPRR 4 is the tear specific high abundant protein that has been reported in the Pterygium patients along with other proline rich proteins1,3,and 5 apart from reduction of it in blepheritis (Koo, Lee et al. 2005; Zhou, Beuerman et al. 2009), primary sjogrens syndrome(Zhou, Huang et al. 2004) and DES associated with contact lens wear (Nichols and Green-Church 2009) as tabulated in table 8.5.

Table 8.5: Expression of the LPRP4 protein in the tear of various type of ocular disease

	Condition	Status of	Reference
1.	Blepharitis	Reduced PRR4	Lei Zhou et al, J proteome research 2004
2.	Pterigium	Report on the presence of 5 types of proline rich proteins 1, 2,3 4 and 5. First report.	Lei Zhou et al, Ann Acad Med Singapore, 2006
3.	Sjogren syndrome	Reduced PRR 4	Tsai et al; The British Journalofophthalmol2006
5.	Contact lens Dry eye	Reduced PRR 4	Nichols et al: Cornea,2009

As seen in table 8.5, the LPRR4 is reportedly reduced in the conditions studied. In the current study we observed the reduction of LPRR4 in all types of dry eye condition i.e dry eye associated with non Sjogren's, primary Sjogren's and in DES - RA. This is the first report on reduction of PRR4 in all types of dry eye conditions (Aluru, Agarwal et al. 2012).This protein was validated by estimating the protein level in the tear by ELISA in the DES and in healthy controls. The clinical correlation was also studied and the structural and functional aspects of LPRR4 were predicted *in silico*.

8.2. MATERIALS AND METHODS

8.2.1. REAGENTS

1. 8M Urea Buffer pH: 7.6
2. Phosphate buffered saline (PBS) pH; 7.2.
3. Schirmer strips
4. ELISA Kit for LPRR4 from USCN company

8.2.2. Collection of tear fluid from controls and Dry eye patients: Reflex tears were collected by using a sterile Schirmer strip. The Schirmer strip placed in the lower cul-de-sac region and allowed to absorb the tear for 5 min in the open eye condition from the inferior prism without any contact with the lower lid, the cornea or conjunctiva. During the process, the person was seated comfortably, head raised and against and without any direct source of light or flow of air. The tear absorbed on to the strip was removed after 5 min, placed in sterile vial and stored at -80°C until analysis.

For the quantification of LPRR4 by ELISA,tear samples were prospectively collected from dry eye cases (mean age: 49 ±16 y, n= 27) associated with NS (mean age: 45 ±20 y, n =9), PSS (mean age: 49 ±20 y, n= 7) and RA (mean age:52 ±9 y, n =11) with age matched controls (mean age: 43 ±10 y,n =16, 10 M, 6 F). Clinical details mentioned in Supplementary Table A.

8.2.3. Standardization of tear protein extraction of for LPRR4 validation:

Protein extraction from Schirmer's was done as for 2D sample prepared briefly,To the tear absorbed strip, 300 µl of 8M urea buffer, 30 µg of protease inhibitor cocktail was added and left at 4°C for 3 hrs with intermittent mixing. After 3 hrs, the strip was removed, centrifuged the solution and the supernatant was used for LPRR4 estimation. Also the protein extraction was done with PBS pH:7.2 as done with urea buffer.

8.2.4. Determination of LPRR4 using ELISA kit: Principle: The microtiter well plate coated with an antibody specific to PRR4. Standards and unknown samples were then added to the appropriate wells in the microtiter plate, with a biotin-conjugated polyclonal antibody specific for PRR4. Avidin conjugated to HRP will be added to this, the colour produced was read at 450 nm upon the addition of TMB substrate. The protocol for LPRR4 estimation is mentioned in chapter 3 section 3.15.

8.2.5. PAS staining of 2D focussed tear proteins for LPRR4: Tear proteins were separated using 2DE as mentioned in chapter 3 section 3.8, and the staining was done as discussed in chapter 3.17.

8.2.6. IHC for LPRR4 in lacrimal gland tissue: Lacrimal gland tissue was fixed in buffered formalin**,** sections were taken and proceeded for IHC as described in Chapter 3.16 and 3.18.

8.2.7. RT-PCR of LPRR4 from normal lacrimal gland tissue: RNA was isolated from LG tissue and converted to, cDNA, proceded for RT-PCR as mentioned in chapter 3.14.

mRNA expression of LPRR4 using RT-PCR

LPRR4 mRNA expression in lacrimal gland tissue was studied. Primers for LPRR4 were designed using genscript website. Forward primer sequence 5′TGCTCTCAGTGGTCCTTCTG3′ and Reverse primer sequence 5′CTTCAGGAGGAGGTCTCTGG 3′. The product base pair size is 144 bp. The PCR conditions used were Initial denaturation temperature of 94°C - 5 min, 94°C – 1 min, annealing temperature of 57°C and extension temperature of 72°C – 1 min, for 30 cycles with final extension at 72°C - 7 min and then at 4 °C.

8.2.8. In silico analysis for LPRR4 strucure and Function prediction

8.2.8.1. 3D Structure Prediction of LPRR4

The 3D structure of LPRR4 is yet to be elucidated. Hence, in this study we attempted a stringent computational approach to predict the structure before predicting the function. As a first step, the amino acid sequence of LPRR4 was retrieved from Uniprot (Acc.no: Q16378) and was BLASTp analyzed against Protein Data Bank (PDB) database(Roy, Kucukural et al. 2010). Since no suitable templates were found in PDB, fold recognition method based modeling was performed through I-Tasser server. Based on overall C-value and stereo chemical quality (PROCHECK) (Laskowski, Rullmannn et al. 1996), the best model was selected and was further refined using Modefiner (Xu and Zhang 2011) .

Molecular Dynamics Simulation was performed on OPEN DISCOVERY Linux Platform with pre-installed GROMACS 4.3 software to analyze stability of the protein. GROMOS96 43al force field was used during the optimization and simulation process. Periodic boundary conditions were applied and 0.9 nm cubic box was used. The protein was solvated with SPC water model which added 7893 SOL molecules to the system. System was neutralized by replacing water molecules with Na^+ counter ions. Steepest Descent algorithm was used for energy minimization which converged in 283 steps. Equilibration of the position restrained ensembles was conducted for 100 ps at 300 k and with 2 fs of integration time step.

And then final MD step was performed for 2 nanoseconds timescale. Finally, the trajectory files were analyzed through GROMACS utilities in order to obtain the Root-Mean-Square Deviation (RMSD) and Root-Mean Square Fluctuation (RMSF) value. The trajectories were visualized using Xmgrace (Bandello, Brancato et al. 2001). The final simulated model was validated by Qmean server (Benkert, Kunzli et al. 2009).

8.2.8.2. Datamining to identify interacting partners of LPRR4

STRING is a Search Tool meant for Retrieval of information on protein-protein Interactions based on direct (physical) and indirect (functional) associations(Szklarczyk, Franceschini et al. 2011). Some of the interacting partners of LPRR4 were identified through querying the STRING (Search Tool for the Retrieval of Interacting Genes) database, wherein, consensus prediction protocol was adopted to achieve high confidence score.

8.3. RESULTS

8.3.1. 2D electrophoresis data of tear protein showed > 95 % reduction of LPRR4 as shown in table 8.6.

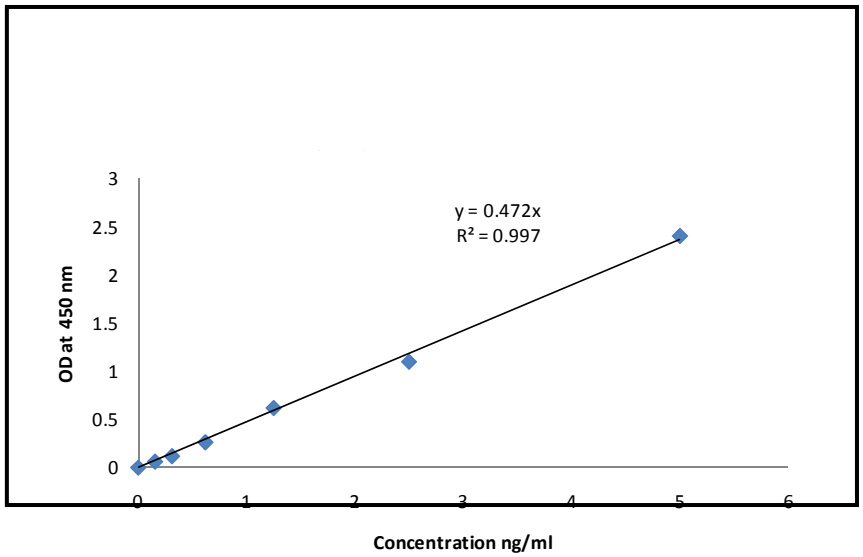
Table 8.6: Down regulation of LPRR4 in DES condition 2DE PD quest analysis

Name	Primary SS (n = 10)	Non sjogren's (n = 27)	DES secondary to RA (n = 27)
% cases down regulated	100	100	96 %
% ↓ Spot intensity	75%	80%	75%

8.3.2. Standardization of LPRR4 using ELISA: 8M urea buffer that was used for 2DE was used for tear protein extraction for LPRR4 estimation. But, it showedthe interference of with the extraction buffer for detection of LPRR4.

Since Urea buffer is interfering for determination, then extraction of proteins done using PBS which showed no interference with protein estimation. Standard graph was constructed and then preceded with the determination in DES samples. The standard graph for LPRR4 is shown in figure 8.1.

Figure 8.1: Standard graph for LPRR4 determination.



The LPRR4 levels in tear fluid of all types of DES shown in Table 8 as mean ± SEM. The values of LPRR4 expressed as µg/ml of tear volume. Table 8 shows the levels of PRR4 protein in the tear as detected by ELISA with a significant decrease in the levels of the protein in all types of DES with a maximal decrease seen in DES associated with RA ($p < 0.00$). A mean LPRR4 level of 6.9 ± 0.78 µg / ml with a range of 2.9 to 15.4 µg / ml range was observed in the normal tear and it was found to be decreased by 4.6 fold to 1.5 ± 0.52 µg / ml in the DES cases with a range of 0.032 to 11.2 µg / ml in DES cases (Table 8.7).

DES-RA showed 70 % reduction of as compared to other DES condition. This was due to more severe cases seen in DES-RA compared to others. More the severity of DES, more the reduction of LPRR4.

Table 8.7: Tear LPRR4 levels from various types of DES by ELISA

Parameter	Control (n=19)	Total DES Cases (n=27)	Non sjogren's (n=10)	Primary Sjogren's (n=7)	DES secondary to RA (n=10)
	LPRR4 (µg/ml)				
Mean	**6.95**	**1.5**	**1.99**	**2.45**	**0.44**
SEM	**0.78**	**0.52**	**0.64**	**0.94**	**0.14**
p value		<0.000	0.001	0.007	<0 .000
Severe cases	**----**	**44%**	**20%**	**43%**	**70%**

8.3.3. Correlation of LPRR4 with the clinical parameters

LPRR4 was reduced in all types of DES. When compared with clinical parameters like Schirmer value, Tear breakup time (TBUT), DES severity and its grade, as the severity increases the levels of LPRR4 were reduced as seen in figure 8.3.

A significant positive correlation between the levels of the LPRR4 protein and the schirmer value (r = 0.55 & p = 0.008) as well as with that of the TBUT values (r = 0.52 & p = 0.005) were observed, indicating the correlation of the protein levels with the severity of dry eye (Figure 8.2)

Figure 8.2: Clinical correlation of LPRR4 with Schirmer's value and TBUT.

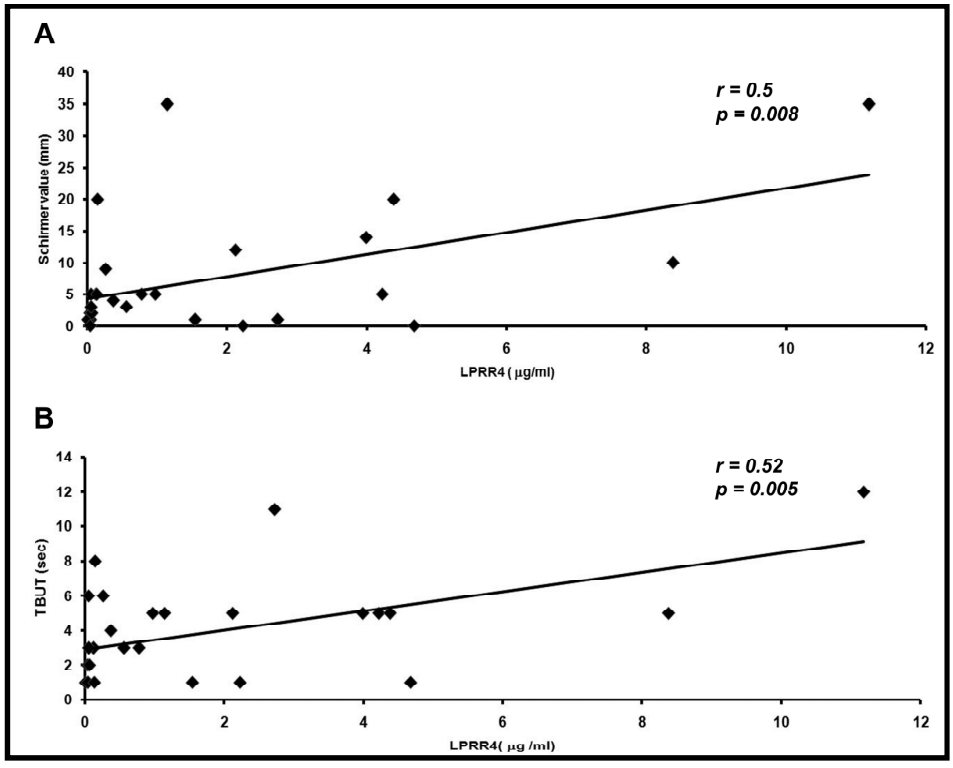


Figure 8.3: LPRR4 levels with DES grade

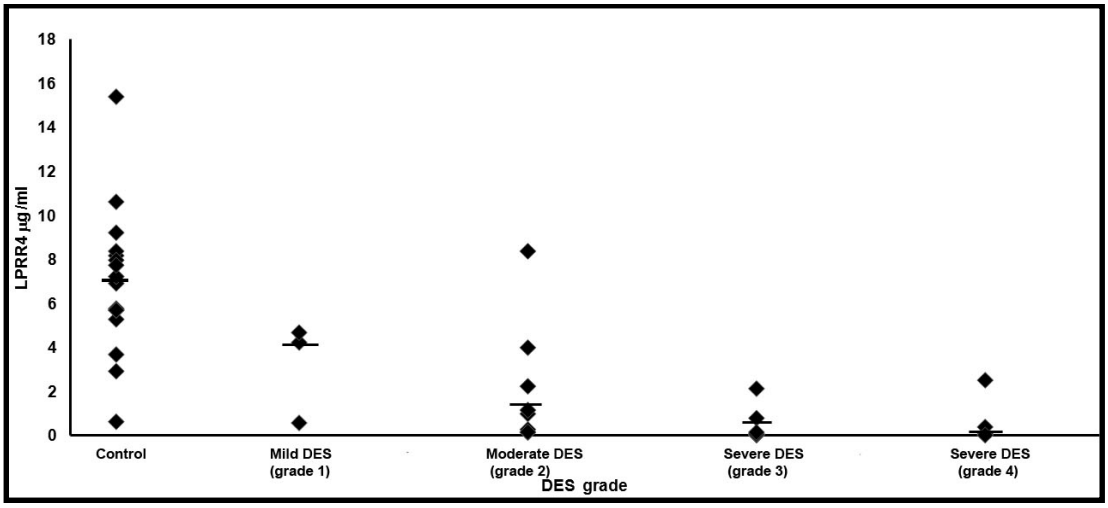


Fig 8.3: LPRR4 levels were reduced as the severity of DES increases. The median showed reduced in severe DES of grade 3 and 4 compared to mild and moderate.

Tear Meniscus Height (TMH) reduced all the cases of DES used for validation. Corneal staining showed filaments, SPKs in most of the cases as shown in the table 9. Other parameters also showed changes (table 8.8).

8.3.4. Immuno histochemistry (IHC) of LPRR4 in lacrimal gland tissue: Lacrimal tissue was processed and IHC of LPRR4 was done. But, IHC showed absence of LPRR4 protein in lacrimal gland tissue as seen in figure 8.4.

8.3.5. mRNA expression study: mRNA expression of LPRR4 was observed in lacrimal gland specifically while the corneal epithelial tissue did not show the expression which indicates the tissue specificity of this protein (Figure 8.5)

Figure 8.4: IHC of LPRR4 in Lacrimal gland

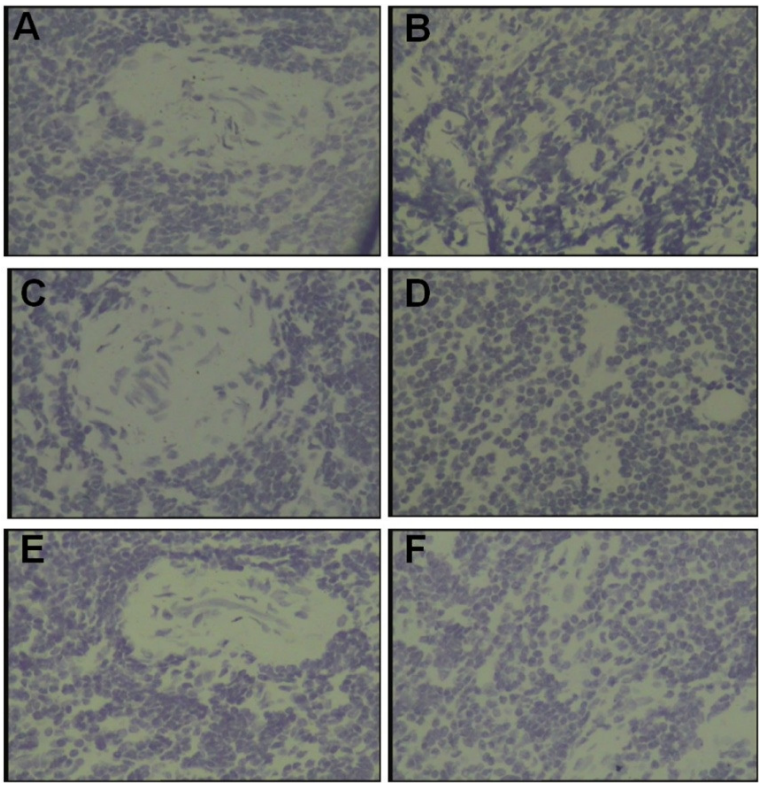


Figure 8.4: IHC of LPRR4 showed absence of LPRR 4 in lacrimal gland tissue. A,B,C and D: LPRR4. E and F: negative control

Figure 8.5: mRNA expression of LPRR4 in ocular tissue.

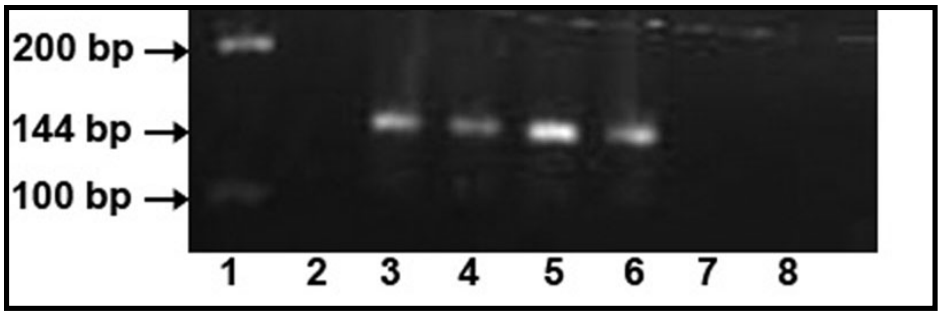


Figure 8.5: lane 1 ladder base pairs 100 and 200 bp. Lane 2: negative control. Lane 3-6: LPRR4 from LG at 144 bp. Lane 7 and 8 – LPRR4 of corneal epithelium (no LPRR4 expression)

8.3.6. Post translational staining: The theoretical molecular weight of LPRR4 is 15 kDa. But during 2D experiments with Tear protein we observed this protein at m.wt of 30 kDa. Therefore possible PTM changes occurred, could be glycosylation. To look for glycosylation PAS staining was done, after the tear proteins were separated on 2D gel. The staining of LPRR4 region indicates the protein glycosylation as seen in pink colour (Figure 8.6) but the type of glycosylation has to be looked into for understanding the further functional aspects of LPRR4.

Figure 8.6: Periodic acid Schiffs staining of tear protein LPRR4.

Figure 8.6: 2DE was done with the tear protein using 3-10 IPG in 1st dimension and then separated on 13% SDS-PAGE. The LPRR4 region showed pink coloured spots indicating glycosylation

8.3.7. 3D Structure Prediction of PRR4: As per Uniprot database entry, PRR4 Protein (Acc.No. **Q16378**) is a protein of molecular weight 15.12 with 134 amino acids of length containing the signal peptide region of 1- 16 amino acid (AA) at the N-terminal followed by the main chain spanning from 17 -134 (AA) residues. Hence, we attempted to predict the 3 dimensional model of the main chain excluding the signal peptide region (Figure 4A). BLASTP search was performed for PRR4 protein against PDB database to identify suitable templates for homology modeling. Since there were no suitable templates available, we implemented an alternative fold recognition approach using I-TASSER software which predicted five possible models for PRR4 main chain. Among the predicted models, the one with significant C- Score (-3.47) was chosen as the best and was further subjected to Geometry optimization and Molecular Dynamics studies.

8.3.8. Model refinement and Structure Validation: The best model was checked for stereo chemical property using PROCHECK Server, in which the Ramachandran plot showed 3.6 % of residues in disallowed regions. Hence, the model was subjected to modrefine refinement which fixes the protein in terms of hydrogen bonds, backbone topology, side-chain positioning, physical quality of local structures and thereby leading to the near native state. Further, the Modrefiner refined model of PRR4 was subjected to geometry optimization using GROMACS package implementing GROMOS96 43al force field through steepest descent method. The potential energy of the protein was initially high (6.50949 Kcal/mol) which got significantly reduced (-1.1674967 Kcal/mol) during the course of optimization. Further, molecular dynamics simulation was performed to analyze the stability and behaviour of the modelled protein under solvated conditions. RMSD trajectory values were calculated to understand the overall structural stability throughout the simulation for 2 nanoseconds. Initially, at 1 nanoseconds the overall RMSD of c-alpha was recorded as 3Å. Subsequently, in the timeframe of 1nano seconds to 2 nano seconds, 4A of RMSD was found to be maintained in the trajectory. RMSF Trajectory infers the flexibility of the protein in a residues-wise manner, wherein, proline rich region (42-95) was found to be more flexible (0.2-0.6nm), when compared to other regions (0.1-0.25nm) (Figure 8.7A)

Q mean score is a composite scoring function, which derives both global (i.e. for the entire structure) and local (i.e. per residue) error estimates on the basis of one single model. Totally, 6 scoring function terms contribute to QMEAN score: torsion, pair wise, solvation, all atoms, secondary structure, and solvent accessibility. The simulated model showed significant refinement with a significant Qmean score of 0.04 bearing no residues in disallowed regions of Ramachandran plot. This indicates that the final 3D model of PRR4 is plausible as it adheres to the optimal geometry. Further, to better understand the active site cavity of PRR4, CastP pocket prediction was performed to map the probable residues (ASP27, PHE30, THR31, VAL35, GLU36, ASP37, SER39, GLN40, ARG41, PRO42, ASP43, GLN44, PRO46, PRO49, GLU52, GLY53, LYS54, PRO56, ARG57, PRO59, GLY60, ASP61, ASN64, GLN65, ASP66, ASP67, GLY68, PRO74, LYS75, PRO76, HIS79, ARG96,

ARG97, GLY98, ARG99, SER103, LEU104, PRO105, ARG106, PRO108, SER109, VAL110,LEU112,GLN113,GLU114) contributing to molecular interactions. The green colored pocket shown in Figure 8.7 B was predicted as the plausible best cavity with an area size of 998.8 and a volume of 1559.4.

Figure 8.7: Predicted 3D structure of LPRR4

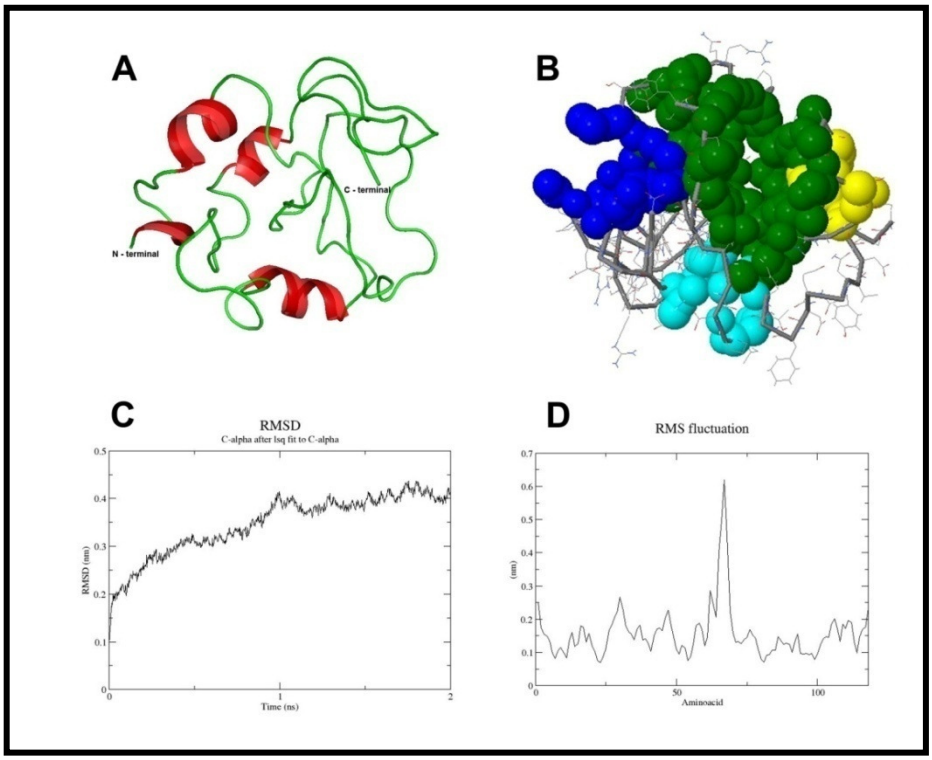


Figure 8.7: **A**: Optimized 3D structure of LPRR4. Red color indicate Helix,Green color indicate Loop. **B**. Active Sites of PRR4 as predicted by CASTP Server. Green colour represents the top ranking potential pocket for binding with other proteins C: C-alpha Backbone residue-wise root mean square deviation (RMSD) of PPR4. D: C-alpha Backbone residue-wise root mean square fluctuations (RMSF) of PRR4.

8.3.9. Speculated Role of interacting partners of LPRR4

Interacting partners of LPRR4 were predicted using STRING server (Figure 8.8). This showed Mucin7 to have direct interaction with LPRR4 as per experimental studies. Mucin7 in turn interacts with statherin and Histatin1 as shown experimentally. The other interacting partners shown in STRING namely, extracellular glycoprotein lacritin precursor, Presqualene diphosphate phosphatase, Proline-rich nuclear receptor coactivator 1, proline-rich protein Hae III subfamily were predicted based on text mining (figure 8.8).

Figure 8.8: Interacting partners of LPRR4 using STRING analysis

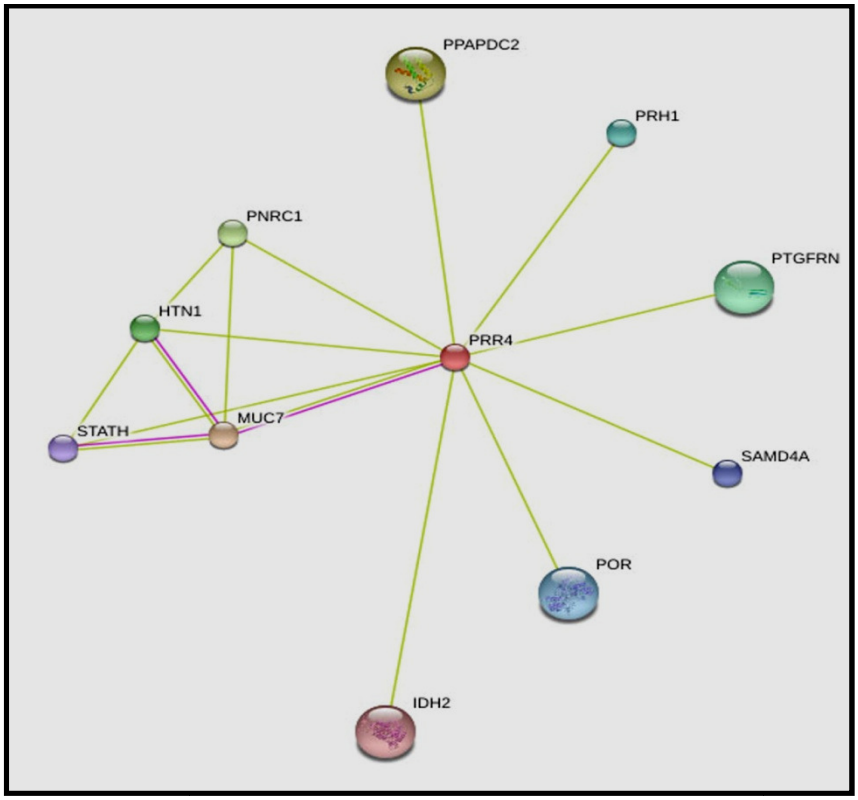


Figure 8.9: Predicted functional partners of PRR4 from STRING. Megenta color represents experimentally proven interaction. Green indicates text mining predicted interactions with LPRR4.

LPRR4 interacts directly with MUC7, while Histatin 1 and statherin are interacting molecules of MUC7 as proven experimentally (Bruno, Li et al. 2005). MUC 7 and Histatin1 precursor have not been reported in the tear**.** However**,** human lacrimal gland synthesizes a spectrum of mucins and the mRNA expression for MUC1, MUC4, MUC5AC, MUC5B, MUC6, and MUC7 is reported in the lacrimal gland. Muc 7 exhibits antimicrobial activity, apart from HT1. The two other interacting protein of LPRR4 namely statherin and PRH1 exhibit calcium binding property and are probably involved in the tear dynamics of the same. Calcium binding proteins namely the S100 proteins are reportedly altered in DES.

Three of the interacting proteins exhibit a regulatory role at transcriptional or translational level. While PNRC1 functions as a transcriptional activator for the

expression of Proline-rich protein, PPAPDC2 down regulates the expression of phosphatidic acid phosphatase that converts Presqualene diphosphate (PSDP) to presqualene monophosphate (PSMP) thereby regulating the levels of PSDP/PSMP which triggers the proinflammatory processes in the cell membrane. The cytochrome P450 namely CYP3A6-expression in rabbit lacrimal gland is reported as drug metabolizing enzyme (Attar, Ling et al. 2005). Normal lacrimal gland function is known to be modulated by androgens. Modulation of CYP3A6-mediated metabolism of testosterone as well as the drug metabolism in the lacrimal gland is reported by this study. Improving the androgen levels will be beneficial in dry eye. Sterile alpha motifs (SAMs) in proteins such as SAMD4A function as a post transcriptional regulator by binding to an RNA sequence motif known as the Smaug recognition element (SRE) and repress it. SAM domains have been implicated in mediating protein-protein interaction. The string analysis also reveals yet another LPRR4 interacting protein namely, prostaglandin F2 receptor (PTGFRN) that activates CXCL8, a chemokine that can result in epithelial cell proliferation. CXCL8 levels are reportedly altered in DES.

8.4. SUMMARY

1. LPRR4 is significantly reduced in all types of DES cases, validated the same by ELISA.

   ELISA also showed the down regulation of LPRR4 in tear samples.

2. DES severity increase showed increased down regulation of LPRR4.
3. mRNA expression showed the ocular tissue specificity of LPRR4 in lacrimal gland which is not present in corneal epithelial tissue.
4. PAS staining of 2D tear profiled gel showed the positive staing of LPRR4 region indicated the glycated protein of LPRR4.
5. Using *in silico* work possible 3D structure, its active site, interacting partners were studied which helps in further functional studies of LPRR4.

CHAPTER 9: VALIDATION OF USP 6 N-TERMINAL LIKE PROTEIN (USP6NL)/ RN-TRE IN TEAR FLUID OF DRY EYE SYNDROME

9.1. INTRODUCTION

DES could be due to various causative factors and one such is Sjogren syndrome (SS). SS is a systemic autoimmune disease, targets primarily the lacrimal and salivary glands, resulting in dry eye and dry mouth disease (Giusti, Baldini et al. 2007). Serum, saliva and synovial fluid protein changes were observed using proteomic studies in primary SS (Giusti, Baldini et al. 2007). Since dry eye is one of the major problem in primary SS, tear protein changes in DES associated with primary SS can give clues to understand the disease mechanism. Tear fluid proteomics from this study showed differentially expressed proteins (chapter 5,6 and 7) in DES associated with non Sjogrens, primary Sjogren's and secondary to Rheumatoid arthritis (RA). As shown in chapter 7, one of the tear proteins that showed up regulation from the proteomic study in DES with primary SS is ubiquitin specific protease 6 N-terminal like protein (USP6 NL) or RN-tre.

This part of the work deals with the validation of USP6 NL in tears samples and then further *insilico* approach to understand the protein role in disease mechanism.

9.1.1. USP6 NL acts as a Rab5-GAP and concurrently as a Rab5 effector

USP6 NL/RN-tre (RN-tre, Synonyms name: USP6NL TRE2NL, KIAA0019) is a Rab5 GTPase-activating protein, whose activity is regulated by the epidermal growth factor receptor (EGFR). RN-tre inhibits internalization of the EGFR. The key regulator for maintaining a healthy cornea and promoting re growth of a wounded cornea is the EGFR.

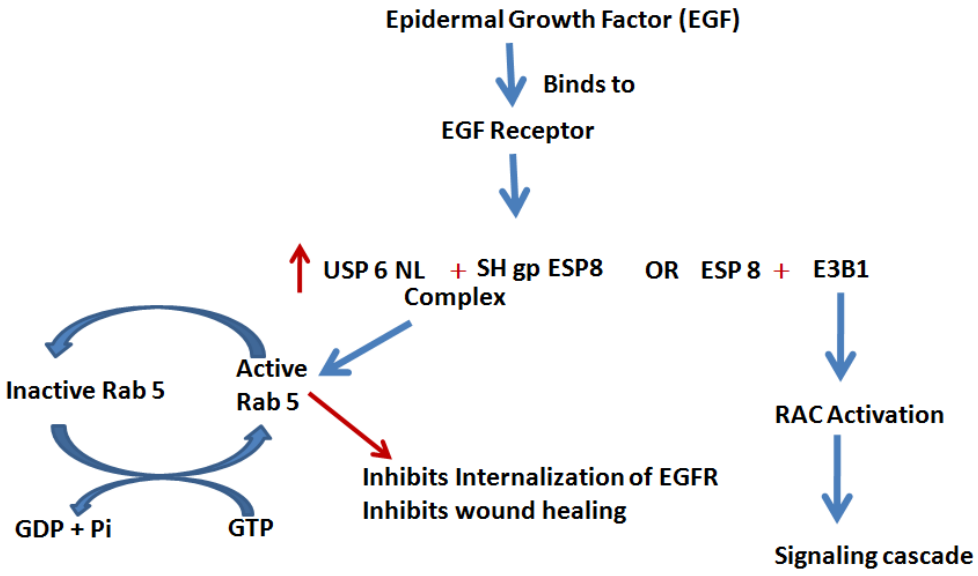
Thus USP6 NL was originally identified as a binding partner for the EGFR substrate Esp8. Eps8 is a substrate for the kinase activity of EGFR. Esp8 interacts with both E3b1 and RN-tre to form a complex, Esp8rab. USP6 NL is an N –terminal –like

protein act as GTPase activating Factors (GAPs) stimulate GTP hydrolysis and induce Rab Proteins (RAB5A). Inactivation and is widely expressed in human tissue (Fig 9.1) (Lanzetti, Margaria et al. 2007). is widely expressed in human tissue (Lanzetti, Margaria et al. 2007).

Eps8 is a substrate for the kinase activity of EGFR. Eps8 interacts with both E3b1 and RN-tre through its SH3 domain (Esp8rab) (Scita, Nordstrom et al. 1999) and helps in further signalling cascade and trafficking respectively. RN-tre is GTPase Activating Factor (GAP) and stimulates GTP hydrolysis inducing Rab5 inactivation (Figure 9.1). The N-terminal region of RN-tre contains a Rab family GAP homology domain. When EGF binds to its receptor EGFR, acts on its substrate ESP8. Esp8 on binding with RN-tre activates Rab5, which inhibits EGFR internalization. If Esp8 binds with E3bl protein, the complex activates RAC protein further signalling cascade proceeds (Figure 9.1).

RN-tre functions as membrane trafficking protein, acting through Rab5, a key GTPase that controls early endosome dynamics and actin cytoskeleton remodelling. Due to its GAP activity, RN-tre is able to inhibit Rab5-dependent functions in vivo, including internalization of both constitutive and ligand dependent growth factor receptors.

Figure 9.1: Role and mechanism of USP 6 NL / RN-tre



Primary Sjogren's syndrome is an autoimmune disease involves inflammation, infiltration of immune cells in exocrine glands such as lacrimal and salivary glands (Nikolov and Illei 2009). This increased lymphocyte infiltration leads to decreased secretary function. Primary Sjogren's syndrome showed decreased EGF, EGFR levels and also decreased Rab30, with an increase in Rab5 (Qian, Xie et al. 2004). This increased rab5 inhibits EGFR internalization and alters membrane trafficking. These factors reduce the ability to secrete proteins leads to dry eye and dry mouth (Damato, Allan et al. 1984). But the role of USP6NL, though it plays major role to inhibit protein internalization not studied so far.

The current study showed the presence of USP 6 NL from the tryptic digestion of total tear protein which is specific protein to pSS. Not much studies are done on the exact mechanism of USP 6 NL / Rn-tre in protein internalization, wound healing, cell proliferation.

Therefore Validation of the USP6 NL protein was done in DES by estimating the levels in tears and correlating clinically. *In silico* analysis also done further to understand the mechanism of the protein in DES associated with primary Sjogrens syndrome.

9.2. MATERIALS AND METHODS

9.2.1 Materials

1. 8M Urea Buffer pH: 7.6
2. Phosphate buffered saline (PBS) pH; 7.2.
3. Schirmer strips
4. ELISA Kit for USP 6 NL from USCN company

9.2.2. Method

9.2.3. Collection of tear fluid from controls and Dry eye patients was done as mentioned in Chapter 2 section 3.2.2.

For the quantification of USP6NL by ELISA, tear samples were prospectively collected from 34 dry eye cases (mean age: 40 ±15 y) associated with NS (mean age: 40 ± 10 y, n =5), PSS (mean age: 39 ± 10 y, n= 17) and RA (mean age:45 ±9 y, n =12) with age matched 27 controls (mean age: 44±16 y)

9.2.4. Determination of USP 6NL using ELISA kit

Principle: The microtiter well plate coated with an antibody specific to USP 6 NL. Standards and tear samples were then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody specific for USP 6 NL. Avidin conjugated to HRP will be added to this, the colour produced was read at 450 nm upon the addition of TMB substrate. The protocol for USP 6 NL estimation mentioned in chapter 2, section 3.13.

9.2.5. *In silico* analysis for USP6 strucure and Function prediction based on identification of interacting partners

To get the homology models for USP6NL and Eps8 (SH3 domain) were constructed using modeller and I-TESSER was used. To check the reliability of models of USP6 NL and Eps8 and validation purposes molecular dynamics tools used. For the indepth idea of structure and functional aspect energy minimization was done using GROMACS software. The possible interacting partners of USP6 NL were done using STRING analysis.

9.3. Results

Insol tryptic digestion of tear protein from DES associated with primary SS showed the presence of USP 6 NL compared to controls and other DES conditions as seen in chapter 6.

Estimation of USP 6NL was done by ELISA method. Figure 2 shows the standard graph. Table 1 shows the levels of tear USP6NL in various DES conditions showed significant increase ($p = 0.009$) of USP6NL levels 0.1225 ± 0.02 ng/ml in DES with primary Sjogrens syndrome compared to controls (0.04 ± 0.01) as wells DES

associated with RA (0.07 ± 0.01), Non SS (0.07 ± 0.03). Figure 9.2 shows the distribution graph of USP 6 NL in DES conditions showed increased levels of the same in DES with primary SS (median 0.108) compared to other DES (median:0.06) and control (median: 0.038) (table 9.1)

9.3.1. Standardization of USP6NL by ELISA

Figure 9.2: Standard graph for USP6 NL

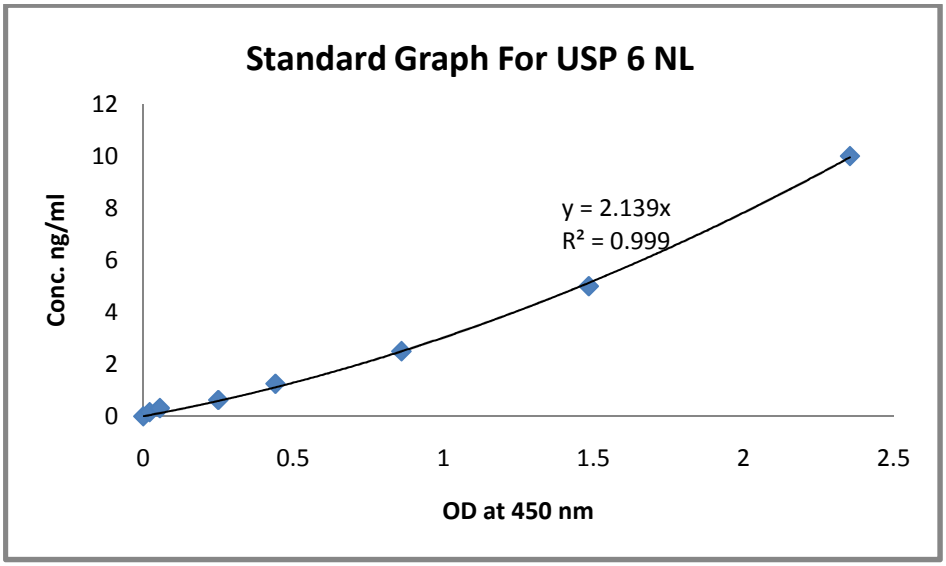
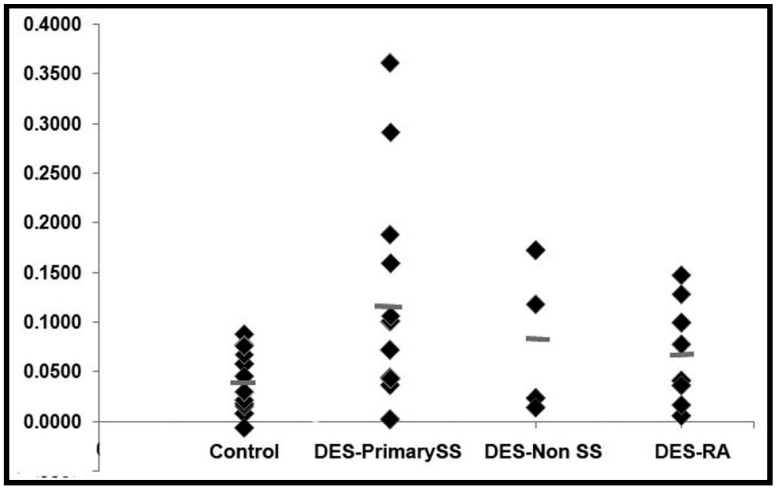


Table 9.1: Tear fluid USP 6 NL levels in DES -Non SS, DES-pSS, and DES-RA.

Groups		Mean ± SEM	*p* value
Control (n = 27)	USP6 NL (ng/ml)	0.04 ± 0.01	
	Schirmer (mm)	27.5 ± 2	
DES Non SS (n = 5)	USP6 NL (ng/ml)	0.07 ± 0.03	0.32
	Schirmer (mm)	11 ± 6	0.009
Primary SS (n = 17)	USP6 NL (ng/ml)	0.1225 ± 0.02	0.009 ↑
	Schirmer (mm)	8.5 ± 2.4	< 0.001
DES-RA (n = 12)	USP6 NL (ng/ml)	0.07 ± 0.01	0.12
	Schirmer (mm)	2.5 ± 0.6	< 0.001

Figure 9.3: Distribution graph of USP6 NL in DES

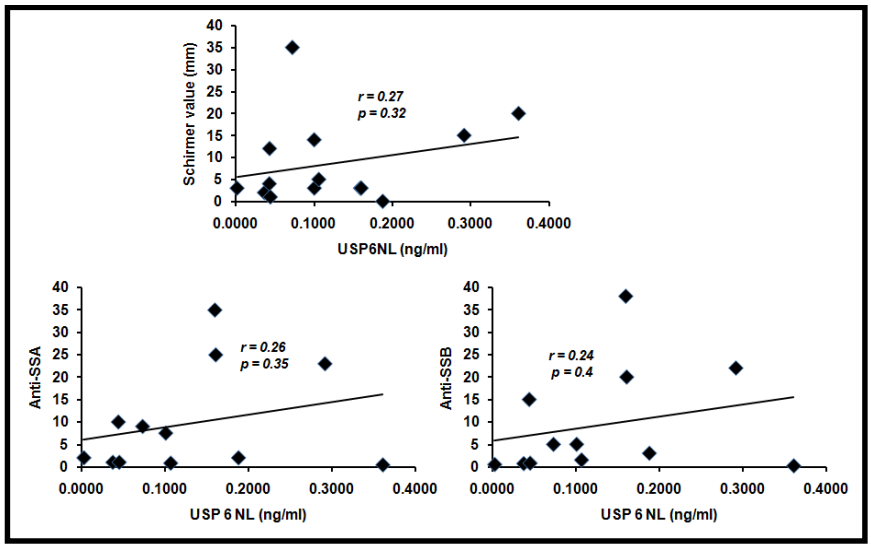


9.3.2. Correlation of USP6 NL with clinical parameters

When correlated with clinical parameters like Schirmer's and TBUT, USP6 NL did not show any significant correlation (*p=0.3, p= 0.9* respectively).

Similarly USP6NL did not show any significant correlation with the serum levels of antibodies to SSA, SSB, DsDNA (p > 0.3) in the DES cases as seen in figure 9.4.

Figure 9.4: Clinical correlation of USP6NL with Schirmer value, Anti SSA and Anti SSB



9.3.3. *In silico* analysis

In order to understand the possible mechanism of USP6NL in ocular pathology, in silico approach was taken to model its structure, functional relationship. Therefore, in this study, the probable the probable interacting proteins of USP6NL,structure of USP6 NL, and interacting proteins like RAB5 protein EPS8 was predicted. The protein-protein interaction of these molecules were looked using docking studies to understand the peptides responsible for the function.

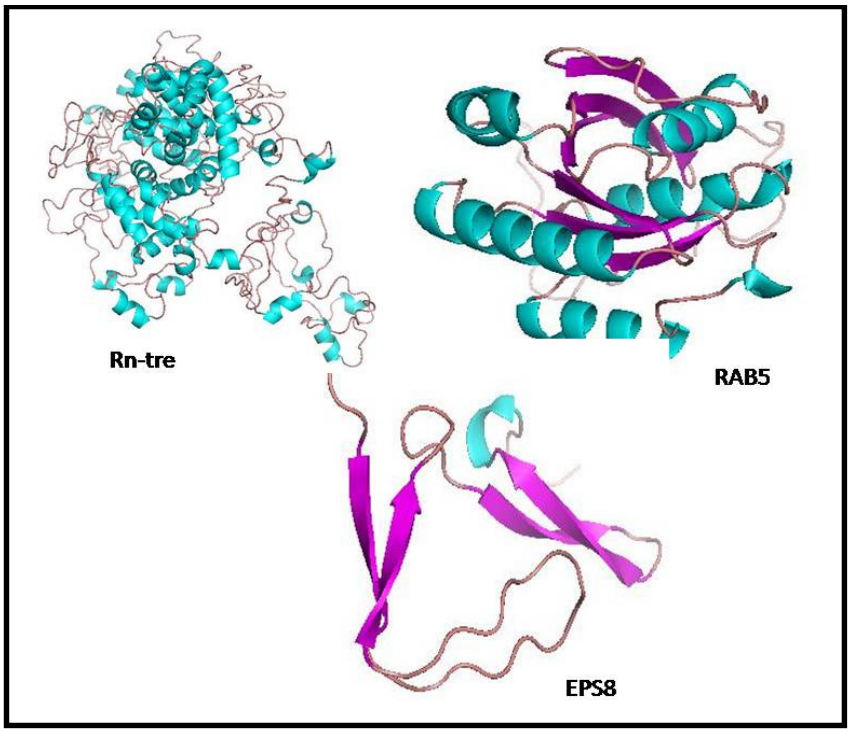
9.3.4. Data sets & Homology modelling

Human protein sequences of Rn-tre and EPs8 were retrieved from uniprokb and the structure of proteins is not available in PDB. BLAST result of Eps8(sh3 domain) from human showed 95% sequence similarity with sh3 domain of eps8 exists as a novel intertwined dimer of Mus musculs (1AoJ) with a resolution of 2.50 suggested that 1A0J as a most suitable template for homology modeling by Modeller9v7 and another protein of RN-tre did not get suitable template.

The best model structure among five from ITASSER which showed best C-Score used for further model-refinement studies (figure 9.5). Three dimensional structures of human RN-tre and Eps8 are not elucidated. The RN-tre protein 3D models structure was predicted based on multiple-threading alignments by I-TASSER server (figure 9.5). This server was generated 5 models for RN-tre with c-scores (-2.81,-3.23,-3.20,-3.32and -3.33) with respectively model 1-5, the lowest c-value score model was selected further studies

The selected models have been subjected to loop refining using “loop.py” script by MODELER9v7.The refined models were subjected to energy minimization using steepest descent carried out in vacuum with OPLS Force field set through GROMACS Soft ware. The stereo chemical properties of the minimized models were checked by Ramchandran Plot using PROCHECK. Further model Proteins validation score were inspected using ProQ predictor. For Eps8 out of 10 structures generated by MODELLER 9v7, the one with the lowest dope score (-1340.67200) value of the MODELLER9v7 objective function was selected as the best model for Eps8 and RAB5 (Figure 9.5).These selected models were refined by loop refinement, minimization and molecular dynamics.

Figure 9.5: Predicted structure of USP6NL, EPS8 and RAB5 by *in silico* approach



9.3.5. MOLECULAR DYNAMICS: After validation, the models were subjected to molecular dynamics simulation to check stability of proteins in water at 300 K temperature and for 1000 pico second using Gromos43a1 force field of GROMACS. The computing facility utilised was High performance cluster for Biological Applications which is based on Intel coreas 7, Dell Precision T-1500 Multicore Clustered Workstation 1TER of memory.

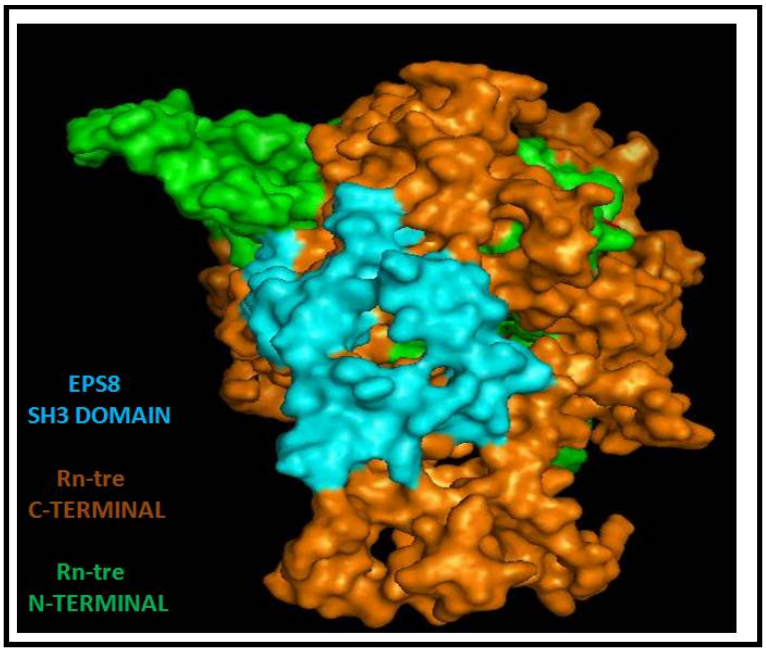
Initially generated models atomic clashes (bumps) removed by rotating side chain torsion angles. After these models has been checked by Ramachandran plot through SAVS. In modelled structures, 77.4%, 94% residues are in most favoured regions and 3.4%, 0.1% residues are in disallowed regions for RN-tre and Eps8 with respectively. Further it was subjected to loop refinement energy minimizing techniques to optimize stereochemistry, remove bumps and stearic clash among non bonded interaction using GROMACS (OPLS force field) using steepest descent algorithms in vacuum condition by GROMACS package. The potential energy of RN-tre and Eps8 were -1.130939and-8.55136 kcal/mol respectively. The overall

stereo chemical properties of RN-tre and Eps8 residues in disallowed regions had been shifted to the generously allowed region and then 79.5%, 96.3% residues are in most favoured regions respectively. Further validation with Pro Q Prediction, results showed that both the structures are very good models based on LG scores, refinement and optimization of the predicted modelled structures shown in 3D structures of Fig 7.

9.3.6. Protein-protein docking: HAD DOCK web server for docking the interacting proteins to predict the protein complex. This server was given input files 3D coordinates of RN-tre and Eps8 in PDB format. The active site region of (725-729) is experimentally proved to be most being favoured for Eps8 (SH3) domain binding in Rn-tre were given one of the additional input to the server.

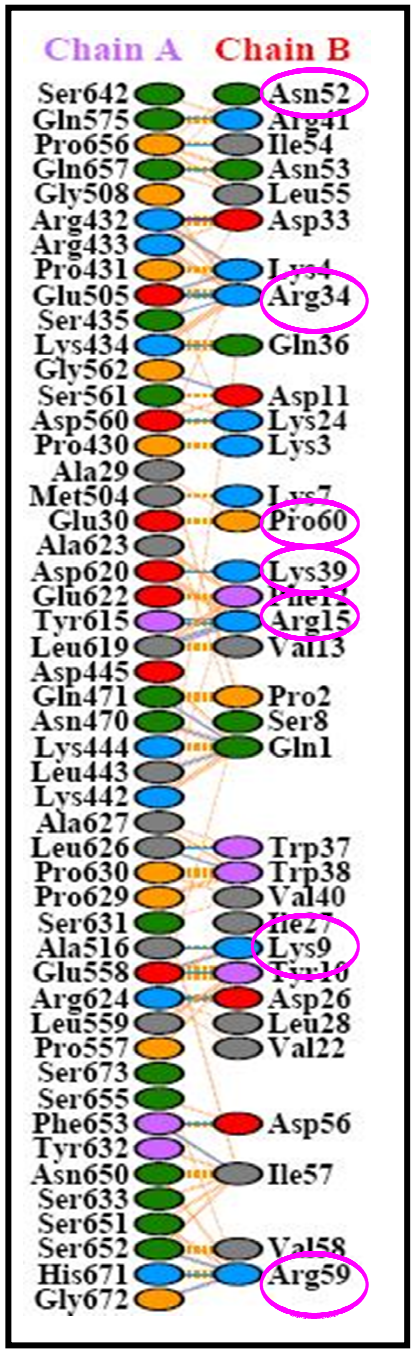
Protein-protein interactions play an important role in a wide range of physiological and functional processes. Here RN-tre C terminal region form a complex with EPS8 (SH3 domain) inhibits internalization of EGFR process.

Figure 9.6: The HADDOCK model for the interaction of Rn-tre C&N-terminal region with EPS8



Docking studies showed RN-tre interaction with Eps8 binding energy of -34272.8kcal/mol, 11 hydrogen bonds and 118 non bond contacts. Docking region of EPS8 also correlated with already wet lab reported regions. It showed significant residues of PRO60, ARG59, LYS39, ARG15, LYS9, ARG34, LEU31, ARG34, ASN52, LYS9 and TYR5 in Eps8 as seen in figure 9.7.

Figure 9.7: The interacting amino acid residues of USP6NL with EPS8.

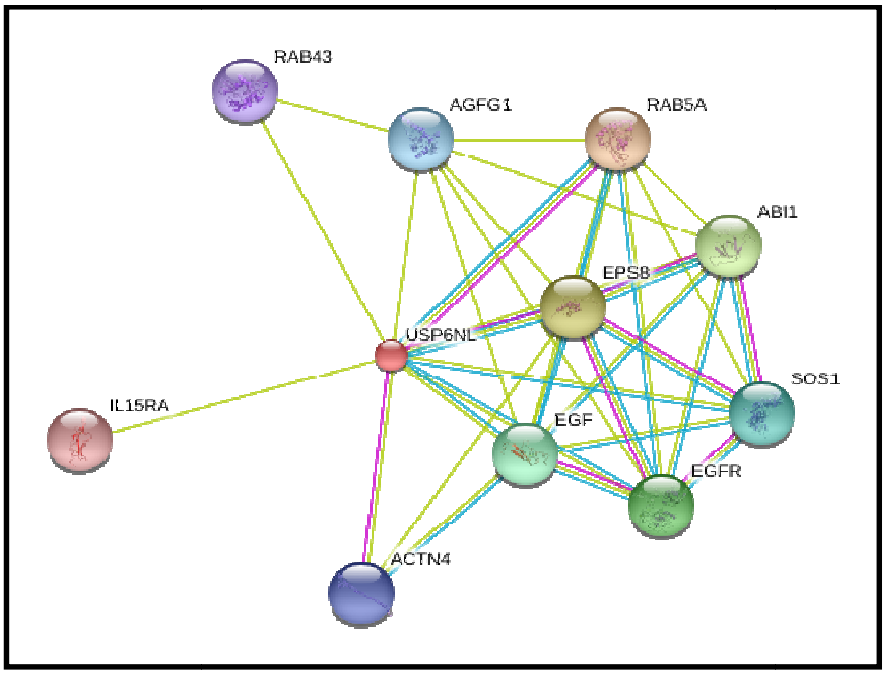


Based on the above data, the possible USP6NL/Rn-tre structure and its interacting molecule ESP8 was predicted, docking studies of these molecules showed the possible residues involved in the process.

9.3.7. STRING analysis of USP6NL protein

STRING analysis of USP6NL showed the proteins that could interact with USP6NL as seen in figure 9.8 and the functions of the interacting proteins are described in table 9.2.

Table 9.8: Interacting partners of USP6 NL using STRING analysis



9.4.8. Function and Relevance of the USP6NL binding partners in disease process of DES-pSS

EGFR one of the interacting protein indirectly regulates MUC 1 expression. Various reports on tear MUC 1 revealed that, MUC 1 in conjunctival cells reduced in DES syndrome (Mantelli, Moretti et al. 2007).

The interacting protein epidermal growth factor showed reduction in DES has been associated with sjogrons syndrome (Pflugfelder, Jones et al. 1999). Based on the above interaction, the predicted mechanism of USP6 NL is lowered EGF binding, which in turn inhibits EGFR and then further inhibits wound healing.

Table 9.2: Functions of interacting parterners of USP6NL using STRING analysis

S.No.	Gene symbol	Name	Function
1	EPS8	epidermal growth factor receptor pathway substrate 8	binding to EGF receptor enhances EGF-dependent mitogenic signals
2	RAB5A	member RAS oncogene family	Required for the fusion of plasma membranes and early endosomes
3	ACTN4	actinin alpha-4	F-actin cross-linking protein. anchor actin to a variety of intracellular structures, vesicular trafficking
4	EGFR	epidermal growth factor receptor	Controls cell growth and differentiation. Phosphorylates MUC1 in breast cancer cells and increases the interaction of MUC1 with SRC and CTNNB1/beta-catenin
5	SOS1	son of sevenless homolog 1 (Drosophila);	Promotes the exchange of Ras-bound GDP by GTP
6	EGF	epidermal growth factor	stimulates the growth of various epidermal and epithelial tissues, Magnesium reabsorption

This protein is needed for exocytosis of secretory proteins in lacrimal gland (Wu, Jerdeva et al. 2006). EPS 8, SOS1 and RAB 5a are not reported in tear fluid so far. Wet lab experiments to validate these proteins in DES with primary SS are to be done for further understanding of the pathology. From the study we hypothesise that in DES the increased levels of USP6 NL may bind to more of EPS8 protein, which can activates EGFR. Alpha actin, is a trafficking protein showed changes in lacrimal acinar cells of SS mice model. Actinin levels may also change which may affect protein internalization.

9.4. SUMMARY

1. USP 6 NL/Rn-tre protein showed significant increase in DES with primary Sjogrens syndrome.
2. It did not show any positive or negative correlation with the clinical parameters like Schirmers value, TBUT also showed no correlation with anti-SSA, anti-SSB molecules.
3. From *in silico* analysis it can be inferred that USP-6 NL may involve in cell proliferation by interacting with molecules ESP8, EGFR, EGF USP-6 NL may involve in cell proliferation by interacting with molecules like ESP8, EGFR, EGF from *in silico* analysis.
4. Structure was predicted to look for its binding partners based on its modelled structure

CHAPTER 10: DETERMINATION OF TEAR FLUID CHEMOKINES IN DRY EYE SYNDROME

10.1. INTRODUCTION

Cytokines are signaling peptides that consist of water-soluble proteins and glycoproteins with a mass of 8 to 30 kDa (Kymionis, Bouzoukis et al. 2008). Cytokines are released by many different types of cells and are important in innate and adaptive immune response, which mediates and regulates immunity, inflammation, and haematopoiesis. Cytokine is a general name, which includes lymphokine produced from lymphocytes, monokine produced from monocytes, chemokine having chemotactic activities (Murphy, Baggiolini et al. 2000), and interleukin produced by one leukocyte and acting on other leukocytes. Cytokines acts in three different ways as autocrine (act on the cells that secrete them), paracrine (acting on nearby cells), and endocrine (acts on distant cells). They are produced *de novo* in response to an immune stimulus (Leonardi, Borghesan et al. 1998). They act over short distances, short time spans and at very low concentration. They play an important role in many diseases (Tishler, Yaron et al. 1998; Pflugfelder, Jones et al. 1999; Pavkova Goldbergova, Pavek et al. 2012; Wieser, Moschen et al. 2013). Cytokines bind to specific cell-surface receptors producing intracellular signalling cascades that modulate sets of gene expression, transcription factors, and other cytokines and cytokine receptors (Ozaki and Leonard 2002). Cytokines are made by many cell populations, but the predominant producers are helper T cells (Th) and macrophages (Zlotnik and Yoshie 2000).

Several cytokines detected in the normal eye (Nakamura, Sotozono et al. 1998; Sack, Conradi et al. 2005). Dry eye (DES) develops from reduced aqueous tear secretion or an increased tear evaporation. DES causes ocular irritation, damage to the ocular surface epithelium (Unlu, Guney et al. 2012). Inflammation of ocular surface observed in dry eye. Increased levels of inflammatory mediators including proinflammatory cytokines and chemokines detected in the tear fluid and conjunctival epithelia of patients with keratoconjunctivitis sicca (KCS) or Dry eye syndrome (Brignole, Pisella et al. 2000). Normal tear fluid contains cytokines such

as IL-1α,β, beta, IL-2, 6 and 8, TGF α and β 1, 2, TNF α, chemokines, interferon γ, growth factors such as, EGF, VEGF, PDGF and HGF. Table 10.1 gives the source of cytokines in eye and its activity. (Nakamura, Sotozono et al. 1998).

Table 10.1: Source cytokines and its activity in Eye

Cytokine	Source	Activity
IL-1	Monocytes,macrophages,corneal epithelium,conjunctival epithelium	IL-2, IL-6, IL-8, TNF production, proliferation
IL-2	Activated T cells	Activation and proliferation
IL-3	T cells, NK cells, keratocytes	Growth and proliferation of hematopoitic cell
IL-4	T cells, Mast cells	Induction of Th2 cells
IL-6	T cells, B cells, Mast cells, Monocytes, macrophages	Growth, antibody prduction
IL-8	Monocytes, macrophages, keratocytes	Chemotaxis
IL-10	T cells, keratinocytes	Inhibition of cytokine synthesis
TNF α	T cells, B cells, Monocytes, macrophages	T cell activation, IL-1 and IL-6 synthesis

Altered levels of cytokines such as IL-1alpha and beta, IL-8, Il-6, IFN-γ observed in tear and conjunctival epithelium of dry eye syndrome. Chemokines such as RANTES, MCP, MIP, IP-10 showed alteration in saliva and conjunctival epithelium of dry eye condition with Non Sjogrens, primary and secondary to RA condition. Based on the available data on cytokines and chemokines in DES, five chemokines namely IL8, RANTES, MIP, MCP and IP 10 levels were chosen and estimated in tear fluid of DES cases associated with non Sjogren's, primary Sjogren's and secondary to RA. The oxidative stress in terms of ROS was also estimated in the same tear samples since DES also associates with oxidative stress.

10.2. MATERIALS AND METHODS

10.2.1. Material

1. Phosphate buffered saline (PBS) pH; 7.2.
2. Schirmer strips
3. Reagents for chemokine determination by FACS analysis - kit from BD biosciences
4. Dichloro fluorescein diacetate (DCF-DA)

10.2.2. Methods

10.2.3. Collection of tear fluid from controls and Dry eye patients: Reflex tears were collected by using a sterile Schirmer strip. The Schirmer strip placed in the lower cul-de-sac region and allowed to absorb the tear for 5 min in the open eye condition from the inferior prism without any contact with the lower lid, the cornea or conjunctiva. During the process, the person was seated comfortably, head raised and against any direct source of light or flow of air. The tear absorbed on to the strip was removed after 5 min, placed in sterile vial and stored at -80°C until analysis.

Tear sample collection: As a prospective age and sex matched case- control study, 27 controls (mean age: 45 ± 11 y, 13 M, 14 F) and 32 DES (mean age: 42 ± 11 y, 28 F, 14 M) were recruited to look for the cytokine changes. Among the 32 DES cases, 22 were DES with RA, 3 were DES with primary Sjogren's (pSS), 5 were DES with non Sjogren's (NSS) and 2 were MGD without any DES.

10.2.4. Analysis of chemokines using FACS

BD CBA (chemokine binding assay) provides a method of capturing a rapid and sensitive soluble analyte or set of analytes with beads of known size and fluorescence, making it possible to detect analytes using flow cytometry. The detailed principle and protocol given in chapter 2 section 3.13.

10.2.5. Tear fluid ROS determination

Tear fluid ROS was determined using DCF-DA method as discussed in chapter 2 section 3.19.

10.3. RESULTS

10.3.1. Standardization of Chemokines by FACS.

All five chemokines IL-8, RANTES, MIP, MCP and IP 10 standardized initially with known concentration of chemokine ranging from 0.625 to 2500 pg/ml. The standard graph of all five chemokines are as provided in figure 10.1 and the cluster representation of the chemokines by FACS seen in Figure 10.2 A along with its graphical representation. From top the 1st cluster represents IL-8, RANTES, MIP, MCP and the last one IP 10. Figure 10.3 B shows separation of control and DES tear 5 chemokines.

Figure 10.1: Standard graph of Chemokines by FACS

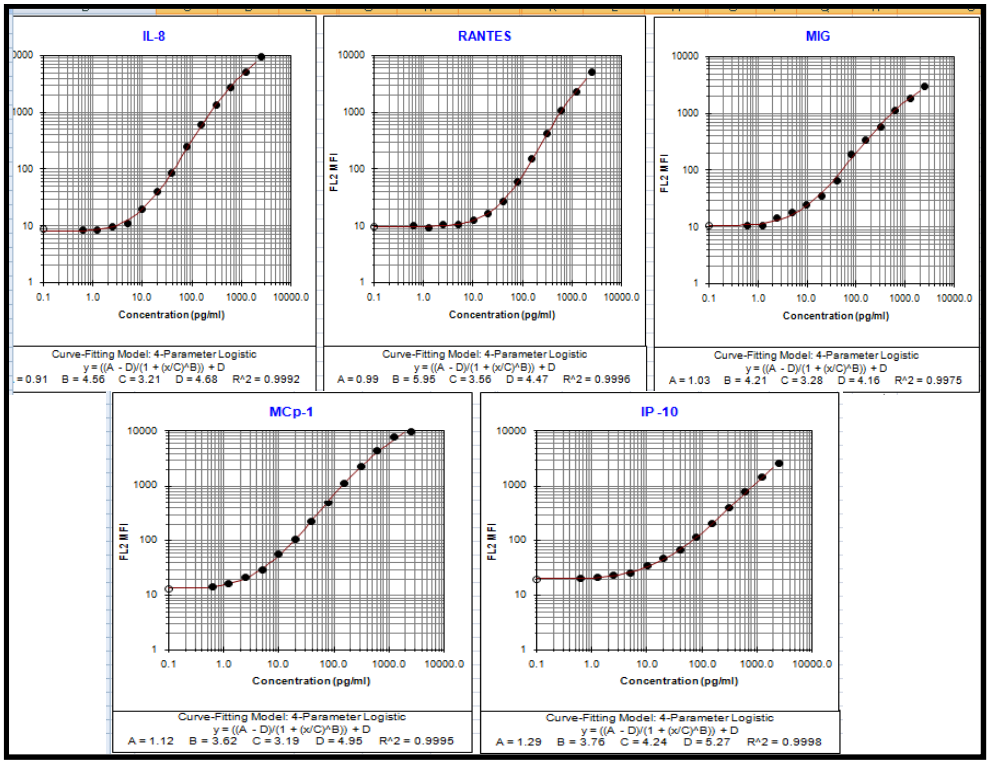


Figure 10.1: Standard graph of 4-parameter curve chemokines IL-8, RANTES, MIP, MCP and IP-10 by FACS. Standards from range from 0.625 – 2500 pg / ml for each chemokines shown in the graph.

Figure10.2A: Representative FACS analysis Standard chemokines

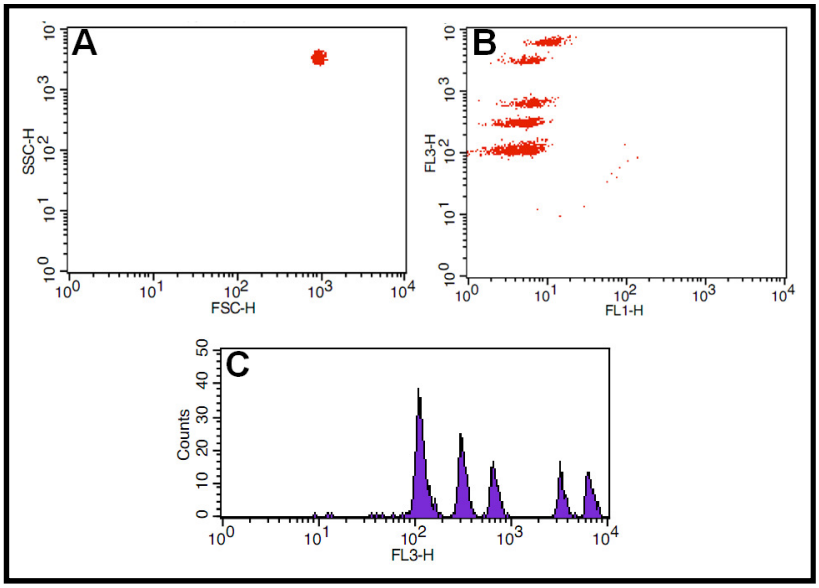


Figure 10.2.A.(**A**).The selection of cluster for chemokines. (**B**).The selected cluster in 2A comprise of all 5 chemokines in tear fluid. 5 clusters represent 5 chemokines IL-8, RANTES, MCP-1, MIP and IP-10. (**C**)**.**The Histogram of 5 chemokines.

Figure 10.2.B. Representative FACS analysis of chemokines in Tear fluid

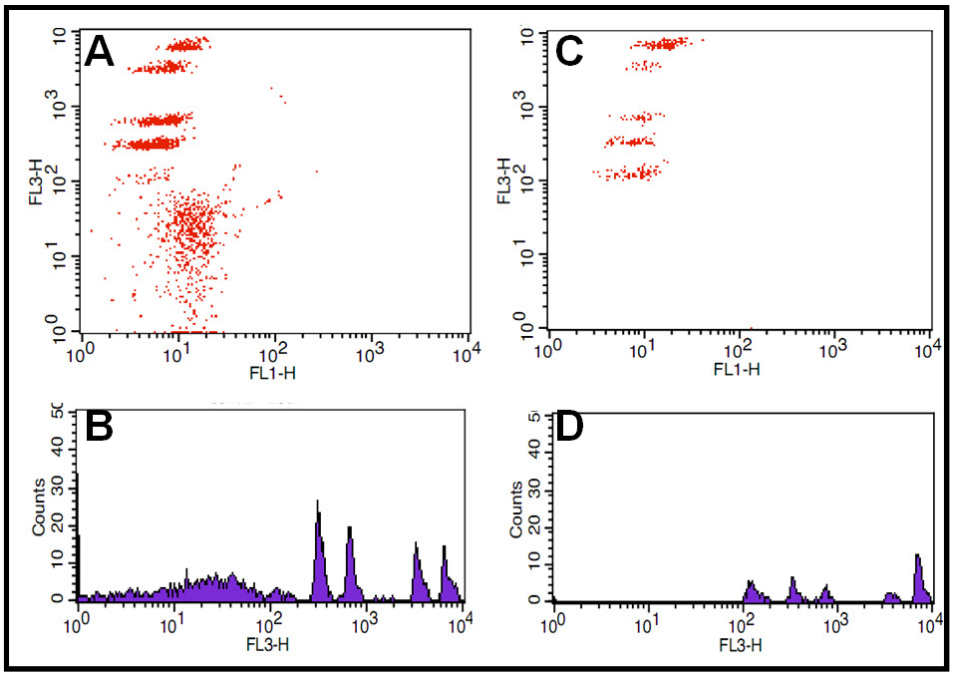


Figure 10.2.B.(**A) cluster represents** 5 chemokines IL-8, RANTES, MCP-1, MIP and IP-10. in control tear **(B)** Histogram of 5 chemokines in control tear. (C) Cluster represents all 5 chemokines in DES tear fluid. (D) Histogram of 5 chemokines of DES tear. DES tear showed change in the chemokine expression compared to control.

9.3.2. Chemokines levels in DES tear fluid

The five chemokines IL-8, RANTES, MIP, MCP and IP 10 were determined by FACS (Table 10.2). The tear IL-8 levels were found to be significantly reduced in all types of DES with a maximum decrease in DES-non Sjogrens cases followed by primary and then secondary to RA. RANTES is decreased maximum in DES-primary Sjogrens. MIP is altered in all types of DES cases. MIP showed increased levels in all types of DES but was not significant. MCP is significantly decreased in RA-DES and not in primary. It is nearly significant in non Sjogrens. IP10 showed no change in all the DES groups.

Table 10.2. Tear fluid chemokines in control *vs* DES groups by FACS

Parameters	IL-8 (pg/ml) (Mean ± SEM)	RANTES (pg/ml) (Mean ± SEM)	MIP (pg/ml) (Mean ± SEM)	MCP (pg/ml) (Mean ± SEM)	IP-10 (pg/ml) (Mean ± SEM)
Control (n = 27)	1173 ± 155	60 ± 9.4	154 ± 89	7.03 ± 1.12	66 ± 11
All DES (n = 32)	175 ± 38$^{***}\downarrow$ *(p < 0.001)*	9 ± 3.3 $^{***}\downarrow$ *(p < 0.001)*	425 ± 136 ↑ *(p = 0.14)*	2.85 ± 0.6$^{***}\downarrow$ *(p = 0.001)*	77 ± 20
DES-RA (n = 22)	250 ± 80$^{***}\downarrow$ *(p < 0.001)*	8.7 ± 4 $^{***}\downarrow$ *(p < 0.001)*	375 ± 157 ↑	2.6 ± 0.6 $^{**}\downarrow$ *(p = 002)*	65 ± 17
DES-pSS (n = 3)	135 ± 44 $^{*}\downarrow$ *(p = 0.036)*	3.4 ± 2.4$^{*}\downarrow$ *(p = 0.05)*	302 ± 134 ↑	4.89 ± 3.4	33 ± 23
DES-NonSS (n= 5)	81 ± 43 $^{**}\downarrow$ *(p = 0.005)*	11 ± 10 $^{*}\downarrow$ *(p = 0.038)*	555 ± 495 ↑	2.20 ± 1.2 *(p = 0.08)*	130 ± 102

p - value comparison of the chemokines between control *vs* the corresponding DES group,

** p - value < 0.05, ** p value < 0.01, *** p value < 0.001*

10.3.3. ROS levels in DES tear fluid

Tear fluid ROS levels of DES increased significantly compared to controls as seen in table 10.4. However DES due to RA showed the maximal increase and DES- non Sjogrens did not show any variation (Table 10.3).

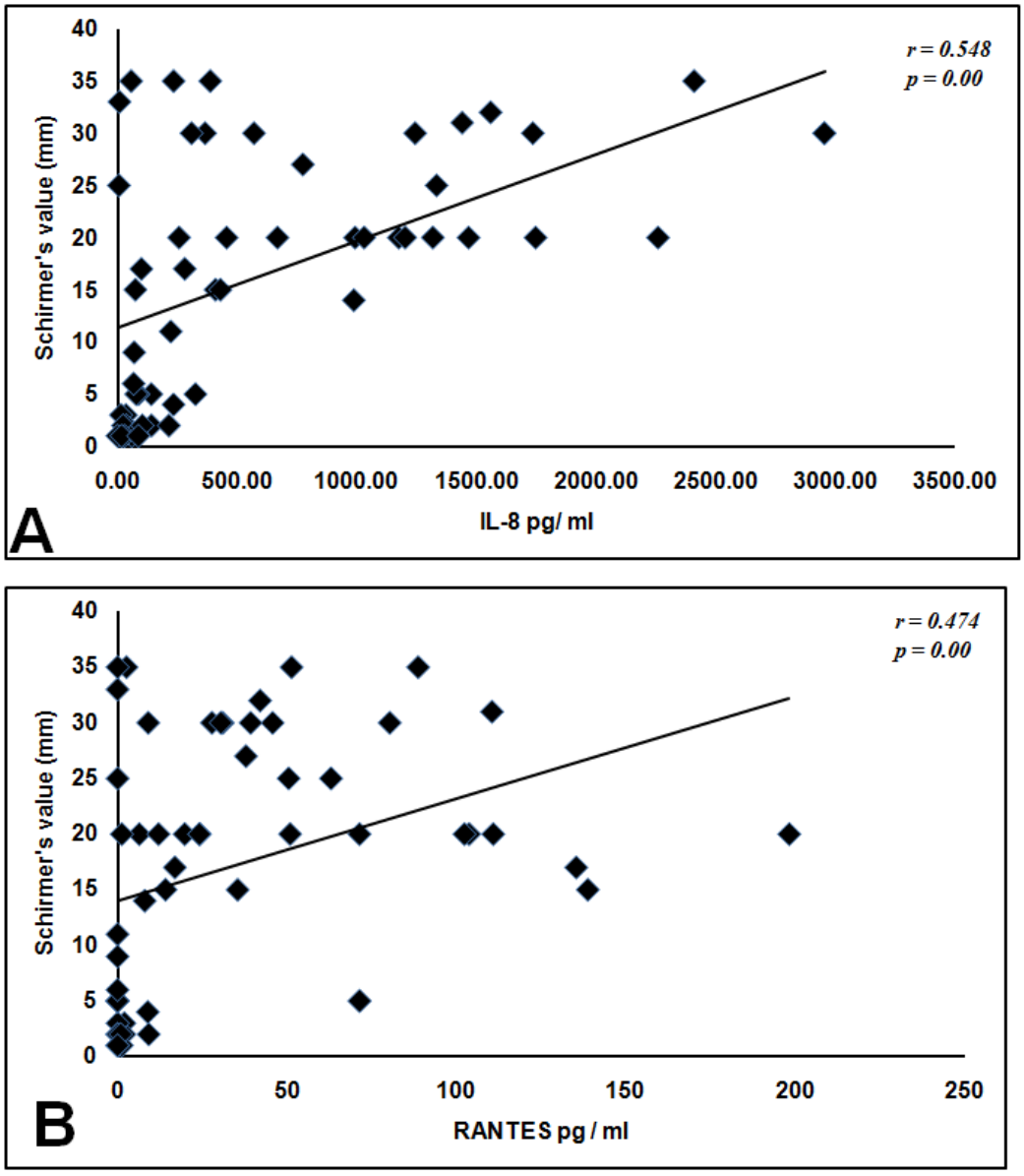
Table 10.3: Tear ROS levels in control *vs* DES groups

Groups	ROS (AFU) (Mean +- SEM)
Control (n = 27)	21 ± 3.6
All DES (n = 32)	108 ± 25* (p = 0.001)
DES-RA (n = 22)	126 ± 33.6* (p = 0.006)
DES-pSS (n = 3)	58.2 ± 36* (p = 0.03)
DES-NonSS (n = 5)	32.7 ± 12.4 NS

10.3.4. Correlation of chemokines with ROS levels of DES tear

Chemokines analyzed did not show any significant correlation with the ROS levels in DES. IL-8, RANTES and MCP-1 did not show significant correlation with the disease severity but IL-8 and RANTES showed significant positive correlation with the clinical parameters Schirmer's value (*r = 0.548, p = 0.00 and r = 0.474,p = 0.000*) respectively as seen in figure 10.3.

Figure 10.3: The correlation graph of chemokines with Schirmer's value



Form the current study, tear anti inflammatory chemokines IL-8, RANTES and MCP-1 reduced significantly in all types of DES, where as pro-inflammatory tear chemokine MIP increased but did not show any significant change in DES. Tear IP-10 is an anti-inflammatory chemokine did not show any significant change. Dry dye is reported to involve oxidative stress, the current study showed significant increase in ROS at tear level.

10.4. SUMMARY

1. Tear fluid chemokines like IL-8, RANTES, MIP, MCP-1 and IP-10 were determined using FACS in all types of DES cases.
2. Chemokines IL-8, RANTES and MCP-1 were reduced significantly in all types of DES, where as IP 10 showed an increasing trend but was not significant.
3. Increased tear ROS was seen in all DES conditions. No significant correlation between the chemokines and ROS levels was observed.
4. Chemokines did not show any correlation with disease severity. But IL-8 and RANTES showed significant positive correlation with DES severity as seen by the clinical parameter Schirmer value.

CHAPTER 11: DISCUSSION

Tear fluid molecular alterations in the form of proteins, lipids, mucins, other metabolites like antioxidant molecules are reported in various ocular pathologies including DES. (Herber, Grus et al. 2001; Kawai, Nakajima et al. 2002; Assouti, Vynios et al. 2006). The study of these molecules helps to understand the DES disease mechanism, which further gives clues for diagnostics as well as for developing therapeutics. Tear specimen is relatively less complex than plasma, tissue specimen and urine in terms of the number of proteins as well as in terms of interferences by abundant proteins (Anderson and Anderson 2002 ; Thongboonkerd, McLeish et al. 2002 ; Green-Church, Nichols et al. 2008). Moreover, the tear specimen is obtained non-invasively unlike other ocular specimen such as vitreous, sub retinal fluid and aqueous humour which are obtained after intervensions. These fluids including tear can reflect the status of the local environment of the eye in the context of the pathology (Grus, Joachim et al. 2007).

Tear proteomics studies have been done in ocular surface diseases like pterigium, blepheritis, microbial keratitis, contact lens wear, meibomian gland disease (MGD), ocular rosacea and evaporative dry eye. Most of the studies have used the capillary tear sample for the 2D and MS analysis.(Molloy, Bolis et al. 1997; Zhou, Huang et al. 2004; de Souza, Godoy et al. 2006; Ananthi, Chitra et al. 2008). However in all these studies only the pooled specimen has been used for the analysis. Capillary tear collection has been used for the 2D electrophoresis in disease conditions such as in diabetic patients (Herber, Grus et al. 2001), blepheritis (Koo, Lee et al. 2005) without pooling the samples. Since the tear secretion is not limiting, capillary collection can be used in such pathological conditions. However since the tear secretion is poor as in dry eye, sterile schirmer strips were used in this study. The comparison of Capillary versus Schirmers method of collection, showed that there was no statistical difference in the expression profile of the protein. 2D profiling of tear proteins using IPG, in the wide pH range of 3 to 10 in 17 cm gel had a good level of agreement between the methods as revealed statistically using Bland-Altman tool. Sample preparation is crucial step in proteomic study. The mode of

tear collection may influence the protein profile. Therefore optimization of the appropriate method of collection, extraction of the tear proteins and 2D profiling for detection of maximum number of discreet spots was done.

The reports on the total number of proteins in the tear are varied. As many as 491 proteins were identified by the *in gel* digestion of pooled capillary tear sample separated by SDS-PAGE and MS analysis, as against 63 in the *in sol* digested as reported by Desouza el al (de Souza, Godoy et al. 2006). Green-Church et al showed a total of 84 Schirmer tear proteins and 66 capillary tear proteins by SDS-PAGE (Green-Church, Nichols et al. 2008). However the data is based on the pooled sample analysis. Compared to the direct MS analysis after *in sol* digestion of tear, the MS of the tryptic digests from 1D or 2D protein bands/spots gives the maximum number of proteins (Koo, Lee et al. 2005; de Souza, Godoy et al. 2006; Green-Church, Nichols et al. 2008). Thus, variations in the number of proteins or spots detected in 2D is based on the method of collection, including pooling of samples, sample concentration methods and the linear/gradient gel is used in the second dimension. Quantitative changes in the protein levels detected based on the collection method using Schirmer has been reported earlier (Stuchell, Feldman et al. 1984). In this study the sample preparation was done with least intervention such as no precipitation of proteins from unpooled samples. The Schirmer extracted tear had still sufficient protein even from dry eye cases. However, a 3 kDa cut off spin column filter was used before profiling as a sample clean up and enrichment protocol.Thus under conditions such as dry eye with various causes as seen in PSS, NSS and SJS where the tear is not adequate, Schirmer strip was the suitable method of collection. The total protein was found to be significantly reduced compared to the controls irrespective of the type of dry eye. This study showed capillary tear protein of 1.5 mg/mL and Schirmer protein of 1.6 mg/mL. DES cases of this study showed reduced protein from 0.4-0.7 mg/ml. The reduction of total protein in DES is also reported by other studies. The protein content in tear reportedly varies from 6 to 10 mg/ml (Lehrer, Xu et al. 1998; Redl 2000). Based on *ingel* and *insol* digestion, a total of 82 proteins were identified from the present study of which 46 were present in the normal healthy tear (table 7.7 and 11.1). The rest of the 37

(figure 7.6) proteins were found in the various types of dry eye. Further validation is required to prove the association of these proteins with Dry eye.

Though all types of DES showed reduction in tear protein the lowering was maximum in DES-RA. However this is probably due to the fact that 89 % of RA patients belong to severe grade of dry eye as against 43% in PSS and 64 % in NSS.

Figure 11.1 shows the tear protein profile based on the peptide spots and table 11.1 gives the differentially expressed in DES. While some of the differentially expressed proteins were reported in previous studies, the proteins namely, LPRR4, Immunoglobulin J, dermicidin, methylenetetra hydrofolate syntahse, SHC transforming isoform 1, ribonuclease P subunit 20, protocadherin, Heterogenous nuclear ribo nucleo protein, Ecto-ADP ribosyl transferase, transcription cofactor ES6, keratin cytoskeletal II protein, Rho related GTP binding protein Q & J showed differential expression, are reported for first time in the present study. Validation of these proteins other than LPRR4 however needs to be done.

Figure 11.2 in the form of venn diagram shows that there were 39 spots that were differentially expressed in DES irrespective of the type compared to the control. These correspond to 13 proteins that are down regulated namely, lacrimal proline rich 4 protein (LPRR4), Immunoglobulin J, Zn-α- glycoprotein, Cystatin, Mammaglobulin B, Lipocalin 1 precursor, cytoskeletal protein 1 and 10, Heat shock protein 1 beta, Lacritin precursor, Lactotransferrin isoform 1 and 2 and Dermicidin. Seven up regulated tear proteins are mammaglobulin B, immunoglobulin heavy and light chains, S100 protein A8, S100, A10 and Methylenetetrahydrofolate synthase domain protein. The proteins that were differentially expressed in 2D were also confirmed using 2D-DIGE.

Most of the studies in DES either Sjogren's syndrome or non Sjogren's syndrome showed changes in the major tear proteins such as lysozyme, lactoferrin, albumin, secretory immunoglobulin A, lipocalins, transferring apart from β 2 microglobulins, calmodulin binding protein, α-amylase, cystatin C and S (Zhou, Beuerman et al. 2009; Versura, Nanni et al. 2010; Srinivasan, Thangavelu et al. 2012). Sjogren's

syndrome showed changes in the tear proteins like peroxidise, aquaporins, goblet cell specific mucin 5A, epidermal growth factor, α 1- anti trypsin (Ferraccioli, De Santis et al. 2010). DES with secondary Sjogren's syndrome to rheumatoid arthritis (RA), DES severity was looked but, tear protein changes are not reported so far (Punjabi, Adyanthaya et al. 2006).

The specific proteins down regulated in DES-RA based on the 2D-DIGE are totally 7, out of which 5 down regulating proteins namely SHC transforming 1 isoform, Ribonuclease protein subunit 20, Protocadherin, Cystatin SN precursor, Lactotransferrin isoform 1 precursor, Heterogeneous nuclear ribonucleoprotein Q isoform 6 and the 2 up regulated proteins namely Keratin type II cytoskeletal protein Ecto-ADP ribosyltranferase-5-precursor and Rho-related GTP-binding protein RhoQ precursor, RhoJ precursor. Based on DAVID annotation tool, all the DES-RA specific proteins are found to be involved in immune response, regulatory function, development, cellular process and in locomotion. RA is an autoimmune disease and is associated with destruction of the lacrimal and the salivary gland. Lactotransferrin and cystatin are antimicrobial proteins that also showed changes in RA synovial tissue. SHC transforming 1 isoform, has regulatory function, also interacts with EGFR (Mantelli, Moretti et al. 2007). The levels of EGFR were reduced in DES condition. EGFR in turn regulates Mucin 1. Mucin 1 levels were also reduced in DES condition. (Caffery,B., M. L. Heynen,et al. 2010). Therefore SHC transforming protein down regulation may regulate other proteins and the increase in disease progression. This has to be further explored. DES-RA specific proteins identified in tear in this study have not been reported in any other specimen associated with RA such as plasma or synovial fluid. However these proteins have to be further validated and disease mechanism studied experimentally. The specific protein changes in primary Sjogren's syndrome identified in this study are Transcription cofactor HES-6, cytoskeletal protein 13.

Table 11.1: Proteins identified in Tear that is differentially expressed in Dry Eye Syndrome

S. No	Name of the protein	M.wt (kDa)	pI	Function	Sources of identification
1	**Dermicidin**	**11**	**7**	**antimicrobial**	**Present study ↓**
2	S100A8	11	5	Regulation of inflammatory process and immune response	Previous study
3	S100A10	11	6.8	Regulation of protein phosphorylation	Previous study
4	Mammaglobin B	11	9.4	Regulation of steroid hormones	Previous study
5	Lacritin	12	4.6	Prosecretory mitogen, helps in proliferation	Previous study
6	Cystatin SN	14	7.5	Cysteine protease, antimicrobial	Previous study
7	**Larimal proline rich 4 protein**	**15**	**6**	**protection**	**Present study ↓**
8	**Ribonuclease P protein subunit 20**	**16**	**9.1**	**Transcription regulator for antimmune antogen**	**Present study ↓**
9	**Immunoglobulin J**	**18**	**5**	**Links monomer igs**	**Present study ↓**
10	Lipocalin	20	5	Transporter proteins, immune response	Previous study
11	**Rho related GTP binding protein Rho Q , Rho J precursor**	**22.6**	**6**	**Regulate cellular responses, epithelial cell polarization**	**Present study↑**
12	**Transcription cofactor HES6**	**24**	**5.1**	**Promotes cell differentiation and transcription repressor**	**Present study ↑**
13	**Ecto-ADP ribosyltransferase 5 precursor**	**32**	**8**	**Catalytic activity at arginine metabolism**	**Present study ↑**
14	Znic alpha glycoprotein	34	5.6	Protective, stimulates lipid degradation	Previous study
15	**Heterogenous nuclear ribonucleoprotein Q isoform 6**	**34**	**8.4**	**transcription regulator at RNA processing level**	**Present study ↓**
16	**SHC transforming 1 isoform**	**62.8**	**6.1**	**Signalling adaptor that couples growth factor receptor with signalling molecules**	**Present study ↓**
17	**Keratin Cytoskeletal II protein**	**67**	**7.5**	**Regulate kinases**	**Present study ↓**
18	Lactotransferrin	78	7	Antimicrobial with ion binding capacity	Previous study
19	**Methylene tetrahydrofoalte synthase**	**102**	**6.8**	**Metabolic activity**	**Present study ↑**
20	**Protocadherin**	**141**	**4.1**	**Regulation of cell growth and cell adhesion**	**Present study ↓**

The study also revealed serological parameters that are significantly altered in primary and secondary sjogrens syndrome due to RA. Anti-CCP, showed increase with the DES grade in RA and also with increased RA severity. Serum anti-SSA and SSB levels increased in both DES-pSS and DES-RA. RF and ESR showed an increase with the increase in severity of the RA. Severe pSS showed significant increase in anti-CCP compared to mild and moderate and therefore could be indicative of progression of the primary Sjogrens into secondary Sjogrens RA condition.

Non Sjogrens DES is mostly caused by aging, viral infection and drugs. SJS is a hypersensitivity reaction that also leads to DES. 2D tear protein profile of SJS cases showed more number of differentially expressed proteins, compared to NSS. DES with NSS showed 6 down regulated and 4 up regulated proteins. One of the up regulated proteins was identified as S100 A8. S100A8 protein showed down regulation in DES-RA and DES-pSS conditions of this study. S100 A8 is calcium binding protein that functions in cell cycle and differentiation as well as in immune response. Other peptide spots that were differential are yet to be identified.

LPRR4 was one of the protein that was significantly down regulated in Dry eye. As it was found to be a novel protein in tear, it was chosen for further validation. LPRR4 was found to be down regulated in DES cases, irrespective of the cause of the dry eye syndrome. Quantitation of LPRR4 was done by ELISA to correlate it with the disease severity. A significant correlation was found between the levels of LPRR4 and the severity of DES. LPRR4 proteins were first reported in tear in pterigium condition (Lei Zhou et al. 2006), and reduction tear PRR4 were reported in contactlens dry eye (Nichols et al. 2009). This study revealed a significant reduction or absence of the LPRR4 protein in all types of dry eye syndrome such as in primary, as well as secondary to rheumatoid arthritis apart from non Sjogrens which included Steven Johnson's syndrome. Reduction of this protein has been reported in Sjogrens as well as in blepharitis conditions (Koo, Lee et al. 2005; Tsai, Evans et al. 2006). However this is the first report to state that decrease in LPRR4 is associated with all types of DES irrespective of the causative factor, since there is lacrimal gland involvement in all the types of DES studied (Aluru, Agarwal et al. 2012).

LPRR4 expression is reported in lacrimal acinar cells (Dickinson and Thiesse 1995). The mRNA expression of LPRR4 showed an ocular tissue specific expression in lacrimal gland when compared to corneal epithelium as seen in this study. However, the limitation of the study is that it is not possible to verify the changes in LPRR4 expression at the level of mRNA in the lacrimal gland of DES cases. A protective function has been assigned for the salivary PRRs (Moreno, Kresak et al. 1982) in protecting the epithelial surfaces (Warner and Azen 1988). The three major functions of salivary PRPs are to act as inhibitors of calcium phosphate precipitation, bind to bacteria to clear potential pathogens as well as bind to minerals or tannins. LPRR4 probably plays a similar protective role in the eye as a modulator of the bacterial flora either by promoting agglutination and clearance of bacteria or by promoting adherence of benign species to the epithelial surfaces thereby eliminating the binding of the other harmful ones (Dickinson and Thiesse 1995). Binding to minerals or tannins may also be important for the protection of the ocular surfaces. The epithelial surface of the eye is exposed to an environment that contains tannins apart from microscopic mineral particles. LPRR4 seems to be an abundant tear protein and may therefore has a protective role. This study showed a significant down regulation of LPRR4 in tear samples of dry eye condition.The structure of LPRR4 is not reported so far. This study modelled the possible predicted structure using *in silico* analysis. The interacting proteins of LPRR4 using string data base showed binding of MUC7, Histatin, lacritin. These interacting partners exhibit anti microbial and anti-inflammatory role as reported. Based on the interacting partners functions, LPRR4 probably has anti inflammatory or antimicrobial activity (Aluru, Agarwal et al. 2012)

USP6NL/RN-tre is another potential biomarker validated in the study. It was found to be differentially up regulated in DES-pSS. USP6NL combines with EGFR regulates downstream signalling cascade. DES-pSS involves inflammation, infiltration of immune cells, leads to decreased secretary function. This study revealed that USP 6 NL/Rn-tre protein shows a significant increase in DES with primary Sjogrens syndrome. However, it did not show any correlation with the clinical parameters like schirmers value, TBUT and with anti-SSA, or anti-SSB

levels in the blood. Structure of USP 6 NL was predicted to look for its binding partners based on its modelled structure. USP-6 NL may involve in cell proliferation by interacting with molecules ESP8, EGFR, EGF. EGFR, one of the interacting protein indirectly regulates MUC 1 expression. Various reports on tear MUC 1 revealed that, MUC 1 in conjunctival cells is reduced in DES syndrome (Mantelli, Moretti et al. 2007). The interacting protein epidermal growth factor showed reduction in DES that has been associated with SS (Pflugfelder, Jones et al. 1999). Based on the above interaction, the predicted mechanism of USP6 NL is that it lowers EGF binding, which in turn inhibits EGFR and then further inhibits wound healing.

Alpha actin, is a trafficking protein that has been showed to change in lacrimal acinar cells of SS mice model. This protein is needed for exocytosis of secretory proteins in association with microtubules and motor proteins lacrimal gland (Wu, Jerdeva et al. 2006). EPS 8, SOS1 and RAB 5A are not reported in tear fluid so far. Further studies to validate these proteins as well as other differentially expressed proteins in DES needs to be done for understanding the pathophysiology.

Oxidative stress plays key role in DES. Increased oxidative stress markers were seen in the corneal and conjunctival epithelium of DES condition *in vitro*. Studies showed, tear ROS levels increased in pathological conditions like keratoconus. (Venkata, Narayanasamy et al. 2009; Saijyothi, Fowjana et al. 2012). This study showed increased ROS levels in the tear of all DES cases associated with DES-pSS, DES- RA and NSS. Various reports showed increase in proinflammatory cytokines and reduction in anti inflammatory cytokines in DES tear as well as conjunctival epithelium observed (Solomon et al.2001; Brignole et al. 2000) This study showed significant decrease in chemokines IL-8, RANTES and MCP-1 in all types of DES. IL-8 and RANTES levels correlated significantly with the disease severity as assessed by Schirmer value. Thus inflammation is a major component of the Dry eye pathology.

Figure 11.1.: The Normal tear protein profile with identified proteins

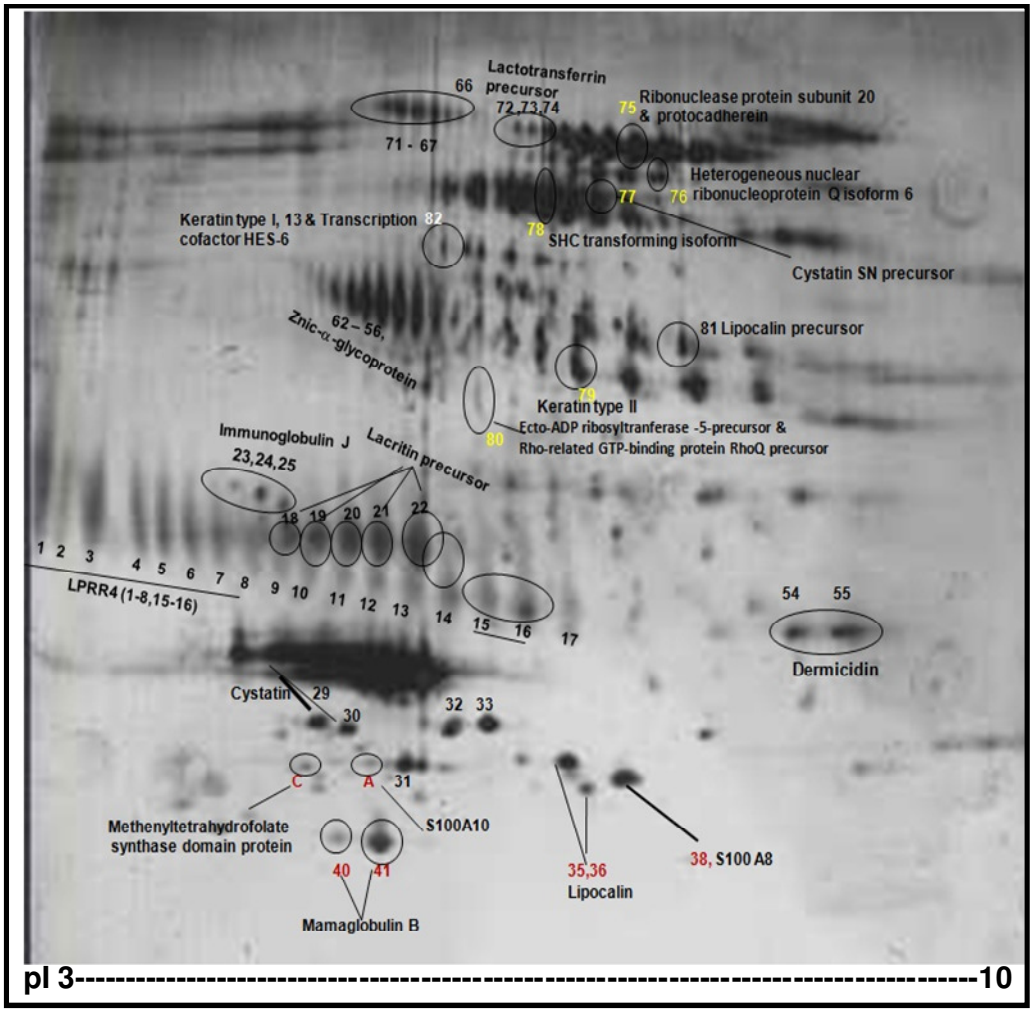
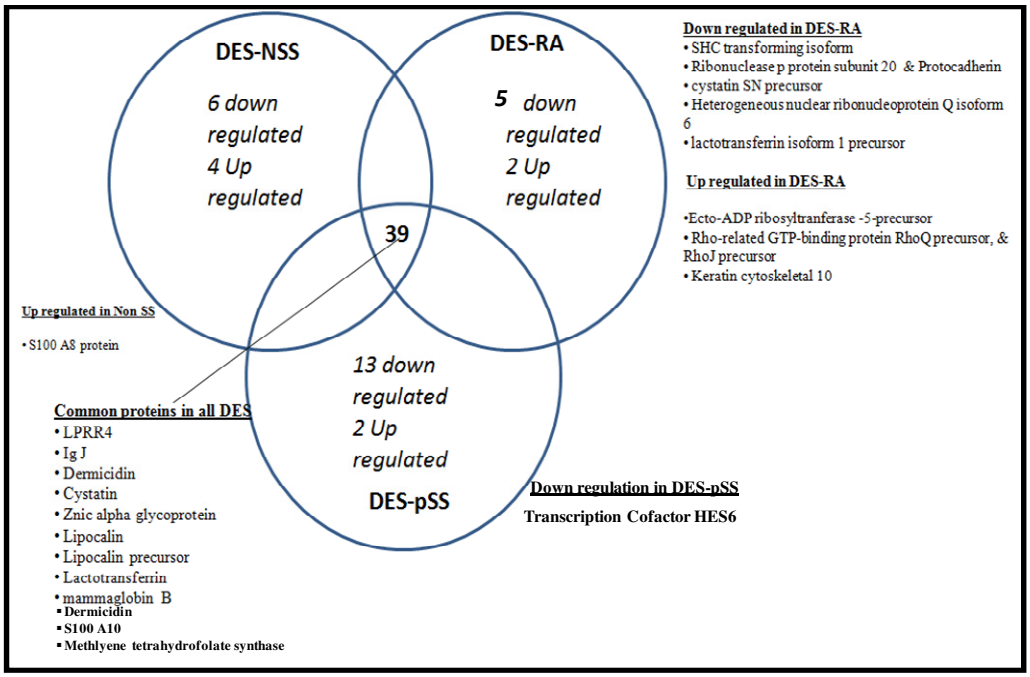


Figure 11.1: 2D electrophtoretic profile of healthy control tear sample showing the peptide spots numbered 1 to 81 corresponding 20 proteins. Spots in red are up regulated in DES. Yellow indicates differentially expressed spots in DES-RA condition. White indicated in DES-pSS. Tear proteins were profiled using 17 cm IPG pH 3-10 and then on 13% SDS-PAGE.

Figure 11.2.: Venn diagram showing the differentially expressed proteins and proteins that are seen in all types of DES and differential to control.



Thus this study for the first time attempts to compare all the type of DES for biomarker discovery in the tear specimen. Un pooled specimen has been used in the study and loss due to immunodepletions has been avoided. Two marker proteins have been validated which is being taken up for further studies to get mechanistic insights.

The limitations of the study include

1. Small sample size in each of the type of DES and larger number representative of the severity required; representation from mild form of DES was poor
2. Identification of all the peptide spots by nano LC-MS/MS analysis is incomplete
3. Validation of more proteins for disease specificity and severity can throw more light on the disease pathology, mechanism and in identifying therapeutic targets.
4. Biomarker identification of sub groups like Steven-Johnson syndrome (SJS) in non sjogrens was not adequate.

Chapter 12: FUTURE SCOPE

1. Proteins that showed differential expression in DES-RA, DES-pSS and DES-NSS based on DIGE need to be further identified using mass spectrometry.
2. Validation of more such potential biomarkers detected in the study characteristic of DES needs to be done.
3. Candidate markers for early detection of DES as to be scrutinized further based on clinical correlations
4. The structure and functional analysis of the proteins detected as potential biomarkers has to be studied using *insilico* analysis wherever reports are not available as well as characterisation of the protein by wet lab studies.
5. Based on functional analysis, the mechanism of the disease involving the disease specific proteins has to be looked into using *in vitro* studies and by animal studies.
6. Possibilities for formulations based on the potential biomarkers that have therapeutic implications for topical treatment of DES needs to be looked into.

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APPENDIX IV

S. No	Chemicals	Company
1	Sodium chloride	Merck,USA
2	Potassium chloride	Merck,USA
3	Disodium hydrogen phosphate	Merck,USA
4	Potassium dihydrogen phosphate	Merck,USA
5	HEPES	Himedia, India
6	EDTA	Biorad, USA
7	Dithiotheritol	Biorad, USA
8	Urea	Biorad, USA
9	CHAPS	Biorad, USA
10	Protease Inhibitor	Sigma,USA
11	Amicon 3 kDa desalting cut-off filters	Millipore,USA
12	Calibrated Capillary tubes	Drummand scientific company, USA
13	Schirmer strips	Conta care, baroda
14	Bradford reagent	Pierce, USA
15	Bovine serum albumin	Himedia, India
16	Sodium carbonate	Merck,USA
17	sodium hydroxide	Merck,USA
18	Tri sodium citrate	Merck,USA
19	Copper sulphate	Merck,USA
20	Folins reagent	Merck,USA
21	Sodium bicarbonate	Merck,USA
22	Bicinchoninic acid	Sigma,USA
23	sodium tartrate	Merck,USA
24	Acrylamide	Biorad, USA
25	Bis acrylamide	Biorad, USA

S. No	Chemicals	Company
26	Tris	Biorad, USA
27	Sodium Deodusyl sulphate	Biorad, USA
28	Ammonium Per Sulphate	Biorad, USA
29	TEMED	Biorad, USA
30	Glycerol	Biorad, USA
31	Beta mercaptoethanol	Biorad, USA
32	Bromo phenol blue	Biorad, USA
33	Glycine	Biorad, USA
34	Methanol	Merck,USA
35	Acetic acid	Merck,USA
36	sodium thiosulphate	Merck,USA
37	Silver nitrate	Biobasic,
38	Formaldehyde	Merck,USA
39	Ampholytes	Biorad ,USA
40	Immobilized pH Gradient (IPG) strips	Biorad ,USA
41	Trichloroacetic acid (TCA)	Merck,USA
42	Mineral Oil	Biorad ,USA
43	Iodoacetaamide	Biorad ,USA
44	Agarose	Biorad ,USA
45	Nanopure water	Biorad ,USA
46	Protean II Xi unit is PAGE apparatus	Biorad ,USA
47	Densitometer GS800	Biorad ,USA
48	Cy3 DIGE fluors	GE Healthcare,UK
49	Cy5 DIGE fluors	GE Healthcare,UK
50	Cy 2 DIGE fluors	GE Healthcare,UK
51	Tris (2-carboxyethyl) phosphine (TCEP)	Sigma,USA
52	N, N-Dimethyl formamide	Merck,USA
53	Trypsin	Promega, USA

S. No	Chemicals	Company
54	Potassium ferricyanide	Merck,USA
55	Ammonium bicarbonate	Merck,USA
56	Acetonitrile	Merck,USA
57	Formic acid	Merck,USA
58	Diethyl Pyrocarbonate	Sigma,USA
59	Chloroform	Merck,USA
60	Isopropanol	Merck,USA
61	Ethanol	Merck,USA
62	3-(N-morpholino)propanesulfonic acid (MOPS)	Sigma,USA
63	Ethidium bromide	Sigma,USA
64	Trizol	Sigma,USA
65	iScript RT-PCR Kit	Biorad,USA
66	Taq polymerase	Bangloregenei, India
67	dNTPs	Bangloregenei, India
68	LPRR4 ELISA kit	USCN, China
69	USP6NL ELISA kit	USCN, China
70	Periodic acid	Merck,USA
71	Pararosaniline	Sigma,USA
72	Sodium disulfite	Merck,USA
73	Sodium metabisulfite	Merck,USA
74	charcoal	Merck,USA
75	Novolink polymer detection kit	Leica Biosystems,USA
76	xylene	Merck,USA
77	FACS CBA chemokine kit	BD Bioscience,USA
78	Dimethyl formamide	Merck,USA
79	Hydrogen peroxide	Merck

LIST OF PUBLICATIONS

1. **Saijyothi Venkata Aluru**, ShwetaAgarwal, BhaskarSrinivasan, Geetha Krishnan Iyer, Sivakumar M. Rajappa, UtpalTatu, PremaPadmanabhan, Nirmala Subramanian AngayarkanniNarayanasamy. Lacrimal Proline rich 4 (LPRR4) protein in the tear fluid is a potential biomarker of dry eye syndrome. ***PLos One,*** 7 (12); 2012: e51979

2. **Aluru Venkata Saijyothi**, JenofarFowjana, Subramanian Madhumathi, MahadevanRajeshwari, MaruthamuthuThennarasu, PadmanabanPrema, NarayanasamyAngayarkanni. Tear fluid small molecular antioxidants profiling shows lowered glutathione in keratoconus. ***Experimental Eye Research***, 103;2012: 41-46

3. RadhakrisnanSelvi, Renganathan Bhuvanasundar, **Aluru Venkata Saijyothi**, KonerirajapuramNatarajanSulochana and NarayanasamyAngayarkanny. Amino Acids Potentiate Insulin Signaling in CHO-K1. ***Archives of Medical Research***, 43; 2012: 173-182.

4. S.Ramakrishnan, R.Selvi, **A.V.Saijyothi**, JyotirmoyBiswas, M.BharatSelvi and N.Angayarkanni. Clinical and Biochemical benefits of administration of vitamins E & C in patients with Eale's Disease. Biomedicine. 32(2); 2012: 162 – 168.

5. **Aluru Venkata Saijyothi,**,Narayanasamy Angayarkanni, Chandran Syama,TatuUtpal, AgarwalShweta, SrinivasanBhaskar, Iyer Krishnan Geetha, Pillai S. Vinay, MaruthamuthuThennarasu, Rajappa M. Sivakumar, PadmanabhanPrema.Two dimensional electrophoretic analysis of human tears: Collection method in dry eye syndrome. ***Electrophoresis***, 2010;31:3420-3427

6. **A.V. Saijyothi**, N.Angayarkanni, Vidhya.S, Geetha.I.K, Ramakrishnan.S, Madhumathi.S, Rajeswari.M. N.Angayarkanni.Tear ascorbic acid levels and

the total antioxidant status in contact lens wearers: A pilot study. ***Indian Journal of Ophthalmology,*** 2009; 57:289-292.

7. Angayarkanni. N, **A.V.Saijyothi**, K.Coral, R.Punithum, R.Krishnakumar, Madhavarao and S.Ramakrishnan Dried Blood Spot (DBS) for Vitamin A analysis – a field friendly method. ***Insight*** vol.XXIII No.1, Jul.2005. (Institutional Journal)

Articles under Review

1. Tear fluid RN-tre levels in Dry Eye associated with Primary Sjogrens Syndrome – accepted with revisions.

Articles under Preparation

1. Tear fluid protein changes In Dry Eye Syndrome associated with Rheumatoid arthritis - A Proteomic approach.
2. Tear fluid Chemokines in dry eye syndrome with rheumatoid arthritis

LIST OF PAPERS PRESENTED IN CONFERENCES

NATIONAL

1. Differential expression of tear fluid proteins in Dry eye syndrome: A proteomic Approach" at **Indian Proteomics conference (IPCON) 2011at New Delhi**.

2. Tearfluid antioxidants profiling by HPLC shows reduced levels of glutathione in keratoconus cases" at **Indian Eye Research Group (IERG) conference, Hyderabad, 2010**

3. Differential expression of tear proteins in Steven-Johnson Syndrome: A proteomic approach" at **Society of Biological Chemists (SBCI) conference, Pune, 2009.**

4. Tear fluid two dimensional gel electrophoresis – Development of sample preparation method: at Emerging trends in Life sciences research **March 2009 at BITS., Pilani.**

5. Determination of ascorbic acid in tear sample of control subjects and contact lens wearers by HPLC – a preliminary study" at Society of Biological Chemists **(SBCI) conference**, **Tirupathi, 2007**.

6. Retinol analysis in Dried Blood samples by HPLC" at Indian Eye Research Group **(IERG) conference, Hyderabad, 2005**

7. Effect of amino acids on labeled glucose uptake & GLUT-4 expression in CHO cells treated with high glucose concentration" at Indian Eye Research **Group (IERG) conference, Chennai, 2004**

INTERNATIONAL

1. Tear two dimensional protein profile in Dry Eye syndrome at **Asia-ARVO**, **at Hyderabad, 2009.**

AWARDS

1. Awarded "**Travel Grant"** to participate in the **Indian Eye Research Group** for the year 2010 held in Hyderabad.

2. Awarded "**WIPRO BIOMED** "for the best out going student in Biochemistry for the year 2003.

BRIEF BIOGRAPHY OF THE CANDIDATE

Ms. A.V. Saijyothi obtained her B.Sc Biochemistry/Microbiogy/Chemistry degree from Priyadarshini degree college, Sri Venkateswara University, Nellore Andhra Pradesh in 2000. She obtained her M.S. (Medical Laboratory Technology) degree from Birla Institute of Technology and Science, Pilani in 2003 with course work at Medical Research Foundation, Chennai. Then she joined the biochemistry research department, SankaraNethralaya as Junior Scientist in 2004 and worked as junior scientist for 4 years. In the year 2008 she was deputed as SRF in project funded by Department of Biotechnology. She registered for Ph. D in Feb 2007 in Birla Institute of technology & Science, Pilani to do research on biomarker identifications in Dry eye syndrome by 2D-DIGE based proteomics in tear. She has made presentations in 7 national and 1 international conferences, comprising 7 poster presentations and 1 oral presentation. She has 3 publications comprising clinical proteomics, 1 comprising cell biology work and 3 clinically in Keratoconus, Eales' disease. Further 1 paper has been accepted and is in process and 2 papers are under preparation related to thesis. She has handled clinical samples during junior scientist and has done special investigations for diagnostics purpose and has exposure to clinical work as part of the thesis work. She has worked in a 2 year clinical study on "Effect of oral amino acid supplementation to patients with NIDDM on the insulin receptor signalling pathway in monocytes – A randomized double-blind placebo controlled Study" before doing her Ph.D. She has received an endowment award in biochemistry during her MSMLT course. She has received the travel grant award for IERG (Hyderabad, 2010) conference. She is also involved in teaching biochemistry and in conducting practical sessions for UG and PG students at Sankara Nethralaya. Her research interests are in clinical proteomics, Biomarker identification in ocular diseases like dry eye syndrome, keratoconus and contact lens and has expertise in HPLC, 2DE, DIGE and other types electrophoretic techniques including Western Blot, FACS analysis, Spectroflourimetry, IHC and PCR.

BRIEF BIOGRAPHY OF THE SUPERVISOR

Dr. N. ANGAYARKANNI is currently the Professor in the department of Biochemistry and Cell Biology, Vision Research Foundation, Sankara Nethralaya, obtained her Ph.D. in 1995 from University of Madras in the field of Biochemistry. She had received the UGC fellowship through the GATE score of 91 percentile and had received the CSIR –SRF and the CSIR-Research Associateship. Apart from teaching biochemistry for the under graduate and post graduate students. She is also currently the guide for 5 Ph.D. students and is involved in mentoring several internships for PG dissertations apart from BITS – PS summer students. She is currently the principal investigator for 6 major projects from DST, DBT, CSIR and ICMR. She has currently 30 publications and 2 books. Her areas of interest include the extra cellular matrix changes in association with homocysteine, MMPs, collagen cross linking oxidative stress and antioxidant as well as in angiogenic and antiangiogenic factors in retinal and vitreo-retinal diseases. She also works on in vitro disease sequels involving retinal pigment epithelial cells and retinal capillary cells. In addition she works on Tear proteomics in Dry eye syndrome and Lipidomics in diabetic retinopathy. She is also involved in patient care as Biochemist and as Quality manager- NABL. She has visited various universities such as, University of Michigan, Kellogs Eye Institute, Singapore eye research institute, University of Mexico, University of Missouri to gain knowledge in the research interests. She has recently received the ICMR overseas fellowship for young biomedical scientist to get trained in animal models of retinopathies at University of Mexico, USA in the department of cell biology and physiology. She is a permanent member of Society of Biological chemists and a current member of Association for Research in Vision and Ophthalmology and periodically presents number of research papers in the national and international conferences on Eye research. She is also member of board of studies for Biochemistry University of Madras and has guideships in SASTRA and MGR Medical Universities other than BITS, Pilani.