Glycobiology of the Human Lacrimal Drainage System

Submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to certify that this thesis entitled "**Glycobiology of the Human Lacrimal Drainage System**" submitted by **Dr Mohammad Javed Ali** bearing registration number **14LBPH08** in partial fulfillment for the award of Doctor of Philosophy in department of Biochemistry, School of Life Sciences is a bonafide work carried out by him under my supervision and guidance.

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Part of the thesis have been:

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DECLARATION

I hereby declare that the work presented in my thesis is entirely original and was carried out by me in the Department of Biochemistry, University of Hyderabad, under the supervision of Prof. N. Siva Kumar and also in Institute of Anatomy, University of Erlangen-Nurnberg, Germany. I further declare that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

Dr Mohammad Javed Ali, MD, FRCS

Prof N Siva Kumar
(*supervisor*)

Date :

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Mohammad Javed Ali

ABBREVIATIONS

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LS – Lacrimal sac

LF – Lacrimal fossa

NLD – Nasolacrimal duct

MCT – Medial canthal tendon

CPF – Capsulo-palpebral fascia

PANDO – Primary acquired nasolacrimal duct obstruction

SEM – Scanning electron microscopy

DCR – Dacryocystorhinostomy

MALT – Mucosa associated lymphoid tissue

LDALT – Lacrimal drainage associated lymphoid tissue

IgA – Immunoglobulin A

IgM – Immunoglobulin M

IgG – Immunoglobulin G

MHC – Major histocompatibility complex

OCT – Optical coherence tomography

FD-OCT – Fourier domain- optical coherence tomography

3D – Three dimensional

CRD – Carbohydrate recognition domain

CTLD - C- type lectin domain super family

MPR – Mannose 6-phosphate receptors

CDMPR- Cation dependent mannose 6-phosphate receptors

CIMPR – Cation independent mannose 6-phosphate receptors

ATP – Adenosine triphosphate

SNP - Single nucleotide polymorphism

RBL – Rhamnose binding lectins

Con A – Concavalin A

TGF-β – Transforming growth factor – β

ERα – Estrogen alpha

Erβ – Estrogen beta

CYP19 – Aromatase

OXTR – Oxytocin receptors

TSTR – Testosterone

PGR – Progesterone

PRL – Prolactin

SSTR – Somatostain receptors

GR – Glucocorticoid receptors

IFN-β – Beta interferons

IL – Interleukins

TNF-α – Tumor necrosis factor alpha

TMEM9B – Trans-membrane protein 9B

MAPK – Mitogen activated protein kinase

TEM – Transmission electron microscopy

PAGE- Polyacrylamide gel electrophoresis

SDS-PAGE – Sodium tetradoecyl sulphate PAGE

MUC – Mucins

TFF – Trefoil factor peptides

SP-A – Surfactant protein A

SP-B – Surfactant protein B

SP-C – Surfactant protein C

SP-D – Surfactant protein D

SP-G – Surfactant protein G

SP-H – Surfactant protein H

SFTA – Surfactant associated protein

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CHAPTER 1:
LACRIMAL DRAINAGE SYSTEM

The lacrimal drainage apparatus begins on the eyelid from a small opening called as lacrimal punctum and courses into the canaliculus, lacrimal sac and nasolacrimal ducts to open into the inferior meatus of the nose (**Figs 1.1-1.3**). This drainage pathway carries tears from the

ocular surface in a unidirectional flow into the nasal cavity. We describe here the anatomy and physiology of the lacrimal drainage system, and also partially refer to its embryology and immunology.

1.1 . Anatomy of the lacrimal punctum

The lacrimal punctum lies on a small fibrous mound, called the “lacrimal papilla” . Diameter of its opening is 0.2-0.3 mm and directs somewhat posteriorly toward the lacrimal lake (Whitnall 1979; Kakiizaki and Vallenzuella 2011; Nirankari and Chadda 1962; Lyons et al. 1993; Burkatt and Lucarelli 2006; Linberg 1988). The puncta are round or oval in youth but often collapses into fish-mouth or slit configuration with age. The inferior punctum lies 0.5-1.0 mm more temporally than the superior one, because the maxillary process in embryonic life grows faster the lateral nasal process. The inner epithelium is non-keratinized stratified squamous epithelium (Kakizaki et al. 2007; 2010). No meibomian glands exists medial to the papillae, and most medial meibomian orifice are situated at 0.5-1.0mm lateral to the puncta (Linberg 1988).

1.2. Anatomy of the lacrimal canaliculus

The lacrimal canaliculus is divided into the vertical and horizontal portions (Whitnall 1979). Its transitional part occasionally dilates to form an irregular dilated cavity or ampulla. The length of the vertical portion is 2mm and that of the horizontal part is 10mm (Linberg 1988). However, it is important to realize that these measurements have been originally deciphered from cadaveric studies. The medial most 2 mm of the horizontal portion mostly forms the common duct or canaliculus (Zoumalan et al. 2011) and more than half of this part runs in the wall of the sac. The punctum and vertical canaliculus are encircled by a similar hard fibrous tissue. This fibrous tissue in the vertical canaliculus contains skeletal muscle fibers, called the muscle of Riolan (Kakizaki et al 2012a). The epithelium of the canaliculus is a non-keratinized stratified squamous epithelium, similar to the punctum. As the canalicular wall contains much elastic fibers, its diameter can be changed to enlarge or shrink as needed. Although the diameter

of the canaliculus is usually 0.3-0.6 mm, it can be expanded to over 1.0 mm. The temporal 4/5 part is directed postero-nasally and surrounded by the Horner's muscle, occasionally called the lacrimal part of the orbicularis oculi muscle. In the nasal 1/5 part, the Horner's muscle directs posteriorly away from the canaliculus. Although the canaliculus usually directs anteronasally after separation from the Horner's muscle, it occasionally directs posteronasally in cases with proptosis. The superior canaliculus courses, in general, almost straight to the internal common ostium, but the inferior canaliculus changes the course superiorly before joining the superior canaliculus. The course of the inferior canaliculus independently emptying into the lacrimal sac has not been convincingly proven.

1.3. Anatomy of the common lacrimal canaliculus

More than 95% of the upper and lower canaliculi join to become the common canaliculus to reach the common internal ostium (Yazici et al. 2000). The canaliculi empty into the sinus of Maier and those independently pouring into the sac are < 2%. Sinus of Maier needs further elaboration. The common internal ostium is the part where the common canaliculus enters into the lacrimal sac. However, the common canalicular cavity does not simply connect with the sac lumen. A laterally bulged portion of the sac, called the sinus of Maier, is occasionally formed around the common internal ostium and some part of the common canaliculus empty into this portion. An expanded common canaliculus is can also be called the sinus of Maier.

The common canaliculus has a non-keratinized stratified squamous epithelium. However, the transitional area with the stratified columnar epithelium of the sac with some goblet cells is occasionally seen in the distal common canaliculus. To the contrary, the stratified squamous epithelium of the common canaliculus sometimes extends to the sac lumen.

A protuberance (fold) is shown, although in only a half of cases, at the junction between the common canaliculus and the sac (Kurihashi et al. 1991). This structure is called the valve of Rosenmüller. The common internal ostium largely opens by temporal traction of the Horner's muscle during eye closing, but there is a nasal movement of the ostium as well (Kakizaki et al

2005). Therefore, the part around the common internal ostium needs a structure dealing with this nasal movement and this may be the real reason for a valvular presence in this region. The sinus of Maier could have also been evolved for the same reason.

1.4. Anatomy of the lacrimal Sac and Its fossa

The lacrimal sac and the nasolacrimal duct are contiguous structures (Jones 1962). The part within the lacrimal sac fossa is called as the "sac", and the part inferior to the superior opening of the nasolacrimal canal is the "nasolacrimal duct". The part of the sac superior to the medial canthal tendon (MCT) is called the fundus, with its vertical length being 3-5 mm. The body of the sac, inferior to the MCT, is about 10 mm in length. The epithelium of the sac is a stratified columnar epithelium (Olver 2002), and contains goblet cells, cilia and serous glands. The epithelial surface shows microvilli (Paulsen et al. 2000; Kurihashi 1996). Although the sac wall is constituted with a cavernous structure, it is fairly thin and less developed than that of the nasolacrimal duct (Bailey 1923; Narioka and Ohashi 2006). The lateral aspect of the sac wall is covered by a fascia, and its posterior portion has a common fascia with the Horner's muscle, which is called the "lacrimal diaphragm".

The lacrimal sac fossa comprises of the anterior frontal process of the maxillary bone and the posterior lacrimal bone. There are ridges anteriorly and posteriorly, which are called the anterior or posterior lacrimal crests, respectively. The suture between the maxilla and the lacrimal bone is situated in various ways, and some take its site close to the posterior lacrimal crest. A process is formed between the inferior portion of the posterior lacrimal crest and the orbital face of the maxilla, which is called the Hamular process. A groove present nasal to the anterior lacrimal crest is called the sutura notha, sutura longitudinalis imperfecta, or pseudo-suture (Kurihashi 1998). It is not considered as a true suture but a vessel groove formed by a branch of the inferior orbital artery.

The superoinferior length of the lacrimal sac fossa is 12-15 mm, anteroposterior 4-9 mm, and the width is 2-3 mm. The lacrimal sac fossa shows shorter anteroposterior length superiorly (Kakizaki et al. 2008; 2012b). As the lacrimal sac fossa opens temporally, the sac lumen is

usually large enough. The long axis of the fossa inclines about 10 degrees posteriorly (Park et al 2012), and directs about 10 degrees temporally. The angle range of the long axis of the fossa is 0-20 degrees posteriorly, and 1-30 degrees temporally (Takahashi et al. 2013a).

1.4A. Structural-Clinical Correlations

1. The orbit is defined as the part posterior to the orbital septum. The lacrimal apparatus is not an orbital tissue as it is located anterior to the orbital septum. However, due to the vicinity with the eyelid as an extra-septal tissue, the lacrimal system is closely related to the eyelid and relies on the eyelid movements for pump functions of the lacrimal drainage. Since the function of the lacrimal apparatus is highly specialized, it is defined as the "lacrimal system" with an independent identity of its own.
2. When an acute dacryocystitis extends around the sac, the inflammation spreads toward the eyelid because of the above anatomical reason. In an advancing stage, it occasionally spreads into the orbital space, since the barriers are not strong enough anymore.
3. The lacrimal bone is too thin with its thickness around 0.1 mm. Therefore, in both external and endonasal dacryocystorhinostomy, an osteotomy is started from the lacrimal bone. In cases of a lacrimo-maxillary suture being situated close to the posterior lacrimal crest, a surgeon occasionally feels difficulty to perform the osteotomy. In an external dacryocystorhinostomy, initial osteotomy sometimes begins at the part around the sutura notha. It is better, however, not to extend the osteotomy towards the ethmoid sinus to prevent bleeding from the ethmoid mucosa.
4. In an endonasal DCR, relationship between the lacrimal sac fossa and the base of the middle turbinate is vital. The base of the middle turbinate, called the "axilla", often corresponds to the lacrimal sac fossa, although there are exceptions. A high sac position is defined as the sac situated superior to the axilla, and a low sac is a position inferior to the axilla. This relative position between the lacrimal sac fossa and the axilla is confirmed with a preoperative CT or intra-operative light pipe inserted from a punctum.

A light cannot be occasionally seen in cases with thick frontal process of the maxilla, posterior location of the lacrimo-maxillary suture, cases with high sac position or cases with anterior protrusion of the ethmoid air cells with wide distance between the lacrimal bone and the lateral wall of the nasal cavity.

5. Endoscopic clinical anatomy reveals that the posterior portion of the lacrimal bone is covered in considerable cases by the uncinate process forming the most anterior part of the ethmoid air cells (Woo et al. 2011). The inferoposterior part of the lacrimal bone tends to be covered largely. A small protuberance called the agger nasi is seen over and medial to the lacrimal sac fossa. Aerated agger nasi (agger nasi cell) can often reach the lacrimal sac fossa.
6. The uncinate process is, as suggested by its name is a bony process with a "hook" (Yoon et al. 2000). This hook part is situated at a considerable depth corresponding to the posterior hiatus semilunaris. As the tail of the uncinate process faces anteriorly, we cannot easily see the "hook" part around the lacrimal sac fossa.

1.5. Anatomy of the nasolacrimal duct (NLD) and canal

The lacrimal sac and the nasolacrimal duct are a continuous tissue, and anatomically speaking, the "nasolacrimal duct" (mucosal portion) is the part inferior to the superior opening of the nasolacrimal canal (bony portion). The nasolacrimal canal is formed by the lacrimal bone superonasally, the inferior turbinate bone inferonasally and the maxillary bone temporally. The superior opening is about 6 mm in diameter and, in general, is an ellipse with a little longer horizontally. The supero-inferior length of the canal is fairly short, about 12mm. Although the longitudinal axis of the canal directs about 20 degrees posteriorly, it directs almost vertically in most cases. The nasolacrimal canal empties into the superior part of the inferior meatus.

The angle range of the long axis of the nasolacrimal canal is 3-40 degrees posteriorly. The frontal view shows the angle range from 12 degrees nasally to 11 degrees temporally and mostly directs vertically around 0 degree. Although a general consensus of the canal course is "temporal", occasionally cases with medial course have been noted.

The nasolacrimal canal does not have a constant diameter throughout its length: some being narrower and others larger (Takahashi et al. 2013b). Two thirds to 3/4 of cases show the narrowest part at the superior opening, but the others have found the narrowest portion at 3.5-5.5mm from the superior opening. These narrowing's may have a bearing on the etiopathogenesis of primary acquired nasolacrimal duct obstructions (PANDO).

Epithelium of the NLD is a stratified columnar epithelium, similar to the lacrimal sac, and contains goblet cells and serous glands. In general, the goblet cells are distributed more inferiorly, but several specimens have also demonstrated considerable number of goblet cells throughout. Although the cavernous structure is shown similar to the lacrimal sac, it is much more developed than the sac. The wall is more thickened inferiorly and most show a funnel shape lumen. Cilia are similar to the nasal mucosal cells. The microvilli on the epithelial surface contribute to reabsorption of the lacrimal fluid.

The NLD occasionally shows folds called as valves of Krause. In addition, septa are sometimes seen in the nasolacrimal duct or the sac. The nasolacrimal duct mostly continues for several millimeters beneath the nasal mucosa after leaving its osseous channel (Cowen and Hurwitz 1996; Takahashi et al 2012; Onogi 2012). This part has a valve called the valve of Hasner. The total length of the nasolacrimal duct is 15-18 mm and it is longer than its bony canal. The shape of the NLD opening into inferior meatus shows 4 types: wide open type (12%), valve-like type (8%), sleeve-like type (14%) and adhesive type (66%), although these were studied in patients with functional epiphora (Onogi 2012). These openings are situated around 30-35 mm posterior to the lateral margin of the nares.

1.5A. Structural-Clinical Correlation

Although the lacrimal sac and the NLD are a continuous structure, and the basic structure is same, their compositions, such as number of goblet cells, development of the cavernous structure and thickness of the wall, are considerably different. In the lacrimal system, roles are shared between these two portions: the lacrimal sac sucks the tears from the ocular surface supported by the lacrimal drainage pumps and the NLD reabsorbs them. This feature is similar to the intestinal canal, which anatomically is a long continuous structure but has different

functions for each portion.

1.6. Mechanism of the Lacrimal Drainage

1.6A. Physiological relationship of lacrimal punctum, lake and caruncle

The lacrimal caruncle derives embryologically from the lower eyelid, and its lateral margin smoothly continues to the lower eyelid margin. As this lateral margin of the caruncle directs inferolaterally, the lower punctum is situated more temporally than the upper punctum.[2] The lacrimal lake is formed adjacent to the caruncle, where the lacrimal papilla faces it in general. This is the normal relationship of the punctum, lake and caruncle. Although the plica semilunaris is formed more temporally to the lacrimal lake, this buffers an imbalance of an ocular and a palpebral movement. When the trinity of the punctum, lake and caruncle is in disproportion, that is to say, when the lacrimal papilla does not face the lacrimal lake, an epiphora may occur.

1.6B. Lacrimal drainage system of the canaliculus

The lateral 4/5 of the lacrimal canaliculus is encircled by the Horner's muscle but not the medial 1/5. The canalicular drainage is easily understood by dividing the canaliculus into 2 parts with relation to the Horner's muscle.

During the eye closing, the Horner's muscle contracts and the temporal 4/5 part of the canaliculus is pressed and closed. The nasal 1/5 part is pulled posteriorly and opens. In this situation, the Horner's muscle moves posteronasally towards the origin of this muscle (posterior lacrimal crest) and this movement begins from the temporal side with shortening of the canalicular length (Doane 1981). Therefore, the lacrimal fluid is effectively transported from temporal to nasal sides, finally reaching the lacrimal sac cavity. During the eye opening, as the Horner's muscle relaxes, the temporal 4/5 part of the canaliculus is expanded and the nasal 1/5 part is pressed and closed via the Horner's muscle and the connective tissues. This canalicular closure is not perfect, though. In this phase, as the whole canaliculus moves anterotemporally and is elongated, the canaliculus can pool more lacrimal fluid in it.

An aspiration from the punctum relies on a capillary phenomenon and/or negative pressure in the canalicular lumen. As stated before, the protuberance on the common internal ostium is thought to be formed to buffer the movement of the common internal ostium. The sinus of Maier may be a similar buffering structure because it is notably seen in eye closing with the Horner's muscle traction. This protuberance has been argued in relation to regurgitation. However, it is difficult to judge this structure formed for prevention of tear regurgitation because almost all patients who underwent dacryocystorhinostomy feel air reflux to the eye during sneezing.

The medial most 1 mm of the common canaliculus runs in the wall of the sac. As the sac wall is constituted by cavernous structure, its thickness could be regulated by an autonomic innervation (Thale et al. 1998). If the intra-sac canaliculus receives an autonomic regulation, then in a sympathetic dominant state, as the sac mucosa shrinks, the intra-sac canaliculus is enlarged and shortened, resulting in more drainage. To the contrary, in a parasympathetic dominant state, as the sac mucosa is thickened, the intra-sac canaliculus is pressed but elongated, resulting in less drainage. However, as its length is only 1 mm and the cavernous structure of the sac is less developed than that of the nasolacrimal duct, it is unclear whether the above phenomenon occurs.

1.6C. Lacrimal drainage system of the lacrimal sac

The lacrimal drainage system of the sac, just like canaliculi can be easily understood if it is divided into two parts in relation to the Horner's muscle. In addition, as the fundus of the sac has a special system, it is explained separately.

The upper part of the sac is directly affected by the Horner's muscle movement. During eye closing (when the Horner's muscle contracts), as the Horner's muscle moves away from the sac, the sac expands temporally. During the eye opening (when the Horner's muscle relax), as the Horner's muscle moves toward the original position and pushes the sac nasally, the sac shrinks with an additional help of its elasticity.

The lower lateral half of the sac is covered only by the lower eyelid capsulopalpebral fascia (CPF). During eye closure, as the CPF takes no tension and the orbicularis oculi muscle pushes the orbital tissues posteriorly, the lower lateral half of the sac is pushed nasally with the tensionless CPF. At the same time, shrinkage of the lower eyelid orbicularis oculi muscle pushes the anterior surface of the sac posteriorly. During eye opening, the CPF is pulled temporally with the lower lateral half of the sac under decreased orbital pressure. Then, the lower eyelid orbicularis oculi muscle is in less tension, resulting in a forward movement of the anterior sac surface.

As the fundus of the sac has an orbicularis attachment, this part is expanded superiorly during eye closure or during an orbicularis oculi muscle contraction (Kakizaki et al. 2004). The orbicularis oculi muscle attached to the fundus is opposed by the medial horn of the levator aponeurosis and relaxes during eye opening to an appropriate muscle length to prepare for the next contraction. As the superoanterior surface of the sac is mostly covered by the orbicularis oculi muscle and a force from the orbicularis contraction being applied only horizontally, an effect to the sac can be ignored. The sac movement stated above does not directly correspond with the tear movement (Amrith et al 2005). With several times blinking, pooled fluid in the sac flows inferiorly as a cluster.

1.6D. Krehbiel flow:

The Krehbiel flow is a special type of lacrimal fluid drainage (Sahlin and Chen 1997; Nagashima 1976). This is a phenomenon in which a lacrimal fluid aspiration from the lacus lacrimalis into the punctum continues for a considerable period during eye opening (without blinking). Although all the cases do not show this phenomenon, 25 % of the lacrimal passage with 45 degrees posterior inclination demonstrate it. According to Prof. Ohashi and Dr .Yamaguchi in Ehime University (Japan), a velocity and a volume of the Krehbiel flow changes with various eye positions (personal communication). The Krehbiel flow is believed to occur by a lower intra-sac pressure against a canalicular pressure, namely by a pressure gradient from the canaliculus to the sac. To decrease the intra-sac pressure, the sac and the

nasolacrimal duct cavities need to be occluded to a certain extent, and the fluid and air need to be absorbed.

1.6D1. Factors favoring Krehbiel flow

1. Long valve of Hasner is necessary for making one-way valve function.

2. The lower nasolacrimal duct should be funnel-like with narrower lumen inferiorly, which should be able to functionally obstruct when needed.

3. The fluid and air need to be reabsorbed by the well developed cavernous structure of the sac and the nasolacrimal duct.

4. In the upper stream, the canaliculus needs to be filled with fluid by continuous tear aspiration with much less air in the lumen.

1.6D2. Clinical Observations on Krehbiel flow

1. When a person takes a lying position or a lower head position, duration of the Krehbiel flow gets shorter or nil. That is to say, an effect by the gravity is only additional.

2. After dacryocystorhinostomy, as the nasal cavity pressure is relatively higher than the preoperative intra-sac pressure, the pressure gradient from the canaliculus to the sac is lost, resulting in no Krehbiel flow.

3. A case with air in the nasolacrimal duct as shown by a CT does not demonstrate the Krehbiel flow (observational finding). Although the common internal ostium is pressed and occluded when the Horner's muscle relax, this closure is not perfect with a little opening. This probably contributes to the simultaneous occurrence of the Krehbiel flow and contribution to tear drainage.

1.6E. Lacrimal drainage system of the nasolacrimal duct:

The nasolacrimal duct does not perform an active lacrimal drainage, but contributes by making the flow smoother and by the way of tear reabsorption. As the cavernous tissue in the sac and the nasolacrimal duct have collagen fascicules, elastic and reticular fibers, which are helically

arranged from superiorly to inferiorly, this complex architecture cooperates with the dynamic lacrimal drainage and the gravity, and helps drain the fluid effectively toward the nasal cavity.

1.7. Mechanism of the Lacrimal Fluid Reabsorption

1.7A. Tissue anatomy in relation to lacrimal fluid reabsorption

The lumen of the sac and the nasolacrimal duct are covered by the stratified columnar epithelium with microvilli. This anatomy enlarges the surface area of the lumen and is advantageous for the lacrimal fluid reabsorption. A lot of vessels exists in the sub epithelial tissue, in which one barrier artery and two types of veins (throttle & capacitance veins) comprise the cavernous structure. This cavernous tissue of the lower nasolacrimal duct continues with that of the inferior meatus. The vessel area of the cavernous tissue is smaller in the sac and larger in the nasolacrimal duct with increasing density as we move inferiorly. More inferior the area, more advantageous for tear reabsorption. As the nasolacrimal duct is embedded in the canal, a change in the lumen width most likely results from a change in thickness of the duct wall rather than a change of the outer diameter. This anatomy creates a greater resistance for the tear drainage, which is advantageous for tear reabsorption.

1.7B. Autonomic regulation of the lacrimal fluid reabsorption

The sub epithelial tissue of the lacrimal sac and the nasolacrimal duct contains a lot of nerves, in which the autonomic nerves regulate mucosal thickness. As the nasolacrimal duct is encircled by bone, the mucosal thickness and the lumen diameter is in inverse proportion. That is to say, in a parasympathetic dominant state, the mucosa is thickened but the lumen gets smaller. At this time, as the drainage resistance becomes higher, the lacrimal fluid flows slower but effect of the tear reabsorption gets increased. To the contrary, in a sympathetic dominant state, because of thinning of the mucosa and enlargement of the lumen, the drainage resistance reduces, the lacrimal fluid flows faster, the tear drainage happens faster but the reabsorption gets lesser.

When a tear secretion is accelerated from the lacrimal gland for example by contact of ocular surface with a foreign body, that is to say, when the lacrimal fluid drainage needs to be blocked

to wash off the foreign body, the autonomic regulation inclines to a parasympathetic dominant. Then, an arterial flow increases but a drainage from the throttle vein decreases with more blood pooling in the capacitance veins. Therefore, the walls of the sac and the nasolacrimal duct are thickened and the lumen diameter gets smaller, which result in lesser flow but more effective tear reabsorption. On the other hand, when the ocular surface needs to be drier like in a situation of fight & flight, the sympathetic system predominates, the arterial flow decreases, drainage from the throttle vein increases with less blood pooling in the capacitance vein. Therefore, the walls of the sac and the nasolacrimal duct are thinned and the lumen diameter gets larger, resulting in acceleration of the tear drainage and keeping the ocular surface relatively drier for clearer vision for fight or flight!

1.8. Immune Mechanism of the Lacrimal Apparatus

1.8A. Immune mechanism in tears

The lacrimal fluid contains numerous anti-microbials like lactoferrin, lysozome and immunoglobulin's etc, and these block proliferation of pathogens by their bactericidal effects (Nakazawa 2012).

1.8B. Structural defense mechanisms

Antigens coming via the ocular surface are dealt with lacrimal fluid and various immune systems on the ocular surface. However, the lacrimal tract also needs to protect itself from a retrograde infection from the nasal cavity.

The notable is the existence of the nasolacrimal duct running beneath the nasal mucosa after leaving the osseous channel. Although all the cases do not show this kind of duct, invasion of pathogens may be considerably prevented mechanically by this structure. With cooperation from the dynamic lacrimal drainage and gravity, collagen fascicules, elastic and reticular fibers helically arranged from superiorly to inferiorly in the cavernous tissues of the sac and the nasolacrimal duct , all contributing effectively to drain the immune-rich lacrimal fluid inferiorly, resulting in defense against pathogens (Perra et al. 1995). However, this mechanism

works in a situation with thinned walls of the sac and nasolacrimal duct. It is hard to apply this theory to a situation with thickened walls of the sac and the nasolacrimal duct. Then, a mucous defense is vital for pathogen blocking. The cilia also contribute to form a one way flow from superiorly to inferiorly to prevent pathogens overgrowth.

1.8C. Mucous defense against pathogens

Density of the goblet cells increases as we descend towards inferior portions of lacrimal drainage system. That is to say, more inferior area can secrete more mucus. When the walls of the sac and the nasolacrimal duct are thick and gaining an appropriate tear velocity to exclude pathogens gets difficult, the mucus can make a functional plug at the lower site of the duct and prevent pathogens from invading retrograde from the nasal cavity. As the mucus contains lactoferrin, lysozyme and immunoglobulins, similar to the lacrimal fluid, defense against pathogens can be performed synergistically.

The mucus is secreted by the goblet cells in the epithelium, and prevents pathogens from adhering to the epithelium. This adhesion block is performed by a simple covering on the epithelium and besieging adhesive agents constituted by glycoproteins and/or glycolipids expressed on the surface of pathogens or toxins.

However, as some pathogens have enzymes which can dissolve the mucus, the pathogen can easily adhere to the epithelium in this situation. In addition, as the degradation products become nutrients for the pathogens, proliferation of the pathogens can get accelerated. Therefore, only a mucus defense is insufficient for pathogens, and humoral and cellular immunities are necessary (Knop and Knop 2001).

1.8D. Humoral and Cellular Immunity

The lacrimal tract contains a mucosa-associated lymphoid tissue (MALT) which is related to an antigen recognition and immune response (Knop and Knop 2001). This tissue functions as the main immune system. The lymphocytes and plasma cells constituting the MALT are sparsely distributed mainly in the lamina propria mucosae but some in the epithelium. This

tissue is thin in the canaliculi, but thick in the sac and the nasolacrimal duct.

The lymphatic follicles in the lacrimal mucosae are, in general, primary without a germinal center, but some show secondary follicles with germinal centers. As the germinal center emerges when lymphocytes with antigen stimulation proliferate actively, the ability of antigen recognition and other immune responses is weak in the lacrimal MALT. However, proliferation and differentiation of the IgA secreting plasma cells do not simply depend on obvious follicles.

The main source of the humoral immunity in the lacrimal tract is the secretory IgA. IgM and IgG, although much less volume than the secretory IgA, are also related to the lacrimal humoral immunity. The immunoglobulin covers the mucosal surface, prevents pathogens from adhering to the epithelium and makes them inactive, resulting in protection from the pathogens. In addition, the immunoglobulin's accelerates opsonisation, a process by which bacteria are altered so that they are more readily and more efficiently engulfed by the phagocytes. As the immunoglobulins in the lacrimal tract need to cover the broad mucosal surface, a secretory mechanism which does not depend on the germinal centers carries an important role against the pathogens. It has also been shown that the lacrimal drainage associated lymphoid tissue (LDALT) is altered in cases of chronic dacryocystitis and discussed the both cellular and humoral derangements that occur (Ali et al. 2013). Further studies on these could provide insights into LDALT and greater immunological understandings and possibly immune factors influencing lacrimal systems in health and disease.

Most lymphocytes in the lacrimal tract are T cells (Sirigu et al 2000). Although volume of the B cells is less than that of the T cells, B cells occasionally form lymphatic follicles. T cells show CD8 positive cells that are inhibitory and cytotoxic. Macrophages exist as well, distributed in the lamina propria mucosae and occasionally in the epithelium. Although the cellular immunity in the lacrimal tract appears less significant than the humoral immunity, the presence of MHC class II positive cells reflects its active role in the capture and presentation of antigens.

1.9. Recent Concepts of Significance

1.9A. Vertical Canaliculus Height (Fig 23)

The widely reported measurement of vertical canaliculus height is 2 mm, however most of these measurements were derived from cadaveric studies early on. That may not necessarily translate to what the actual measurements are in living individuals. Wawrzynski et al performed optical coherence tomography (OCT) of the proximal lacrimal drainage system and reported the mean vertical canalicular height to be 753 microns with a standard deviation of 247 microns with a wide range of 392-1242 microns (Wawrzynski et al. 2014). A normative database of the proximal lacrimal drainage morphometry using the Fourier Domain OCT (FDOCT) with En-Face imaging was published (Kamal et al. 2015). The maximum vertical canaliculus height in their series of 103 eyes was 1310 microns. However as compared to Wawrzynski, they reported higher mean values (890.41 microns), less standard deviation (154.76 microns) and less wide ranges (547-1310 microns). This disparity could be explained secondary to the active tone of the orbicularis muscle which may shorten the height of the vertical canaliculus in the living as compared to cadavers. Detailed studies of embryological development of the lacrimal system is likely to shed light on many of such newer concepts (El Shaarawy, 2014)

1.9B. Bony Lacrimal Fossa and NLD

Numerous racial variations are known to influence bony structure surrounding the lacrimal drainage apparatus (Gore et al. 2015; Bulbul et al. 2016; Paulsen et al. 2016; Maliborski et al. 2014; Takhashi et al. 2014; Young et al. 2014). Gore et al radiologically assessed the differences between black Africans (n=42) and Caucasians (n=30). The vertical height of the lacrimal fossa (LF) was significantly lesser ($p<0.001$) in Africans (mean 11.4 with SD of 1.5 mm) as compared to the Caucasians (mean 12.4 with SD of 1.3 mm). The maximum thickness of the frontal process of maxilla was significantly greater ($p<0.01$) in Africans as compared to Caucasians. Yong et al assessed the bony

nasolacrimal parameters and found no difference between different ethnicities (South Asian, Southeast Asians and Occidental races) in relation to the vertical lacrimal fossa diameters, anterior lacrimal crest thickness and narrowest portions of the nasolacrimal duct. However, the Southeast Asians had a wider inter-frontozygomatic suture distance than the other two groups. Decreased inter-frontozygomatic suture distance was directly correlating with the narrower nasolacrimal ducts. Takahashi et al classified bony NLD into two types based on their morphological configuration as 'funnel type' and 'hourglass type' . They found that patients with primary acquired nasolacrimal duct obstruction (PANDO) more often demonstrate a funnel type of bony NLD. The distance from the entrance of the bony NLD to its narrowest width was significantly shorter in patients with PANDO. Although most of these studies reflect on a possibility of NLD diameters as one of the causal factors for PANDO, a direct relationship has not yet been convincingly proven.

1.9C. Elasticity of lacrimal walls and sinus of Maier

Lacrimal wall elasticity has been proposed to be a possible factor that facilitates the lacrimal drainage functions. The distribution of the elastic fibers was recently studied (Kakizaki et al. 2014, 2015b) and it was found that it was quite variable based on anatomical locations. The area occupied by the elastic fibers was more in the intramuscular portions and Horner's muscle fascia as compared to the extra-sac extramuscular portions. The nature of the elastic fibers was different in areas with and without Horner's muscle fascia. The intramuscular portions of the canaliculi are thus hypothesized to be playing an important role in generating and maintaining various intra-canalicular pressures to facilitate tear flow from the ocular surface.

Sinus of Maier as explained earlier in the text is occasionally present near the opening of the common internal ostium into the lacrimal sac. The terminology and anatomical types have been confusing because of its use in different contexts by various authors (Kakizaki et al. 2015). Two types of Sinus of Maier have been well demonstrated

anatomically and histologically. The first type is akin to diverticula with larger diameter (1.29 mm) into which the canaliculi contribute separately. The second type had a smaller diameter (0.51 mm) and was solely contributed by terminal dilatation of the common canaliculus. This work could pave way for further understanding of the Sinus of Maier and its possible role in the tear flow dynamics (Mito et al. 2014, Takahashi et al. 2014b; Kakizaki et al. 2015b; Shams et al. 2016;).

1.9D. 3D Volumetric assessment and PANDO

Nasolacrimal diameters and their association with PANDO as causal factors have been controversial with literature supporting as well as refuting the associations (Estes et al. 2015; Janssen et al. 2000; McCormick et al 2009; Fasina et al. 2013; Ramey et al. 2013). Although NLD was measured three dimensionally earlier, Estes et al[60] showed a clear technique of 3D volumetric measurements of the nasolacrimal duct. It is interesting to note that they did not find any difference (p=0.23) between the patients (0.411±0.18 cm^3; n=35) and controls (0.380±0.13 cm^3; n=35) in their volumetric analysis. Although women had smaller volume as compared to men and male patients had smaller volume than male controls, it was not on expected lines to find the female patients had a larger volume as compared to female patients. The attempt to link NLD volumes with PANDO has been discouraged by this study. Nonetheless, it has paved way for further 3D volumetric analysis of both the bony and soft tissue lacrimal drainage system.

1.9E. Demonstration of Lacrimal Drainage Dynamics

The lacrimal drainage system's movements were demonstrated in real time following a Moh's excision for a medial canthal basal cell carcinoma (Shams et al. 2016). They demonstrated in real time with an open sky view, how with each blink, the canaliculi moved medially and the lacrimal sac laterally. Dacryoendoscopy has been recently used to view the canalicular and lacrimal sac movements with positive and negative pressures (Takahashi et al. 2014b; Kakizaki et al. 2015b). It was noted that there was a

consistent dilatation of the canaliculi with positive pressures and contraction with negative pressures. The common canalicular portion was more dynamic than the proximal canaliculi. The lateral wall of the lacrimal sac moved outwards with positive intraluminal pressures and inwards when subjected to negative pressures. These findings have contributed significantly in our understanding of lacrimal drainage dynamics

1.9F. Electron microscopy of the normal lacrimal passages

Electron microscopy is a very useful modality to study the anatomical ultra structure of the lacrimal drainage system (Ali et al. 2014). Scanning electron microscopy (SEM) of healthy lacrimal systems has shown demonstrable anatomical junctions between the distal portion of the punctum and the proximal most portion of the vertical canaliculus (**Fig 1.4**). Such anatomical junction was also noted between the lacrimal sac and nasolacrimal ducts. The mucosa of the canaliculus was occasionally thrown into folds with the surface showing rugae as compared to the normal smooth architecture (**Fig 1.5**). These are likely to represent the valvular structures of the lacrimal system. In the vicinity of the canaliculi, the orbicularis fibers were found to be very well organized in bundles (**Fig 1.6**). The fundus of the lacrimal sac showed very peculiar glands not found elsewhere and whose function is unknown.

Importance of Glycoproteins

Several tissues in the human body have been earlier characterized with respect to identification of specific proteins, their localization and functions. A large number of soluble proteins in different tissues have been shown to be glycoproteins that play an important role in various physiological and biochemical processes in the cells and several diseases have also been linked in humans due to lack or malfunctioning of specific glycoproteins.

My research objectives at the L.V. Prasad Eye Institute has been Dacryology, an area

which specifically deals with the lacrimal drainage system or the tear conduits from the eye to the nose. I head the Govindram Seksaria Institute of Dacryology. Numerous disorders of the lacrimal drainage system have not been understood with regards to their etiopathogenesis and clinical behaviours. We felt, Glycobiology is an area which has not been studied much with regards to the lacrimal drainage system. Therefore the present study is a first step towards understanding the structural-functional relationship of this system in relation to the presence of various glycoproteins and has the objectives as mentioned below in the scope of investigation. At the end of the thesis, the conclusions drawn on the interesting results obtained in this study which would provide us with leads for further experimentations and explorations.

1.10. SCOPE OF THE PRESENT INVESTIGATION

The core aim of the present thesis is to explore the Glycobiology of the human lacrimal drainage system. In this context we planned to outline our research work into five parts as follows:

A. Exploring the Surfactant proteins in the human lacrimal canaliculus.
B. Ultrastructural features of lacrimal sac and nasolacrimal duct mucopeptide concretions.
C. Qualitative hormonal receptors analysis of the human lacrimal drainage.
D. Exploring the presence of Mannose 6 Phosphate receptors and lysosomal enzyme assay within a non-diseased lacrimal drainage pathway.
E. Exploring the various major glycoproteins in the lacrimal drainage tissues.

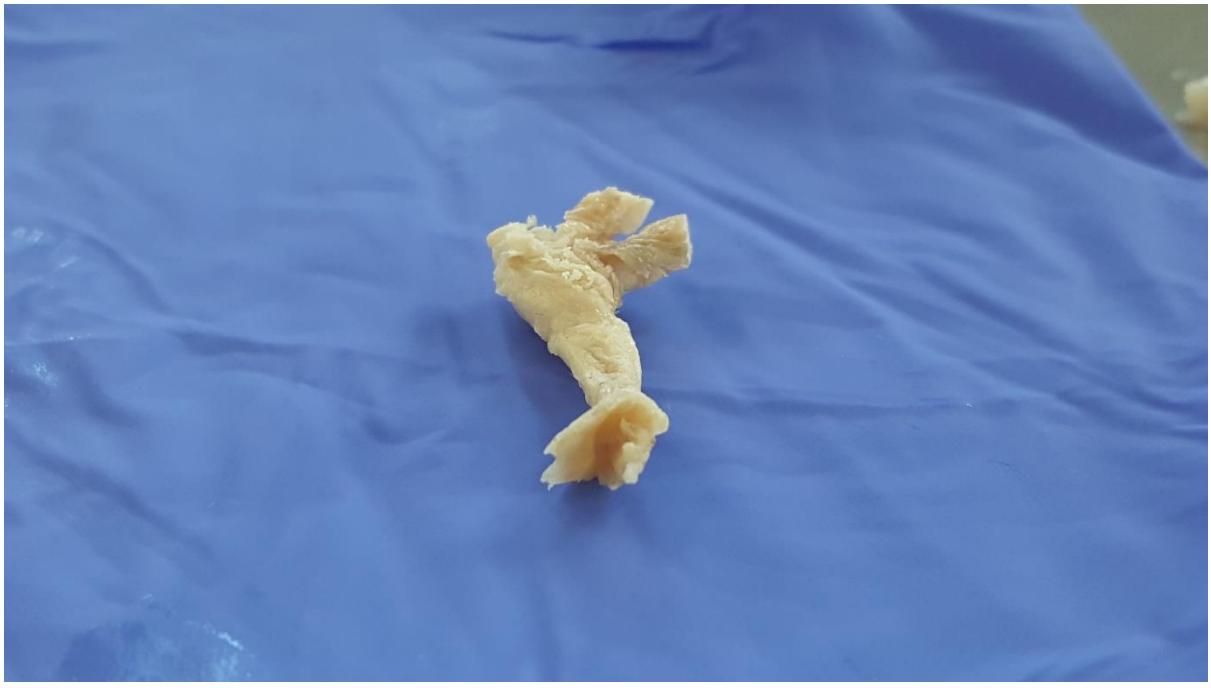


Fig 1.1: Gross Cadaveric specimen of the entire lacrimal drainage system from the canaliculus to the nasolacrimal duct opening.

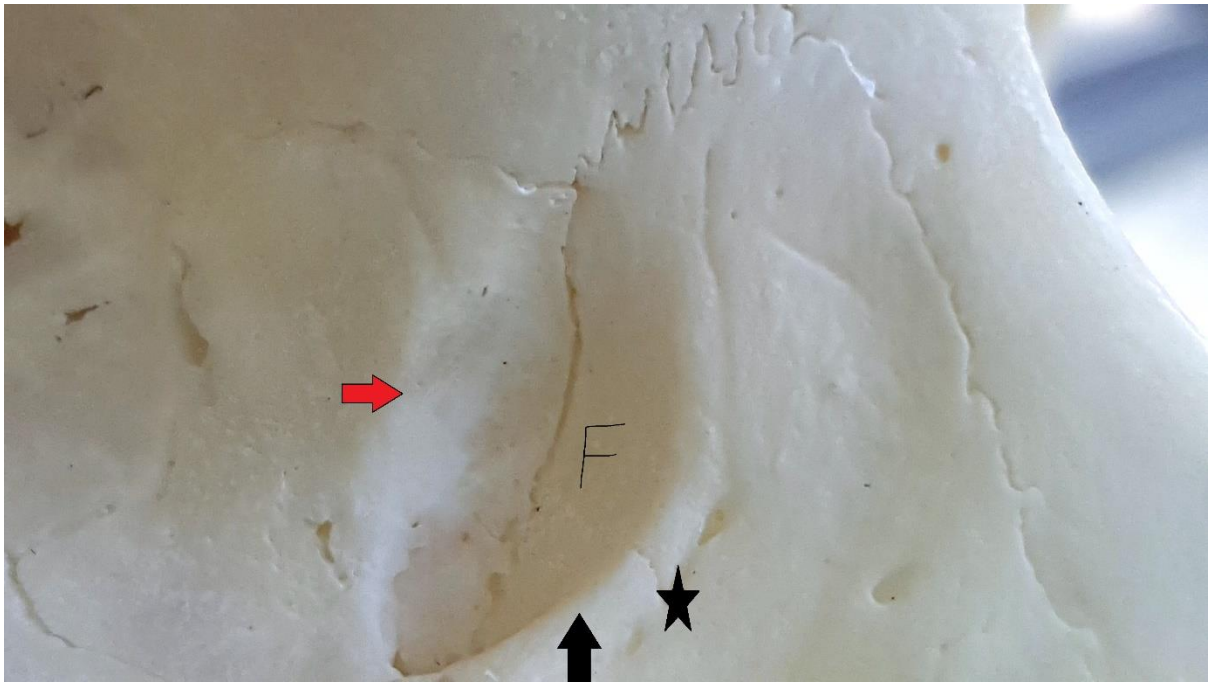


Fig 1.2: High magnification image of a skull showing the bony lacrimal sac fossa. Note the sutura notha (black star), anterior lacrimal crest (black arrow), posterior lacrimal crest (red arrow) and bony fossa (F).

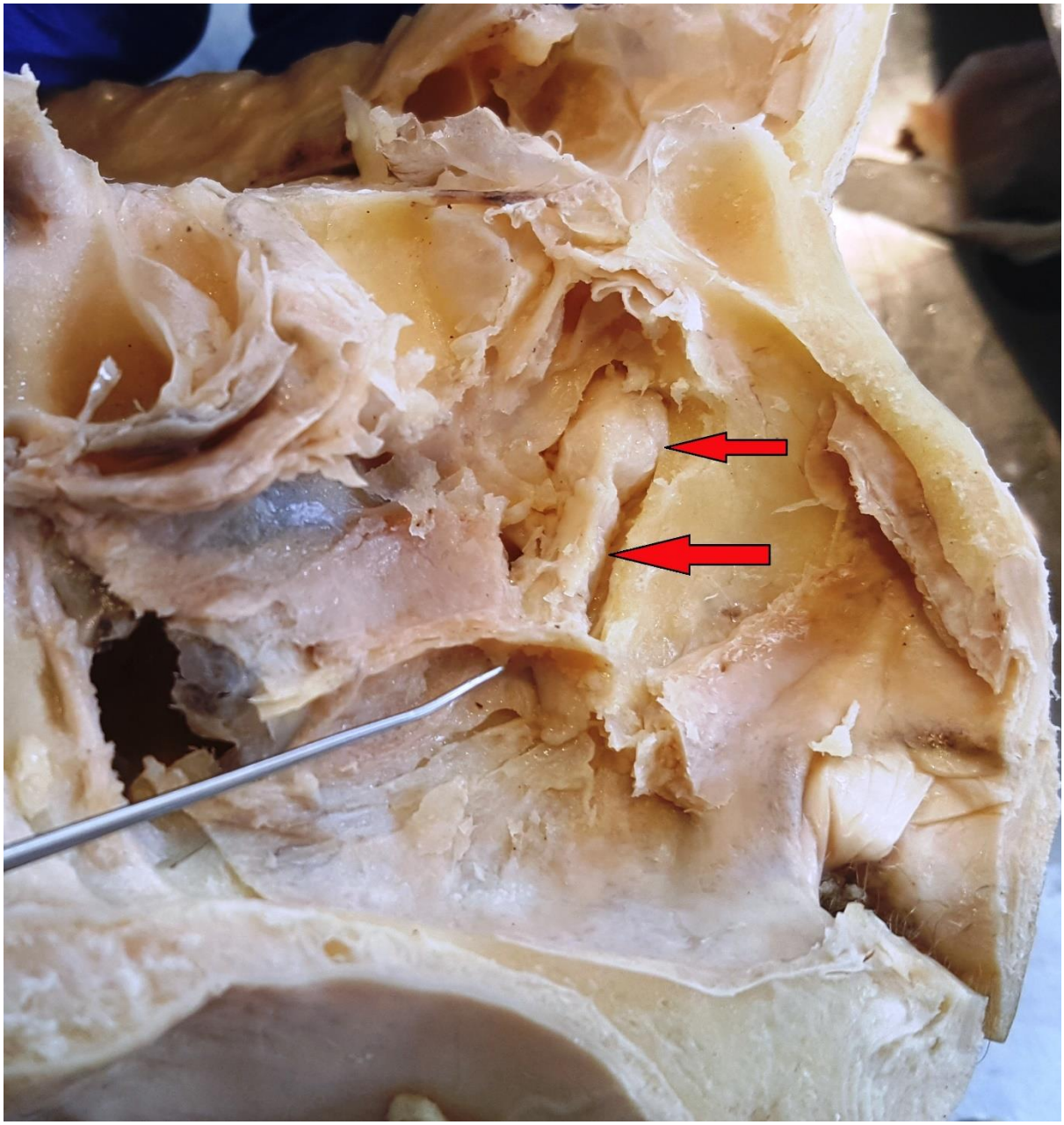


Fig 1.3: Cadaveric lateral nasal wall image demonstrating the lacrimal sac (thin red arrow), nasolacrimal ducts (large red arrow) and nasolacrimal duct opening into the inferior meatus (instrument pointing it).

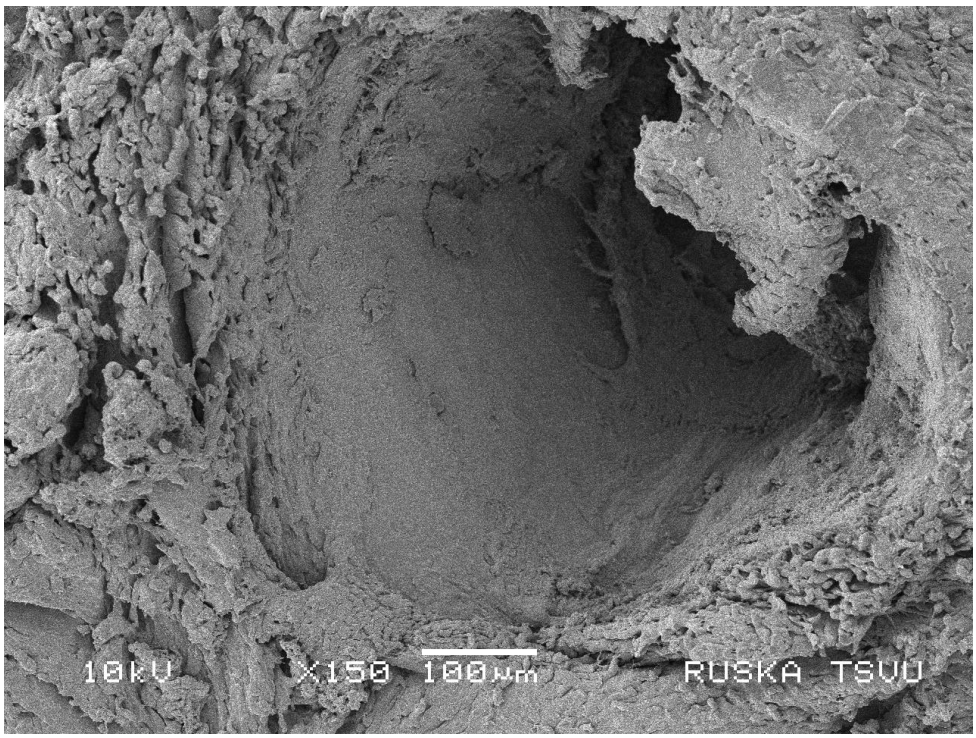


Fig 1.4: Scanning electron microscopic image of the punctum. Note the junction between the external and internal punctum (SEM x150).

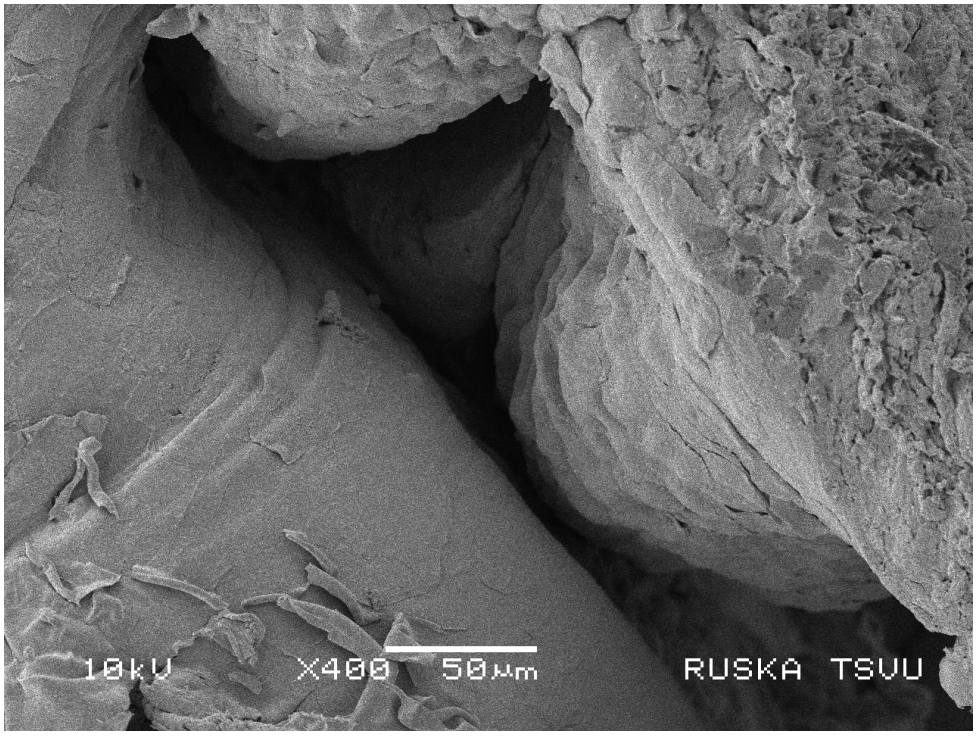


Fig 1.5: SEM image of the horizontal canaliculus. Note the mucosal folds (SEM x400)

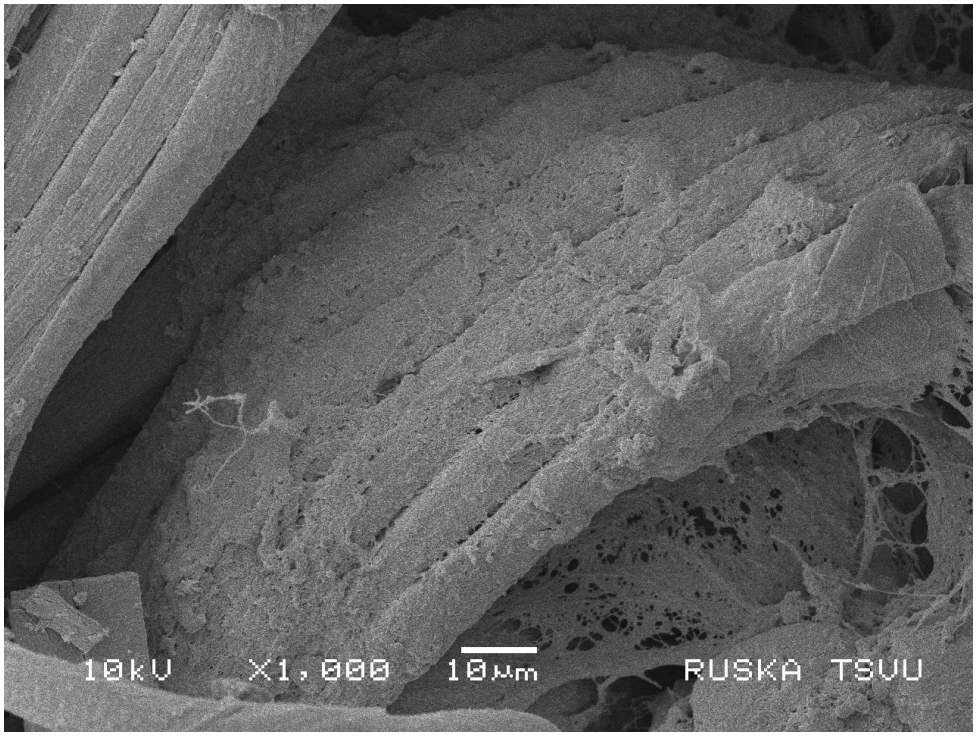


Fig 1.6: SEM image of the pericanalicular area demonstrating the well structured Horners muscle (SEM x1000).

CHAPTER 2: CANALICULAR SURFACTANTS

2.1. Introduction

Surfactant is a complex mixture of dipalmitoylphosphatidylcholine (DPCC), other lipids and proteins produced by type II alveolar epithelial cells and stored in specific intracellular lamellar bodies (Yu et al. 2003; Halliday et al. 2008). Extensively studied in the lungs, the surfactants are crucial for pulmonary physiology and mainly function to reduce the surface tension and

prevent alveolar collapse (Amigoni et al. 2017; Halliday et al. 2008). They have also been isolated from extra-pulmonary tissues like larynx, Eustachian tube, oral cavity, brain, digestive system, kidney cell lines, testis, ocular surface, tears and nasolacrimal ducts (Sheats et al. 2016; Beileke et al. 2015; Schob et al. 2013; Bräuer et al. 2007a, 2012; Schicht et al. 2010, 2013; Schröder et al. 2017). Four major surfactant proteins, SP-A, SP-B, SP-C and SP-D are well studied with SP-A and SP-D demonstrating additional immunological functions (Nathan et al. 2016; Whitsett et al. 2005; Kingma et al. 2006;). Recently whole genome sequencing and bioinformatics sequence analysis have revealed the presence of additional two novel putative surfactant proteins; SP-G or surfactant associated protein 2 (SFTA2) and SP-H or surfactant associated protein 3 (SFTA3) (Rausch et al. 2012, 2014; Schicht et al. 2014). Although the specific roles of these are yet unclear, SFTA3 is known to mediate defense mechanisms like phagocytosis (Diler et al. 2014; Tschernig et al. 2016). The current study looks into the expressions and distribution patterns of surfactant proteins in the canalicular system and attempts to hypothesize their potential roles in tear flow and lacrimal drainage defense mechanisms.

2.2. Methods

The study was performed on eight fresh frozen cadaveric canaliculi (2 males, 2 females; age range 57-74). Cadaveric samples were obtained from bodies donated to the department of Anatomy, Friedrich Alexander University Erlangen-Nürnberg, Germany, after obtaining appropriate consents and ethical approvals. All methods for securing cadavers and their subsequent dissection were humane and complied with the Tenets of Declaration of Helsinki. Prior to dissection, the detailed medical history of each cadaver was studied. None of the cadavers had a history of lacrimal or nasal disorders, trauma or nasal surgery. The canaliculi were dissected in toto and immediately frozen at -80 ^{0}C for molecular biological investigations. Cryo-sectioned human lacrimal canaliculi were subjected to antigen retrieval techniques. Each primary antibody (1:50 – 1:100) was applied overnight at room temperature. The secondary antibodies (1:1000) were incubated at room temperature for at least 4 hours. Visualization was

achieved with diaminobenzidine (DAB) for at least 5 min. After counterstaining with hemalm, the sections were mounted in Aquatex (Boehringer, Mannheim, Germany). For immunofluorescence the secondary antibodies, respective fluorescein conjugated antibodies (Alexa 488, green and Rhodamine, red) diluted 1:1000 with TBS were used. Sections of human lung were used as the positive control. The slides were examined with a Keyence Biozero BZ81000E microscope.

2.2A. Antibodies

The antibodies used were as follows: mouse anti-human surfactant protein A (SP-A) monoclonal antibody, 1:50 (MAB3270; Chemicon-Millipore, Hampshire, UK); mouse anti-human surfactant protein B (SP-B) monoclonal antibody, 1:50 (MAB3276; Chemicon-Millipore, Billerica, USA); rabbit anti-human SP-C (proSP-C) monoclonal antibody, 1:50 (AB3786; Chemicon-Millipore, Billerica, USA); rabbit anti-human SP-D monoclonal antibody. 1:50 (BM4083; Acris, San Diego, USA); rabbit anti-human SP-G polyclonal antibody, 1:100 (Seqlab, Göttingen, Germany); goat anti-human SFTA3,1:100 (sc-248576, Santa Cruz Biotechnology, Dallas, USA). Secondary antibodies: anti-rabbit*/anti-mouse** IgG, respectively, conjugated to horseradish peroxidase (*P0448; Dako, Glostrup, Denmark/**80807; Dianova, Hamburg, Germany), anti-rabbit*/anti-mouse** IgG, respectively, conjugated to Alexa-Fluor 488 and Rhodamin-Red (*31665, **A11001, Thermo Fisher Scientific/life technologies). All antibodies mentioned were used as specified by the manufacturers. The antibodies used were highly specific and have been successfully used earlier (Sheats et al. 2016; Beileke et al. 2015).

2.2B. Western blot analysis

Samples of fresh frozen canaliculi were used for Western blot techniques as described by Sheats et al[4] earlier. In brief, following homogenization, the protein content was measured using the Bradford assay. 30 µg of proteins were analyzed by Western blot and they were resolved by using 15% SDS polyacrylamide electrophoresis and were electrophoretically

transferred at room temperature onto 0.45 µm nitrocellulose paper. Bands were detected by primary and secondary antibodies mentioned above applying chemiluminescence (ECL plus; Amersham Pharmacia, Uppasala, Sweden). The molecular weights of the detected protiens were estimated using standard proteins (Pre-stained Protein Ladder, Fermentas, Germany). Human lung tissue was used as control and were treated and incubated at same conditions as that of experimental samples.

2.3. Results

Fluorescence double staining with DAPI and surfactant proteins showed distinct immunoreactivity for SP-A, SP-B, SP-C, SP-D and SP-H/SFTA3 (**Figs 2.1-2.3**). The expression of proteins was noted across all the layers of the epithelium especially on the surfaces and superficial cytoplasm of the superficial and deep epithelial cells but not within the sub-epithelial structures. However, there were subtle differences in the distribution among the various surfactant proteins. SP-A expression was noted in all the epithelial layers but very few basal cells showed positivity (**Figs 2.1A-2.1C**). SP-B expression was noted in the superficial layers and absent in the basal epithelial layer (**Figs 2.1D-2.1F**). SP-C (**Figs 2.2A-2.2C**), SP-D (**Figs 2.2D-2.2F**) and SP-H (**Figs 2.3D-2.3F**) revealed expression across all epithelial layers. A uniformly negative immunoreactivity was noted across the canalicular system for SP-G/SFTA2 (**Figs 2.3A-2.3C**). Western blot analysis showed distinct bands for SP-A at 60 KDa, for SP-B at 40 KDa, for SP-C at 21 and 26 KDa, for SP-D at 43 KDa, for SP-H (SFTA3) at 36 KDa on samples from fresh frozen canaliculi (TC) (**Fig 2.4**). The bands for SP-C were a little blurred owing to the hydrophobicity of the protein. There were no distinct bands at 30 KDa for SP-G (SFTA2) (**Fig 2.4**). Lung tissues (LU) were used as positive controls and showed distinct bands at the same molecular weights as the canaliculus samples.

2.4. Discussion and Hypothesis

This study has provided a proof of principle of the presence of multiple surfactant proteins in the canalicular system. Their presence, differential expression and distribution patterns have led to potential insights into the tear flow dynamics and defenses of the proximal lacrimal drainage system, which needs to be further investigated in detail.

SP-A and SP-D are large hydrophilic surfactant proteins and belong to C-type lectin family and are known to interact with the carbohydrate moieties on the surfaces of different organisms predisposing them to opsonization and phagocytosis (Nathan et al. 2016; Whitsett et al. 2005; Kingma et al. 2006). SP-B and SP-C are small hydrophobic proteins which regulate the insertion of new lipids into an existing system and hence essential for stability of lipid monolayers at air-fluid interfaces (Rausch et al. 2014). They decrease the surface tension of the surface and maintain the structures which they coat. In contrast SP-G and SP-H are small hydrophobic proteins like SP-B and SP-C but are made of different domains (Rausch et al. 2014; Schicht et al. 2014). Although SP-G and SP-H contribute to surface stability by reducing the surface tensions, the latter (SP-H) is being increasingly recognized for its role in immunological pathways (Diler et al. 2014; Tschernig et al. 2016).

Molecular biology investigations in the past have demonstrated SP-A, B, C and D in the tear fluid as well as the nasolacrimal duct (Bräuer et al. 2007a, 2007b). They were demonstrated in the cytoplasm of the high columnar epithelial cells of lacrimal sac and nasolacrimal duct without any differences. Reactivity was negative in goblet cells and intra-epithelial mucinous glands. The current study investigated the previously unexplored canalicular system with all the known six surfactant proteins and found strong expressions of all except SP-G.

The canalicular system is an important transit pathway for tears from the wide ocular surface to the narrow lacrimal drainage system. The widespread presence of surfactant proteins on the epithelial face of the canaliculi could play a very important role in the flow of tears towards the lacrimal sac. The tear spread on the ocular surface is not only the function of surfactant proteins but as a result of many other molecular and mechanical factors. Hence tear surfactants alone may not be able to overcome the surface tension of the canalicular system and the localized SP's here may help in formation of additional lipid monolayers. This in turn could prevent tiny

individual droplet formation and enhance flow of tears as a stream distally towards the lacrimal sac.

All the three surfactant proteins (SP-A, SP-D and SP-H) involved in the innate immune mechanisms have been found in the canalicular system. The hypothetical role of these proteins in lacrimal drainage defenses should be assessed in the light of their major regulatory effects on opsonization, modulation of phagocytosis, release of pro-inflammatory cytokines and reactive oxygen species and peri-natal immune modulation (Schicht et al. 2014; Diler et al. 2014; Sano and Kuroki 2005). Since most of these pathways lead to microbial clearance, it is believed that SP's especially SP-H (SFTA3) may be involved in mucosal protection during mucosal inflammation (Tschernig et al. 2016). It is also important to note that surfactant proteins are well demonstrated at sites of barriers of immune privileged organs like testis (Beileke et al. 2015) and this may partly reflect on their immune surveillance roles.

Animal models of lung infections have shown selective alterations in the levels of SP-A and SP-D and similar increased expression was also noticed in patients with periodontal infections and rhinosinusitis as compared to healthy individuals, further cementing their possible roles in mucosal defenses (Atochina et al. 2000, 2001; Schicht et al. 2015). In addition, SP-A knockout animal models have shown poor phagocytosis, poor microbial clearance and susceptibility to infections (Veith et al. 2014). Hypothetically, canalicular system could mount the first line innate defense against micro-organisms and allergens that gets washed into the lacrimal drainage from the ocular surface and SP's could be of paramount importance in these mucosal defenses as seen in oral and nasal mucosa.

The limitations of the current study include lack of quantitative analysis and the current speculative nature of the hypotheses that needs further validation. However, the strengths of the study include wide assessment of all known surfactant proteins and providing the proof of principle of surfactant microenvironments.

In conclusion, surfactant proteins from the lung are expressed in the human canaliculus. It is important to understand that the proposed roles of surfactants are only one of the many factors that influence the lacrimal drainage defenses and the tear flow dynamics. The widespread

presence of surfactants within the lacrimal drainage system opens up exciting newer avenues for further exploration.

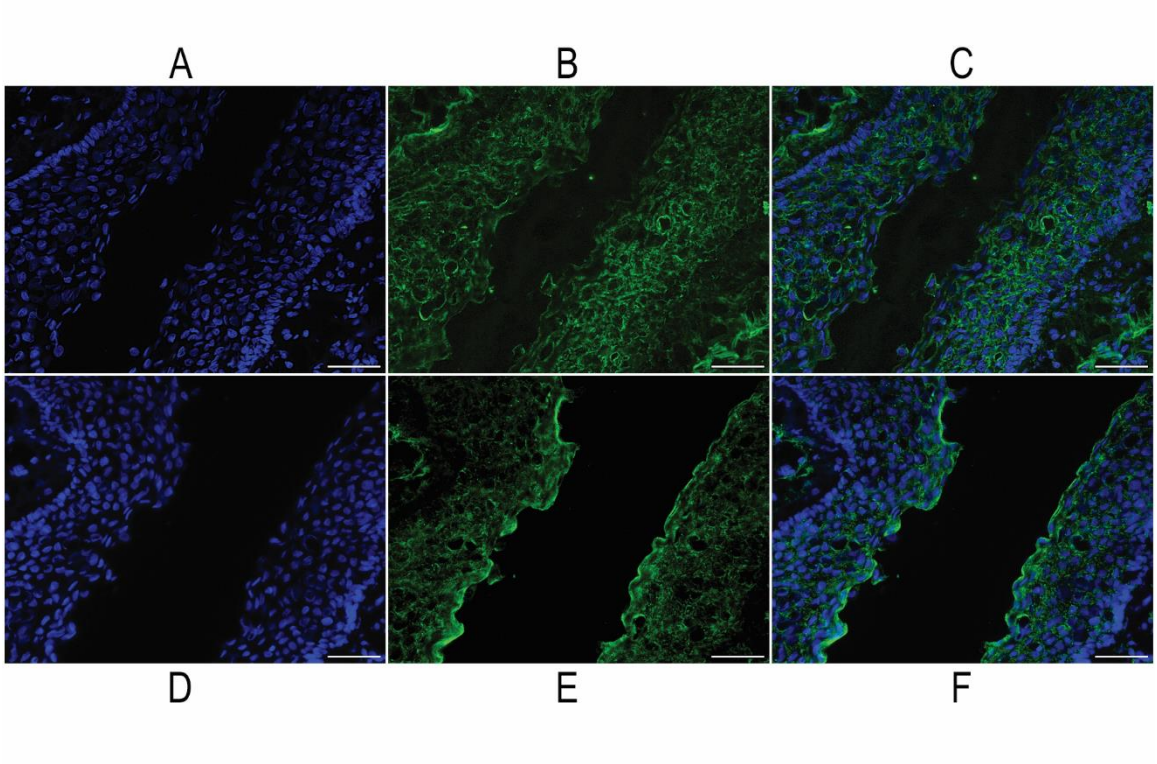


Figure 2.1: Canalicular expression of SP-A and SP-B: Panels A, B and C represent SP-A and Panels D, E and F represent SP-B. Microphotographs showing fluorescence double staining with DAPI (blue) (Panels A and D) and surfactant protein (green) (Panels B and E) and merge (Panels C and F). The merge microphotographs clearly demonstrate the strong immunostaining on the surfaces and cytoplasm of the epithelial cells. Also note that very few basal cells express SP-A and none express SP-B. (x400, bar - 50µm).

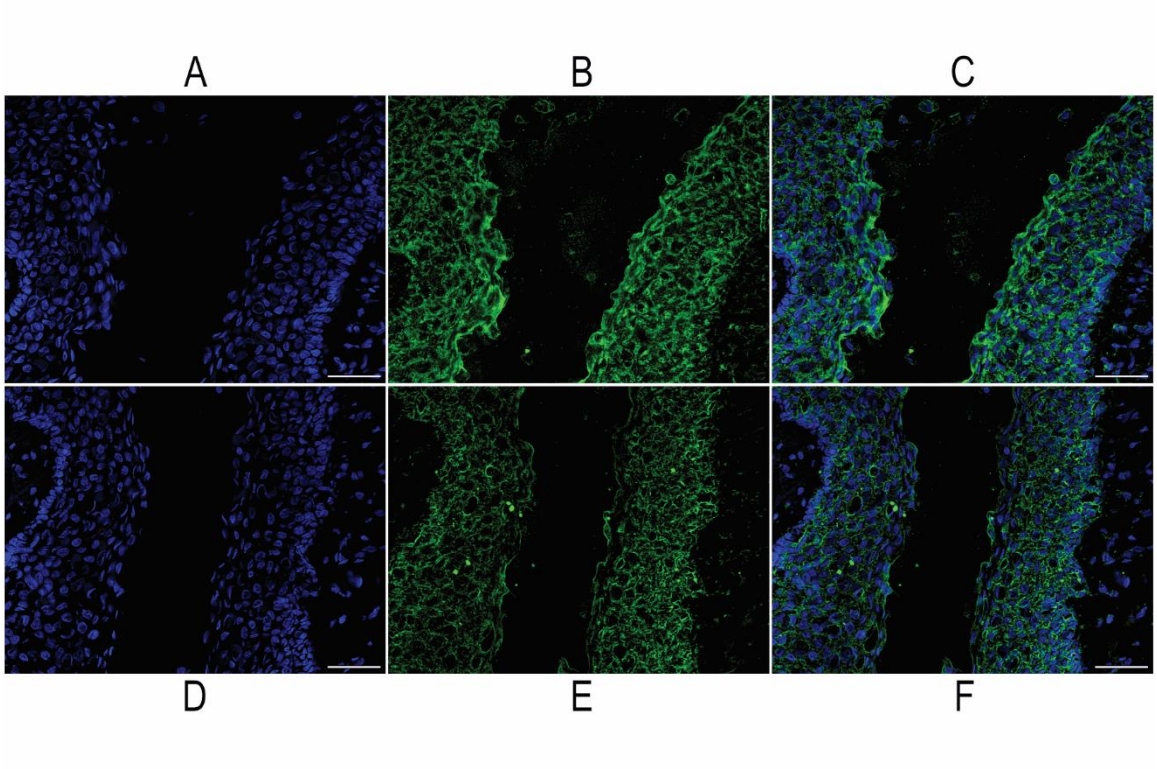


Figure 2.2: Canalicular expression of SP-C and SP-D: Panels A, B and C represent SP-C and Panels D, E and F represent SP-D. Microphotographs showing fluorescence double staining with DAPI (blue) (Panels A and D) and surfactant protein (green) (Panels B and E) and merge (Panels C and F). The merge microphotographs clearly demonstrate the positive immunostaining on the surfaces and cytoplasm of the all the epithelial cells including the basal cells (x400, bar - 50µm).

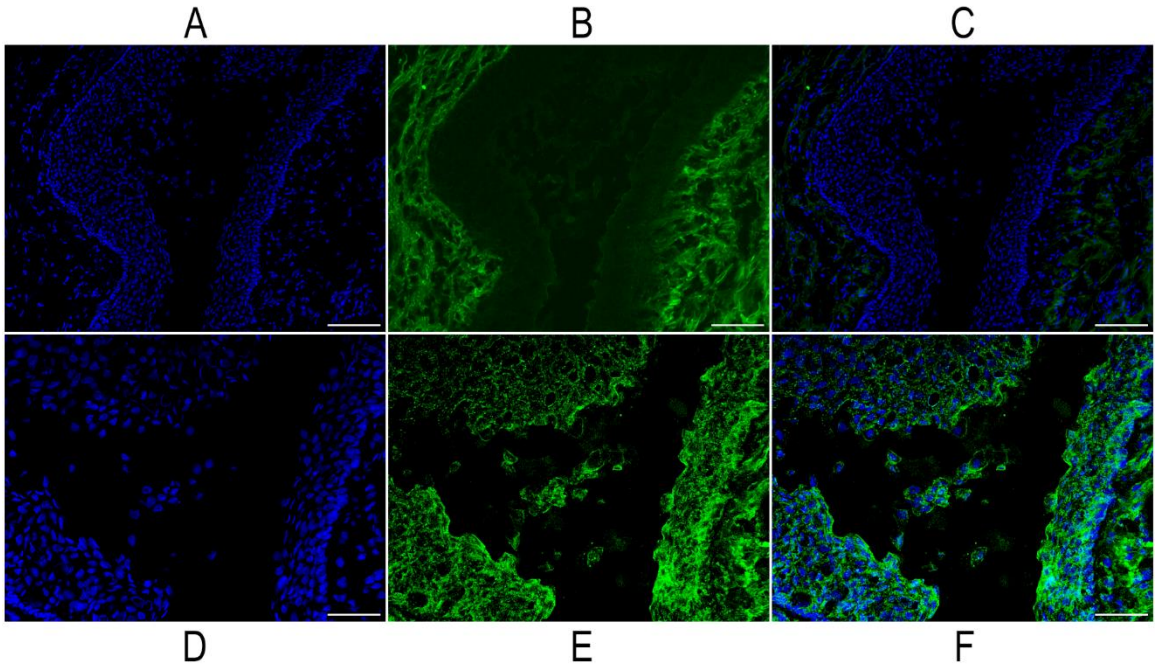


Figure 2.3: Canalicular expression of SP-G (SFTA2) and SP-H (SFTA3): Panels A, B and C represent SP-G and Panels D, E and F represent SP-H. Microphotographs showing fluorescence double staining with DAPI (blue) (Panels A and D) and surfactant protein (green) (Panels B and E) and merge (Panels C and F). The merge microphotograph clearly demonstrates the uniformly negative immunostaining for SP-G/SFTA2 across the epithelium (Panel C) and strong positive immunostaining for SP-H/SFTA3 on the surfaces and cytoplasm of the epithelial cells including the basal cells (Panel F) (Panels A-C x200, bar - 100µm; Panels D-F x400, bar - 50µm).

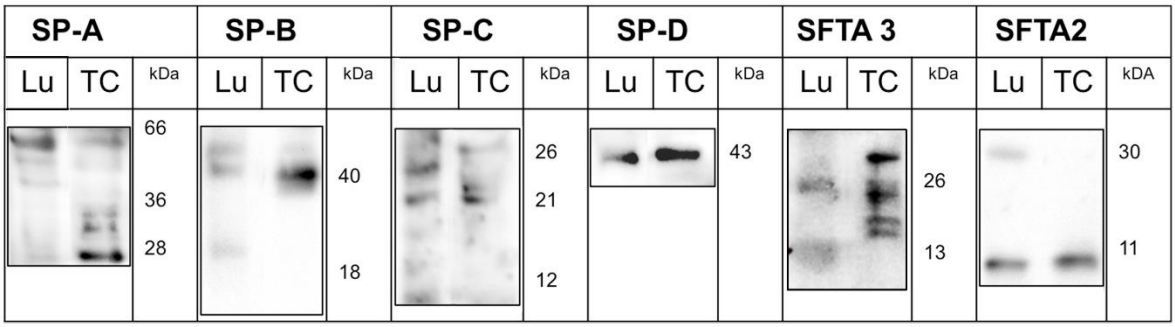


Figure 2.4: Western blot for SP-A to SP-H. Western blot analysis showing distinct bands for SP-A at 60 KDa, for SP-B at 40 KDa, for SP-C at 21 and 26 KDa, for SP-D at 43 KDa, for SP-H (SFTA3) at 36 KDa after reducing SDS polyacrylamide electrophoresis using samples of fresh frozen canaliculi (TC). There were no distinct bands at 30 KDa for SP-G (SFTA2). Lung tissues (LU) were used as controls.

CHAPTER 3: ULTRASTRUCTURE OF MUCOPEPTIDE CONCRETIONS

3.1. Introduction

Dacryolithiasis is not a very uncommon occurrence in the lacrimal drainage system with the reported rates of its presence ranging from 6-18% amongst patients undergoing endoscopic dacryocystorhinostomy (Mishra et al. 2017; Repp et al. 2009; Kominek et al. 2014). The lacrimal concretions can be broadly divided into mucopeptide and bacterial types based on their location, histomorphic, histologic and microbiological assessment (Perry et al. 2012). Ultrastructural features of lacrimal concretions are not very well studied and limited to two studies that used scanning electron microscopy (Orhan et al. 1996; Kominek et al. 2014). To get deeper insights into the etiopathogenesis of dacryolithiasis, we focused in the present

investigation on the ultrastructural features and demonstrated detailed features of both transmission and scanning electron microscopy. For this we studied bulk of mucopeptide concretions (earlier called as dacryoliths) obtained from the lacrimal sac during a dacryocystorhinostomy (DCR).

3.2. Methods

3.2A. Study design and sample collection

Institutional review board approval was obtained prior to the commencement of this study. Mucopeptide concretions (earlier called lacrimal sac dacryoliths) obtained from the lacrimal sac during a dacryocystorhinostomy (n= 10, 9 females and one male, mean age 46.6 years; range 24-73 years) were immediately fixed for scanning and transmission electron microscopic analysis. The surfaces were studied separately and longitudinal and transverse ultra-thin sections were obtained at different levels and all were studied using standard protocols.

3.2B. Scanning electron microscopy (SEM) protocol

For scanning electron microscopy (SEM) specimens were fixed in a solution of 4% formalin before being processed as previously reported (Priebe et al. 2006). Specimens were then impregnated with tannic acid for 2 days. Counter-fixation in 2% osmium tetroxide for 4 hours was followed by dehydration in ethanol and drying in a critical point dryer (Leica EM CPD 300; Leica, Germany). The preparations were coated with gold (Leica EM ACE 600, Leica, Germany) and examined with a scanning electron microscope (JEOL JSM-IT300LV, JEOL Ltd, Tokyo, Japan).

3.2C. Transmission electron microscopy (TEM) protocol

Mucopeptide concretions were processed for TEM, according to a protocol reported previously (Priebe et al. 2006). In brief, specimen were fixed in Ito fixative (2.5%

glutaraldehyde, 2.5% paraformaldehyde, and 0.3% picric acid dissolved in phosphate-buffered saline, pH 7.3) and embedded in Epon. Semi-thin sections (1µm thick) were cut with a microtome (Ultracut E; Reichert Jun, Vienna, Austria) and subsequently stained with toluidine blue. Sections were viewed with an epifluorescence microscope (Aristoplan; Enrst Leitz, Wetzlar, Germany) and imaged (DC 500 camera; Leica Microsystems, Wetzlar, Germany). Ultrathin sections were stained with uranyl acetate and lead citrate and viewed with a transmission electron microscope (JEM-1400 Plus electron microscope, JEOL Ltd, Tokyo, Japan).

3.3. Results

Mucopeptide concretions based on their extent takes the shape of the lacrimal sac and nasolacrimal duct (**Figs 3.1A and 3.1B**). The external surfaces and cut sections show mostly areas of homogenous, amorphous acellular deposits with occasional intervening rough heterogeneous areas (**Figs 3.1C and 3.1D**). The external surfaces, abutting the lacrimal sac walls, on higher magnification were rougher and less contiguous whereas those within the nasolacrimal ducts were smoother and contiguous (**Figs 3.1E and 3.1F**). Cut sections revealed numerous craters on the surface, more obvious in the heterogeneous areas (**Fig 3.2A**). Most of the craters had multiple, non-continuous perforations with few cells within its realms (**Figs 3.2B and 3.2C**). However, some of the small craters did not show any perforations or blood cells within them (**Fig 3.2D**). Numerous vacuoles and fissures appear to be more of artifact than any metabolic process as speculated earlier (Kominek et al. 2014). These could be ascribed to either cutting artifacts (**Fig 3.2E**) or drying artifacts (**Figs 3.2F and 3.3A**). The core of the concretions is made up of extensive networks of fibrillary tangles filled predominantly with granular material and red blood cells with occasional presence of granulocytes and epithelial cells (**Figs 3.3B to 3.3F**). No organic fibers of fungal filaments were noted within the concretions. There was no evidence of any bacterial biofilms other than few focal areas of scattered bacteria.

3.4. Discussion and Hypothesis

The current study examined the SEM and TEM features of mucopeptide concretions obtained from the lacrimal sac during dacryocystorhinostomy. Distinct features of surfaces, cut sections and internal architecture were elucidated uniformly in this study. The widespread presence of sub surface vacuoles and focal fissures could represent cutting and drying artifacts during preparation for histology and electron microscopy and not metabolic gas vacuoles as speculated earlier.

Dacryoliths or mucoliths or canaliculoliths or calculi are the various names attributed to concretions obtained from the lacrimal drainage system. An earlier detailed work proposed the discontinuation of these terms in favor of more descriptive terms like mucopeptide or bacterial concretions (Perry et al. 2012). The reasons for this change are the lack or minimal presence of hard mineralized components like calcium and hence inadequate description using the old terminology (Iliadelis et al. 2006). However, it is important to keep in mind that there are few conflicting reports, albeit anecdotal, which found significant proportion of inorganic components (Ozer et al. 2012; Kaye-Wilson et al. 1991). Mucopeptide concretions predominantly originate in the lacrimal sac and are composed of amorphous acellular material arranged in lamellae. The surface is mostly smooth and moulds to the lacrimal sac walls (Perry et al. 2012). The concretions are soft when freshly explanted but may become hard following chronic retention or fixation for histopathology. They are generally bereft of cellular components and contain numerous sub-surface vacuoles. In contrast the bacterial ones are located predominantly in the canaliculi, are friable, and composed of masses of gram positive bacteria, filamentous bacteria like *Actinomyces* and occasionally fungal filaments (Perry et al. 2012). Acute or chronic inflammatory cellular infiltrates are usually noted in and around a bacterial concretion. Although the current ultrastructure study on mucopeptide concretions confirmed most of the earlier described histological features, it found that the core internal architecture was not bereft of cellular components but instead had a dense packing of predominantly red blood cells.

Paulsen et al studied the dacryoliths obtained from the lacrimal sac (mucopeptide concretions) of patients during dacryocystorhinostomy applying immunohistochemistry with

numerous antibodies and found that the major components are mucins (MUC) as well as trefoil factor peptides (TFF) (Paulsen et al. 2006a). There was augmentation of all the three TFF peptides; TFF1, TFF-2 and TFF-3. Types of mucins detected in dacryoliths were MUC 1, 2, 3, 4, 5AC, 5B, 6, 7, 8 and 14, although type 14 was the most predominant. Lymphocytes, macrophages and secretory phospholipase A2 were seen only in one of the 15 dacryoliths studied. It is important to note that these mucins and TFF peptides are components of not only the tears and the tear film (produced by ocular surface structures and glands like lacrimal and accessory lacrimal glands) but also produced by the lacrimal sac and nasolacrimal ducts (Paulsen et al. 2006b). They are known to play roles in mucosal epithelial integrity, rheological functions as well as in antimicrobial defenses (Paulsen et al 2006a, 2006b, 2006c).

The internal core architecture of all the mucopeptide concretions showed an extensive network of fibrillary tangles densely packed with predominantly red blood cells. Since this finding was uniformly noted across all the concretions studied, it is hypothesized that micro trauma to the luminal surfaces, secondary to mechanical or chemical factors may be the triggering agent for their formation. These areas of trauma with the leaked blood could then act as nidus. The epithelial trauma may then trigger a cascade of protective events resulting in enhanced mucin and peptide production, which progressively gets deposited around the core nidus leading to formation of a mucopeptide concretion. These mucin and peptide deposits could originate from their upregulation in the tears as well as from the lacrimal drainage epithelia itself. The alterations of rheological properties secondary to Mucin and TFF peptide changes can facilitate more deposition. In addition, washed cellular debris from the ocular surface, local lacrimal cellular debris and dirt particles in tear films could also deposit around the nidus and contribute to concretion formation.

Orhan et al studied SEM features of a single dacryolith and found them to be composed of lobes and lobules around an amorphous organic core. Kominek et al studied the SEM features of 7 dacryoliths and found them to take the shape of internal lumen of the lacrimal sac and nasolacrimal duct. However, unlike Perry et al, they found the surfaces to be rough with

ridges and notches caused by lacrimal mucosa. The SEM showed dacryoliths to be composed of core of amorphous organic matrix with lobular surfaces. Three of the seven dacryoliths showed organic fibers in the central areas. An electron microprobe with an energy dispersive detector could identify rare dispersion of inorganic components within the organic matrices (Kominek et al 2014). They also found numerous variably sized spaces and hypothesized them to be either gaseous by-products of metabolic processes or artifacts. Our present study like that of Kominek et al found smooth homogenous and rough heterogeneous surfaces, however did not show any organic fibers within the dacryolith, as found earlier. In addition, two distinct types of craters were found, mostly within heterogeneous areas. TEM features were more suggestive of the sub-surface vacuoles actually being cutting artifacts rather than gas bubbles and the fissures to be drying artifacts. However, the possibility of them being tear fluid inclusions secondary to altered rheology, cannot be entirely excluded (Paulsen et al 2006c).

In conclusion, ultrastructural features of mucopeptide concretions from the lacrimal sac help in better understanding of their etiopathogenesis and tissue interactions. The current study proposes a hypothesis of their genesis, which needs to be validated by further experimentations and explorations. It is proposed that uniform scientific terminologies like mucopeptide concretions and bacterial concretions should be used.

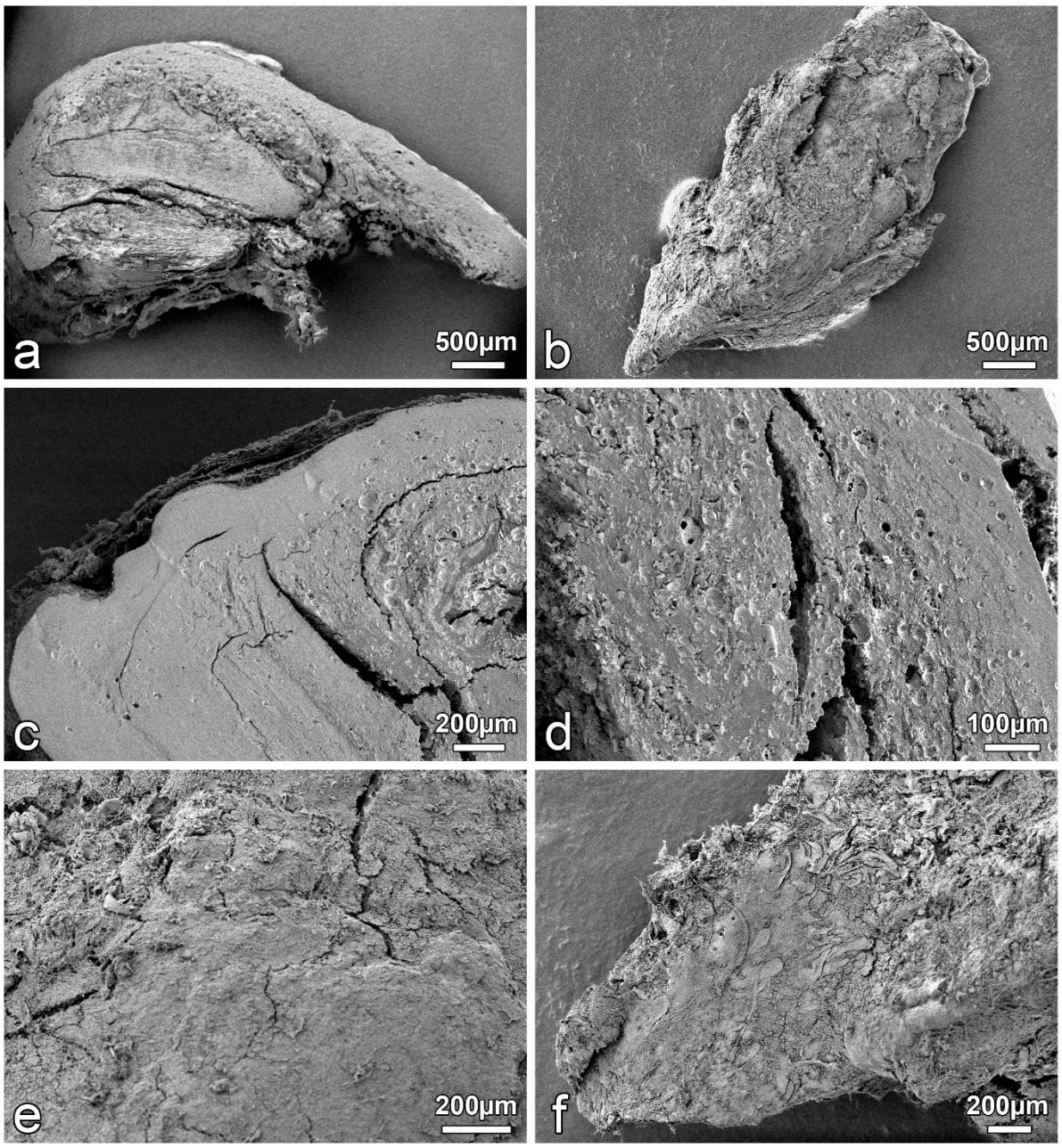


Figure 3.1: Ultrastructure of Mucopeptide concretions. Scanning electron micrograph (SEMG) showing concretions taking the shape of the lacrimal sac and the nasolacrimal duct (**Figs 1A and 1B**). Cut sections of the concretions showing homogenous areas of amorphous material (**Fig 1C**) and heterogenic rough areas (**Fig 1D**). SEMG of the rough external surface abutting the lacrimal sac walls (**Fig 1E**) and the relatively smoother surfaces of the portion in the nasolacrimal ducts (**Fig 1F**).

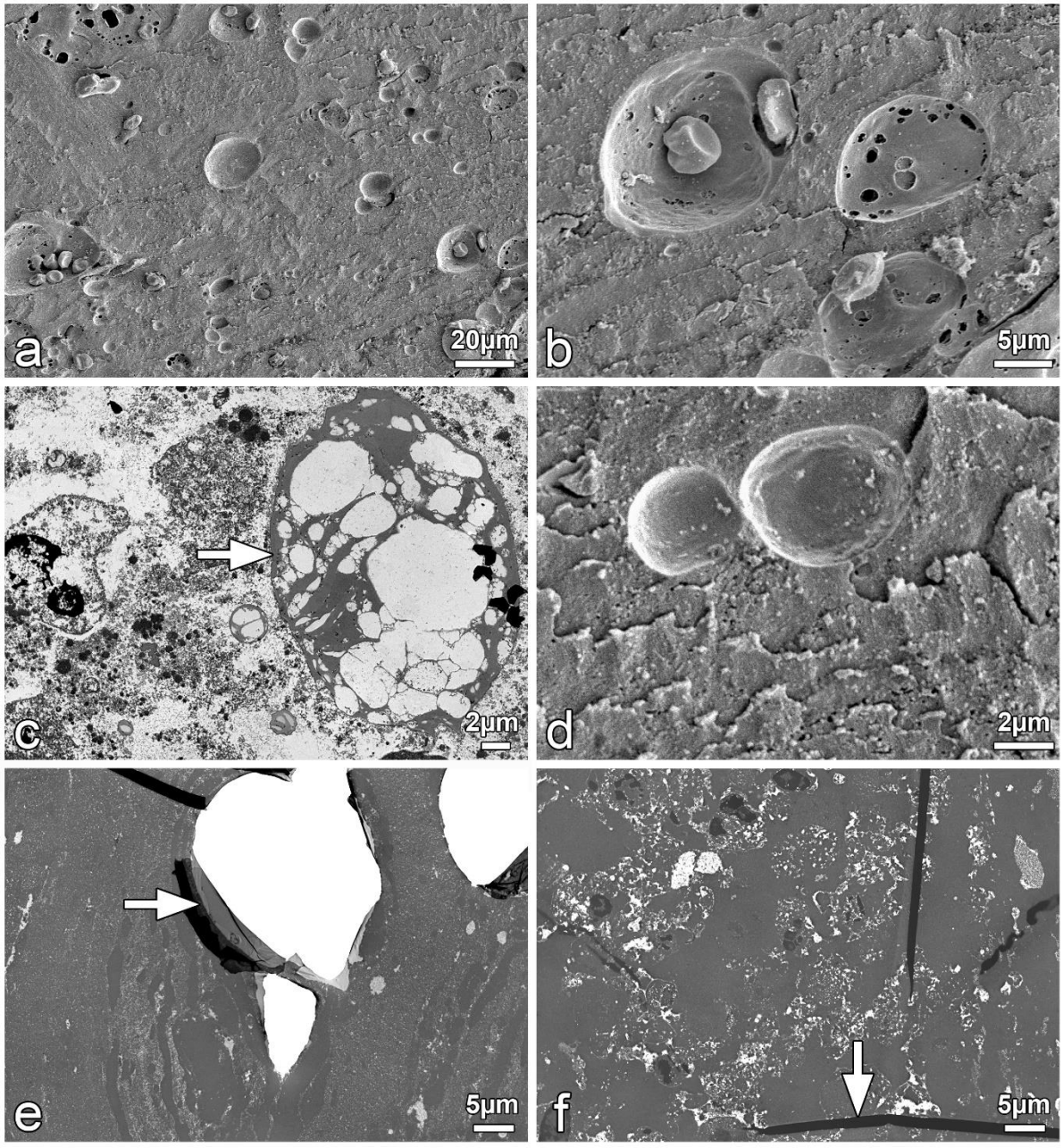


Figure 2: Ultrastructure of Mucopeptide concretions. SEMG showing numerous shallow and deep craters (**Fig 2A**). Higher magnification SEMG of a crater with numerous non-contiguous perforations and red blood cells within them (**Fig 2B**). Transmission electron micrograph (TEMG) of a crater (arrow) with numerous perforations (**Fig 2C**). SEMG image of a crater without any perforations or cellular elements (**Fig 2D**). TEMG of the area of vacuoles. Note the cut and folded edge of the vacuole, representing the rolled up tissue in that area and hence a cutting artifact (**Fig 2E**). TEMG of well-defined fissure (empty area, arrow) of drying artifact during sample preparation (**Fig 2F**).

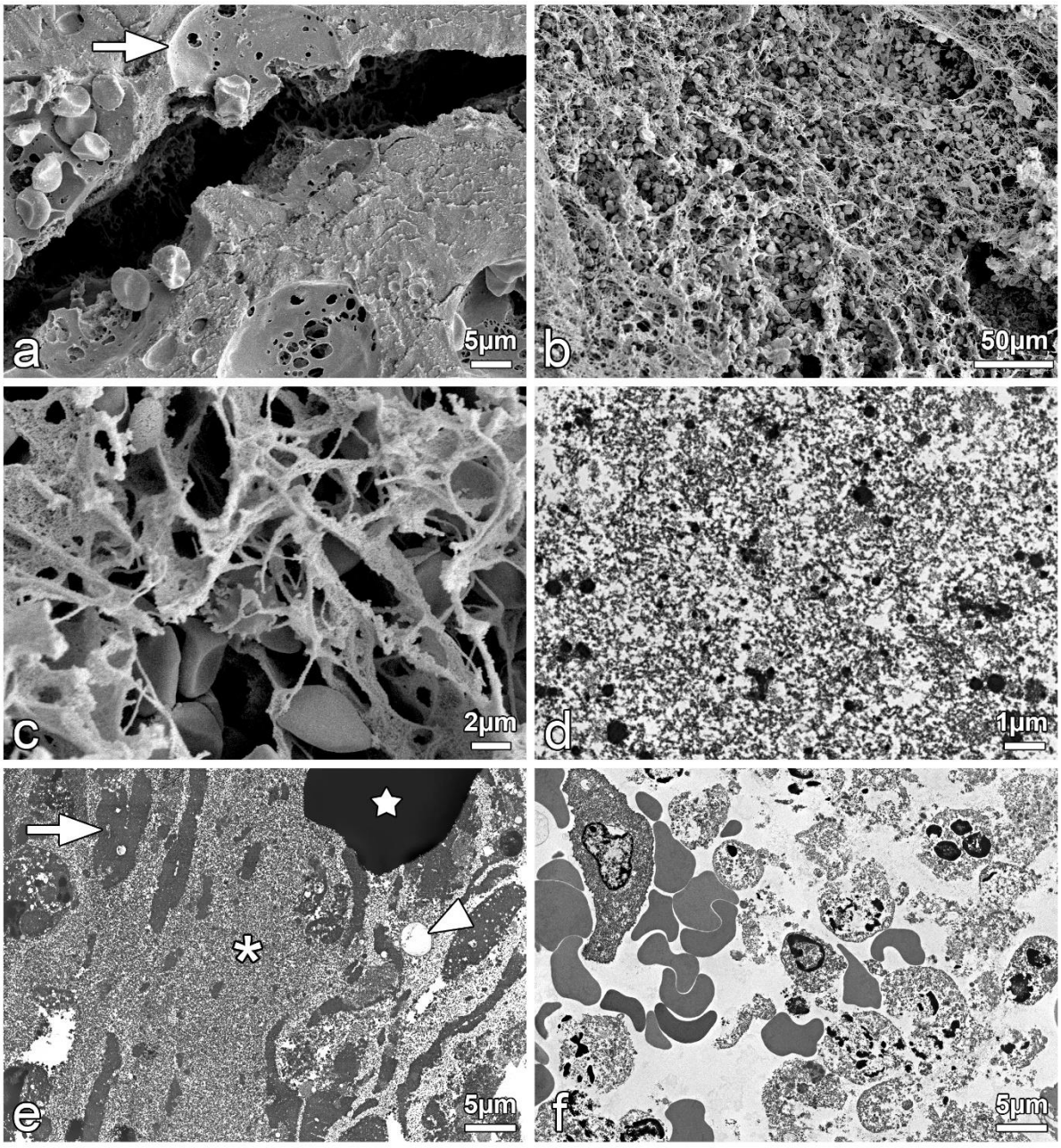


Figure 3: Ultrastructure of Mucopeptide concretions. SEMG of a large fissure representing a drying artifact. Note the splitting of the crater (arrow) into unequal halves (**Fig 3A**). SEMG of the core internal architecture made up of extensive network of fibrillary tangles packed densely with red blood cells (**Fig 3B**). High magnification SEMG of the core internal architecture showing the extensive fibrillary network (**Fig 3C**). High magnification TEMG showing the details of the fibril network (**Fig 3D**). TEMG image showing the lamellar deposits (arrow), the core granular material (thin armed star), fissure (star) and vacuole (triangle) (**Fig 3E**). TEMG showing on occasional area of cellularity of red blood cells, sparse granulocytes and epithelial cells (**Fig 3F**).

CHAPTER 4: HORMONAL PROFILING OF LACRIMAL DRAINAGE SYSTEM

4.1. Introduction

Primary acquired nasolacrimal duct obstruction (PANDO) was initially described as a clinical syndrome comprising of a usual onset of epiphora after the age of 40 years, female preponderance, idiopathic inflammation, fibrosis and obstruction of nasolacrimal duct and the subsequent development of associated symptoms and signs of chronic or acute dacryocystitis

(Linberg and McCormick 1986). The etiopathogenesis appears to be multifactorial and include the roles of osseous nasolacrimal canal, infections, sinusitis or nasal inflammation, loss of lacrimal drainage associated lymphoid tissue (LDALT), malfunctions of cavernous bodies, mucins and trefoil factor peptides (TFF) (Paulsen et al. 2000b, 2003a, 2003b; Perra et al. 1995; Ali et al. 2013; Ohtomo et al. 2013). However, one of the most striking features of PANDO is the female preponderance (Tarbet et al. 1995; Ali et al. 2015b, 2015c; Woog et al. 2007; Feng et al. 2011; Roussos et al. 1973) and this led to speculations about a possible hormonal basis in the etiopathogenesis.

The presence of estrogen and androgen receptors in the lacrimal gland and ocular surface is well known today and is believed to influence the inflammatory pathways leading to dry eyes (Rocha et al. 2000; Fuchsjäger et al. 2002; Versura et al. 2015; Suzuki et al. 2006; Mostafa et al. 2012). However, such information is not clear with regards to the lacrimal drainages system (Gupta et al. 2012; Kashkouli et al. 2010; Minsel et al. 2009; Ulrike et al. 2012). The current portion of the research work looks into the detailed expressions and distribution patterns of eight hormones and their further subsets and attempts to assess one of the angles amongst the many in the etiopathogenesis of PANDO.

4.2. Methods

The study was performed on frozen cadaveric (3 males, 3 females; age range 57-78) and clinical samples (PANDO) of lacrimal drainage system (3 females, 2 males; age range 51-67). Cadaveric lacrimal systems were obtained from bodies donated to the department of Anatomy, Friedrich Alexander University Erlangen-Nürnberg, Germany, after obtaining appropriate consents and ethical approvals. All methods for securing cadavers and their subsequent dissection were humane and complied with the Tenets of Declaration of Helsinki. Prior to dissection, the detailed medical history of each cadaver was studied. None of the cadavers had a history of lacrimal or nasal disorders, trauma or nasal surgery. Clinical samples were obtained from dacryocystectomy (Sac and part of the nasolacrimal duct, performed for PANDO) sample archives. This was chosen so as to provide an opportunity to study the entire lacrimal sac and

its wall contents as against a focal area of a DCR flap. Immunohistochemical labeling was performed for assessing the presence and distribution of receptors of estrogen alpha (ERα), estrogen beta (ERβ), aromatase (CYP19), testosterone (TSTR), progesterone (PGR), oxytocin (OXTR), prolactin (PRL) and somatostatins 1 to 5 (SSTR1, SSTR2, SSTR3, SSTR4, SSTR5). The details of each antibody used are listed in **Table 4.1**. Paraffinized human lacrimal drainage systems were subjected to antigen retrieval techniques. Immunoperoxidase detection system was performed using the Avidin-biotin method. Following standard treatments with 3% hydrogen peroxide, citrate buffer (pH 6) boiling and Tris buffered saline with Tween 20 (TBST), the samples were treated by overnight incubation with primary antibodies and later with secondary antibodies. Sections of placenta, testis, breast, adrenal glands and pancreas were used as positive controls. The immunohistochemistry stains were scored as positive or negative based on its presence or absence and the distribution patterns in the canaliculus, lacrimal sac and nasolacrimal duct (NLD) were assessed based on the intensity of staining as strong or poor. Differences in hormone receptor expression between the healthy cadaveric lacrimal systems and the diseased clinical samples were assessed.

Table 4.1: Details of Antibodies used

Antibody	Host	Source	Clonality	Dilution
ERα	Rabbit	Santa Cruz	Poly	50
ERβ	Rabbit	ThermoFischer	Poly	50
OXTR	Goat	Biozol	Poly	50
CYP19	Mouse	Acris	Mono	50

TSTR	Rabbit	Genetex	Poly	100
PRG	Mouse	Novus	Mono	100
PRL	Rabbit	Abcam	Mono	50
SSTR1	Goat	Santa Cruz	Mono	Pre-diluted
SSTR2	Goat	Santa Cruz	Mono	Pre-diluted
SSTR3	Goat	Santa Cruz	Mono	Pre-diluted
SSTR4	Goat	Santa Cruz	Mono	Pre-diluted
SSTR5	Goat	Santa Cruz	Mono	Pre-diluted

4.3. Results

4.3A. Estrogen alpha (Figs 4.1a to 4.1h): The immunoreactivity was strong with ERα and was uniformly noted across the canaliculi, fundus, body of lacrimal sac and the nasolacrimal ducts. The ERα receptor expression was maximum in the epithelium and localized to the cytoplasm and perinuclear regions. Non-diseased post-menopausal females had a lower signal intensity of ERα as compared to non-diseased males of comparable age. Diseased samples from PANDO did not show any expression of ERα across the lacrimal drainage system.

4.3B. Estrogen beta (Figs 4.2a to 4.2f): The immunoreactivity was strong with ERβ in the regions of lacrimal sac and nasolacrimal ducts, however, the canaliculi demonstrated poor expression of ERβ receptors. The expression in the lacrimal sac and nasolacrimal ducts was most prominent in the cytoplasmic and perinuclear regions of the epithelial cells. Non-diseased post-menopausal females had a lower signal intensity

of ERβ as compared to non-diseased males of comparable age. There was no expression of ERβ in cases of PANDO.

4.3C. Aromatase/CYP19 (Figs 4.3a to 4.3f): Aromatase expression was noted in the epithelium of the entire lacrimal drainage system and also in the sub-mucosal glands of the lacrimal sac and nasolacrimal ducts. The expression was mostly restricted to the cytoplasmic regions of the lining epithelial cells and perinuclear regions of the ductal epithelial cells. Like the ERα and ERβ, aromatase expression was poor in cases of PANDO. The expression in the sub-mucodal glandular cells reduced notably in the diseased as compared to the normal cases.

4.3D. Oxytocin (Figs 4.4a to 4.4f): Oxytocin receptor expression was strong in the lacrimal sac and nasolacrimal ducts. Like ERβ, the canalicular expression of OXTR was poor. OXTR expression was prominently observed in the cytoplasmic areas of the lacrimal sac and NLD epithelium and by the numerous vascular walls of the lacrimal sac. Non-diseased post-menopausal females had a lower signal intensity of OXTR as compared to non-diseased males of comparable age. OXTR expression was negative in cases of PANDO.

4.3E. Testosterone (Figs 4.5a to 4.5g): The testosterone receptor expression was strong on the cell surfaces and in the cytoplasm of superficial epithelial cells of the lacrimal sac and nasolacrimal ducts. Canaliculi showed weak expression. The normal post-menopausal females showed more intense staining of the basement membrane as compared to the male counterparts. The immunoreactivity was negative for both the epithelium and the basement membrane in diseased cases.

4.3F. Progesterone (Figs 4.6a to 4.6h): The progesterone receptors were not observed in the canaliculi but showed high expression on the cell surfaces, cytoplasm and supranuclear regions of the lacrimal sac and nasolacrimal duct epithelia. Similar to testosterone, normal post-menopausal females demonstrated intense staining of the basement membrane and moderate staining of the sub-mucosal glands. All these expressions were negative in cases with PANDO.

4.3G. Prolactin (Figs 4.7a to 4.7f): Prolactin receptor expression was strong in the lacrimal sac and nasolacrimal ducts. The canaliculi showed sparse expression. The expression was prominent in the cytoplasmic areas of the lacrimal sac and NLD epithelia and also the sub-mucosal glands. This expression was less in normal post-menopausal females in comparison to their male counterparts. The immunoreactivity reduced in the epithelium of diseased (PANDO) cases and was absent in the glandular epithelial cells.

4.3H. Somatostatin (Figs 4.8a to 4.8h): Somatostatin expression was assessed for its five different receptors (SSTR1, SSTR2, SSTR3, SSTR4, SSTR5). In the normal lacrimal systems, only the expressions of SSTR2 and SSTR4 were noted and this expression was restricted only to the most superficial epithelial surfaces (adluminal villus surfaces) of the lacrimal sac and NLD epithelia. There was no expression of any of the five SSTRs in cases with PANDO.

4.4. Discussion and Hypothesis

This study has provided a proof of principle of the widespread expression of sex hormone receptors in the lacrimal drainage system. Differential expression and distribution patterns were observed between the genders and when compared to the diseased tissues. This has led to formulation of few hypotheses. These provide potential insights into the multifactorial etiopathogenesis and gender predilection of primary acquired nasolacrimal duct obstruction, each of which needs to be further investigated in detail.

Estrogen, progestins and androgens are the main classes of sex steroids and exert their actions by modulating gene transcriptions through their specific cytoplasmic and nuclear receptors. Following menopause, there is a dramatic reduction in the secretion of the sex steroids by the gonads, although little estrogen production from androgen conversion continues in the adipose tissues (Versura et al. 2015). There is also a

longitudinal decline in the androgens as males' age; however the process is less dramatic.

Presence of androgen and estrogen receptors and steroidogenic enzymes has been demonstrated in the lacrimal gland, meibomian glands and the ocular surface and hence an intracrine mechanism of sex-steroidogenesis is likely to exist in these tissues (Rocha et al. 2000; Luu-The. 2013; Schirra et al. 2006). The lacrimal drainage system has strong anatomical and functional relationships with the ocular surface and it should not be a surprise if such enzymatic machinery exists within the tear ducts as well. Although the systemic contribution of sex-steroids from these tissues may not be significant, the importance of their local effects cannot be overlooked. The influence of sex hormones in the causation of dry eye is being increasingly recognized. In-vitro studies demonstrated effects of ovariectomy or anti-estrogen treatment to induce lymphocytic infiltration, acinar cell disruption, apoptosis and glandular tissue loss with estrogen administration correcting most of these (Mostafa et al. 2012; Azzarolo et al. 1995, 1999). There is also an increasing evidence of the protective role of androgens against dry eyes. Androgens are known to exert anti-inflammatory effects via reduction in interleukin-1β and tumor necrosis factor alpha (TNF-α) (Mostafa et al. 2012; Beauregard et al. 2004; Azzarolo et al. 1995).

The current study found that there was a specific pattern of immunostaining of the lacrimal sac and nasolacrimal duct with sex hormones. In the post-menopausal age (the age more predisposed to PANDO), the expressions of ERα, ERβ, Aromatase, OXTR, PRL (not TSTR and PGR) were more intense in normal males as compared to normal females with negative expression or gross reduction in cases with PANDO. The first hypothesis that generates out of this finding suggests that it is possible that the lower expression of these hormone receptors in the normal females as compared to their male counterparts predisposes their lacrimal drainage system for lymphocytic infiltration, inflammation, cellular degradation, loss of lining epithelium, luminal fibrosis and ultimately an obstruction. This is also indirectly supported by in-vitro evidence in

lacrimal gland where β-estradiol had greater effect on the opening of the voltage gated K^+ channels in acinar cells in male mice versus the females (Suzuki et al. 2004). This hypothesis is also supported by the lack of gender differences in expression patterns within the canaliculus of all the studied hormones. Hence, most retain canalicular patency even in the presence of NLDO and chronic dacryocystitis.

The second hypothesis is with regards to OXTR. Oxytocin receptors were predominantly noted in the vascular walls of the lacrimal sac and nasolacrimal ducts with normal males showing higher expressions than females. Fundamental work by Paulsen et al has shown the potential of malfunctions of the cavernous bodies (of lacrimal sac and NLD) to induce reactive hyperemia and mucosal edema, triggering a cycle that culminates into a NLDO (Paulsen et al. 2000b, 2001, 2003c). It is possible that decreased expression of OXTR in post-menopausal females predisposes their cavernous bodies to malfunctions and this can also be supported by negative vascular expressions noted in cases with PANDO.

The third hypothesis is with regards to the expression of prolactin, which has demonstrated trophic action on the lacrimal glands in-vitro (Azzaralo et al. 1995). Other than lactotrophs, PRL has been demonstrated to be secreted by the lacrimal gland, where it regulates the traffic of secretory vesicles in the acinar cells (Tan et al. 2012; Wang et al. 2007). In hypophysectomized female rats, treatment with prolactin had partially restored the amount of proteins in the glands and the Na^+-K^+-ATPase activity. It fully restored the cholinergic neurotransmitter receptors and alkaline phosphatase activity (Azzaralo et al. 1995). Prolactin is also known to augment effects of testosterone in accessory sex organs in male mice (Keenan et al. 1981) and is increasingly being recognized for it immunomodulatory effects (Ferrag et al. 1994). The hypothesis generated from the current study suggest that the sub mucosal glands of the lacrimal sac and NLD could be potential sites of PRL synthesis. Post-menopausal decrease in PRL may hamper the glandular functions of the lacrimal drainage system, interfere with the physiological functions of motility and secretions modulated by the

cholinergic pathways, and loss of immunomodulatory effects; thus predisposing females for a risk of PANDO. In addition, decreased PRL may in turn reduce the protective abilities of androgens.

Somatostatin receptors have been demonstrated on the ocular surface and nasolacrimal ducts and relative expression of SSTR1 and SSTR2 were differentially modulated by pro-inflammatory cytokines and bacterial components, suggesting their role in immunomodulation (Minsel et al. 2009). In addition they are suspected to be important in neurotransmitter feedbacks to lacrimal gland and supporting the androgen production (Sullivan et al. 2004; Paulsen et al. 2003c). The demonstration on SSTR2 and SSTR4 on the villus surfaces of the lacrimal sac and nasolacrimal duct epithelia and their loss in diseased states could potentially reflect their role in epithelial defenses and prevention of infections.

The more intense staining of the basement membranes (BM) of the lacrimal sac and nasolacrimal duct epithelia in normal post-menopausal females is interesting and needs further exploration. Androgens have been demonstrated to play a role the maintenance and distribution of extracellular matrices in rats accessory organs like prostate (Terry et al. 1996). Collagen basement membrane type IV and chondroitin sulfate proteoglycans are influenced by testosterone interactions (Terry et al. 1996; IIio et al. 2000). The loss of BM staining in the diseased samples could possibly reflect loss of supportive role of these hormones, which may in turn negatively influence the stability of lacrimal sac and NLD epithelia.

Gupta et al assessed immunohistochemical features of lacrimal sac epithelium using antibodies to various cytokeratins, epithelial membrane antigen, estrogen and progesterone receptors (Gupta et al. 2012b). They did not find any hormonal receptors. However, it is to be noted that they used only the diseased samples obtained during a dacryocystorhinostomy. Kashkouli et al assessed serum levels of follicle stimulating hormones (FSH), luteinizing hormone (LH), estrogen, progesterone and testosterone in cases and controls and found them to be normal for their age and hence concluded that

they do not play a role in the pathogenesis of PANDO. However, it is important to note that the serum contribution of locally synthesized hormones is negligible and such conclusions cannot be drawn from assessment of serum concentrations alone. In addition, the findings and discussion of this study reflects the significance of local synthesis and receptor interactions in the potential cellular and sub cellular mechanisms involved in etiopathogenesis.

The limitations of the current part of the research work includes lack of quantitative analysis, lack of comparison with the samples of normal lacrimal system in younger populations and the current speculative nature of the hypotheses that needs further validation. In addition, the diseased samples used were of lacrimal sac and nasolacrimal ducts and not of the canaliculi. However, the strengths of the study include wide assessment of multiple hormones, use of large variety of samples and providing the proof of principle of localized hormonal microenvironments' in targeted post-menopausal groups, where the incidence of PANDO is high.

It can also be argued that the observed decrease in the expression of the hormonal receptors in the diseased samples can be the result of the process of inflammation, epithelium destruction and scar, as is the PANDO pathogenetic sequence, rather than a primary event in the cascade. However, in addition to attributing the loss of receptor expression to the pathogenic sequences of PANDO, well preserved epithelial and sub-epithelial areas of the diseased samples were also studied, which indicate a possible primary derangement in the hormone cascade.

In conclusion, each of the multiple hypotheses suggested in the current study needs further validation. It is also important to understand that hormonal factors are only one of the many factors that could explain the female preponderance to PANDO. The widespread presence of hormonal receptors within the lacrimal drainage system opens up exciting newer avenues for further exploration to demystify the etiopathogenesis of PANDO.

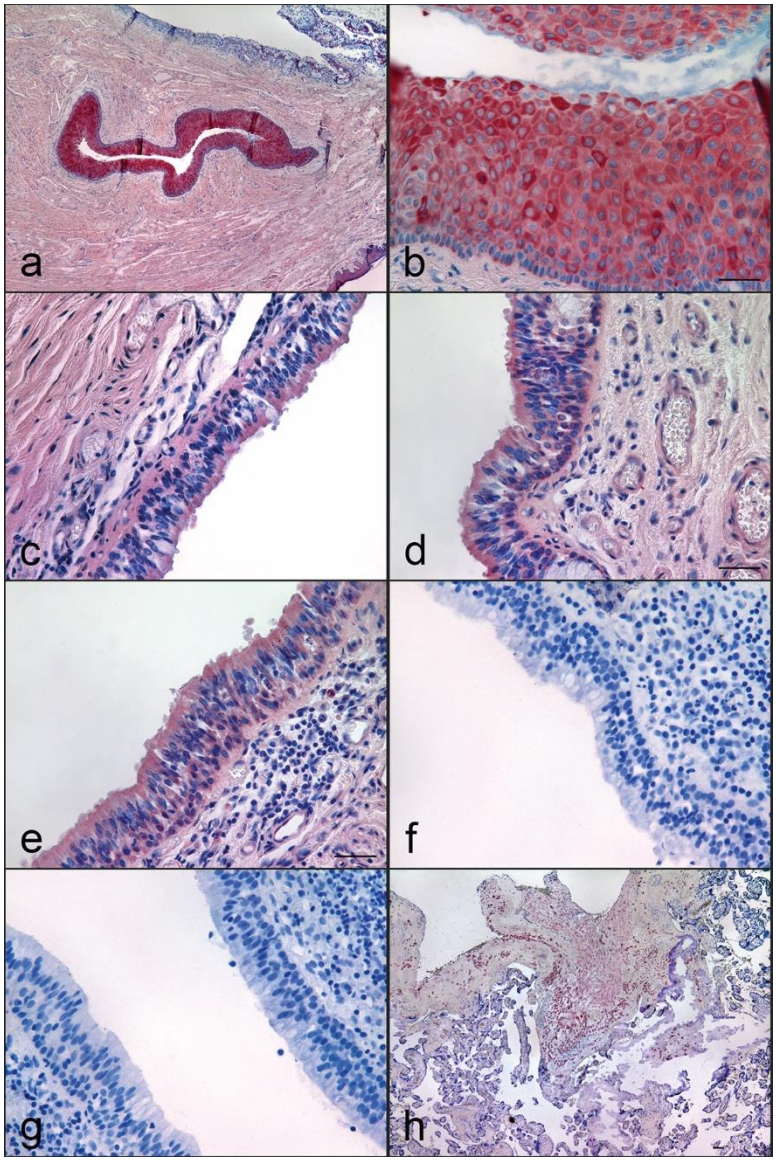


Fig 4.1: Estrogen receptor alpha expressions: Strong ERα expression in the entire canaliculus (cut section, superior canaliculus) (**Fig 1a, ERα x40**), cytoplasmic and supranuclear ERα expression in the stratified squamous epithelium of the canaliculus (**Fig 1b, ERα x400**). Expression of the ERα in the lining epithelium of the fundus (**Fig 1c, ERα x400**), lacrimal sac (**Fig 1d, ERα x400**) and the nasolacrimal duct (**Fig 1e, ERα x400**). Microphotograph of the diseased (PANDO) lacrimal sac (**Fig 1f, ERα x400**) and nasolacrimal duct (**Fig 1g, ERα x400**) showing no expression of the ERα receptors. Microphotograph of a positive control; placenta (**Fig 1h, ERα x200**).

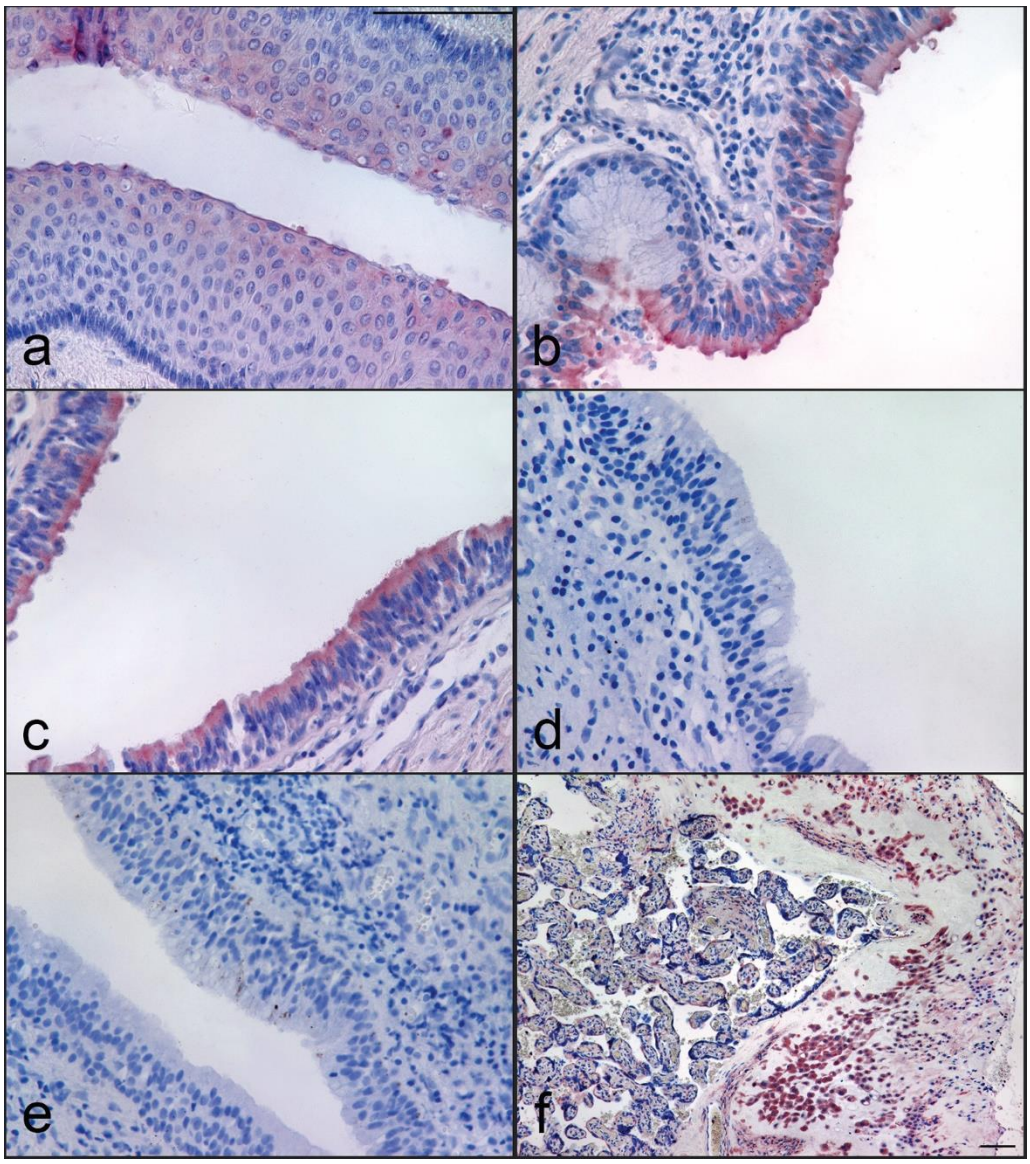


Fig 4.2: Estrogen receptor beta expressions: Weak ERβ expression in the canaliculus (**Fig 2a, ERβ x400**). Compare it with Fig 1a. Strong ERβ expression was noted in the lining epithelium of the lacrimal sac (**Fig 2b, ERβ x400**), and the nasolacrimal duct (**Fig 2c, ERβ x400**). Microphotograph of the diseased (PANDO) lacrimal sac (**Fig 2d, ERβ x400**) and nasolacrimal duct (**Fig 2e, ERα x400**) showing no expression of the ERβ receptors. Microphotograph of a positive control; placenta (**Fig 2f, ERβ x200**).

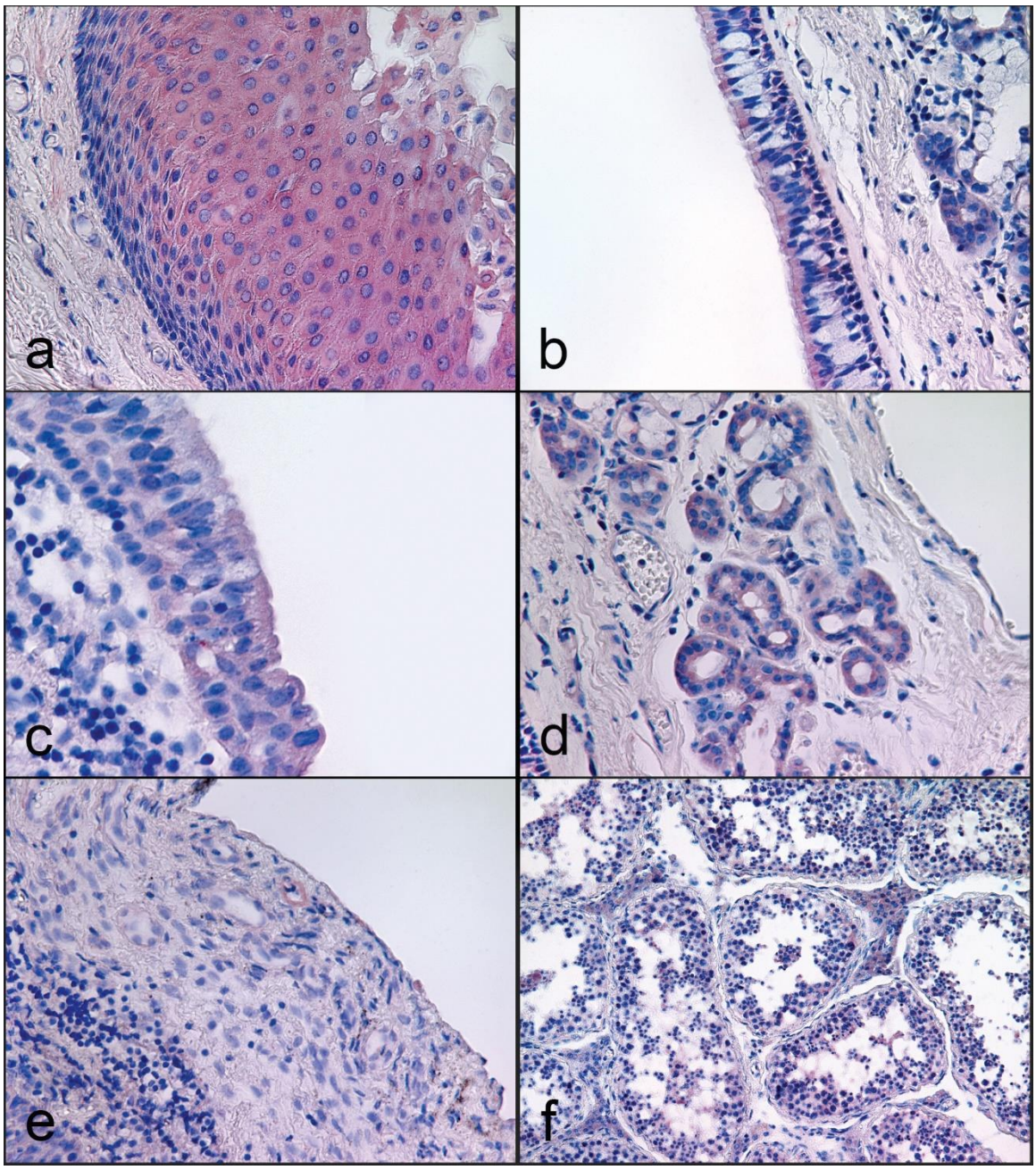


Fig 4.3: Aromatase (CYP19) expressions: Good Aromatase expression in the canaliculus (**Fig 3a, Aromatase x400**). Strong aromatase expression was noted in the lining epithelium of the lacrimal sac (**Fig 3b, Aromatase x400**), the nasolacrimal duct (**Fig 3c, Aromatase x400**) and submucosal glands (**Fig 3d, Aromatase x400**). Microphotograph of the diseased (PANDO) lacrimal sac (**Fig 3e, Aromatase x400**) showing no expression of aromatase. Microphotograph of a positive control; testis (**Fig 3f, Aromatase x200**).

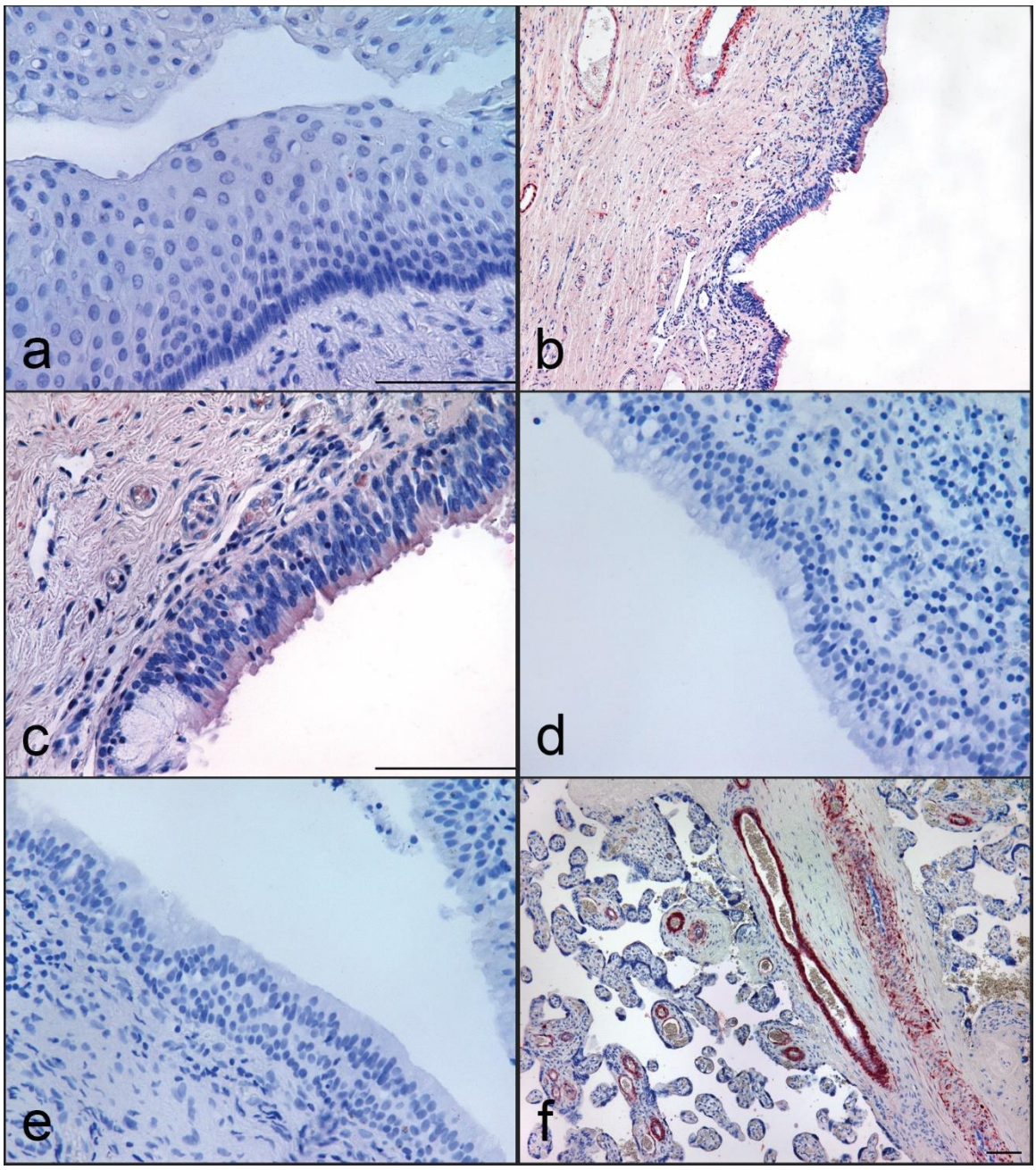


Fig 4.4: Oxytocin receptor expression: Negative OXTR expression in the canaliculus (**Fig 4a, OXTR x400**). Compare it with Figs 1a and 2a. Strong OXTR expression was noted in the lining epithelium of the lacrimal sac and large sac wall vessels (**Fig 4b, OXTR x100**). Microphotograph of the lacrimal sac showing good expression of OXTR in the cytoplasm of the adluminal epithelium (**Fig 4c, OXTR x400**). Microphotograph of the diseased (PANDO) lacrimal sac (**Fig 4d, OXTR x400**) and nasolacrimal duct (**Fig 4e, OXTR x400**) showing no expression of the OXTR receptors. Microphotograph of a positive control; placenta (**Fig 4f, OXTR x200**).

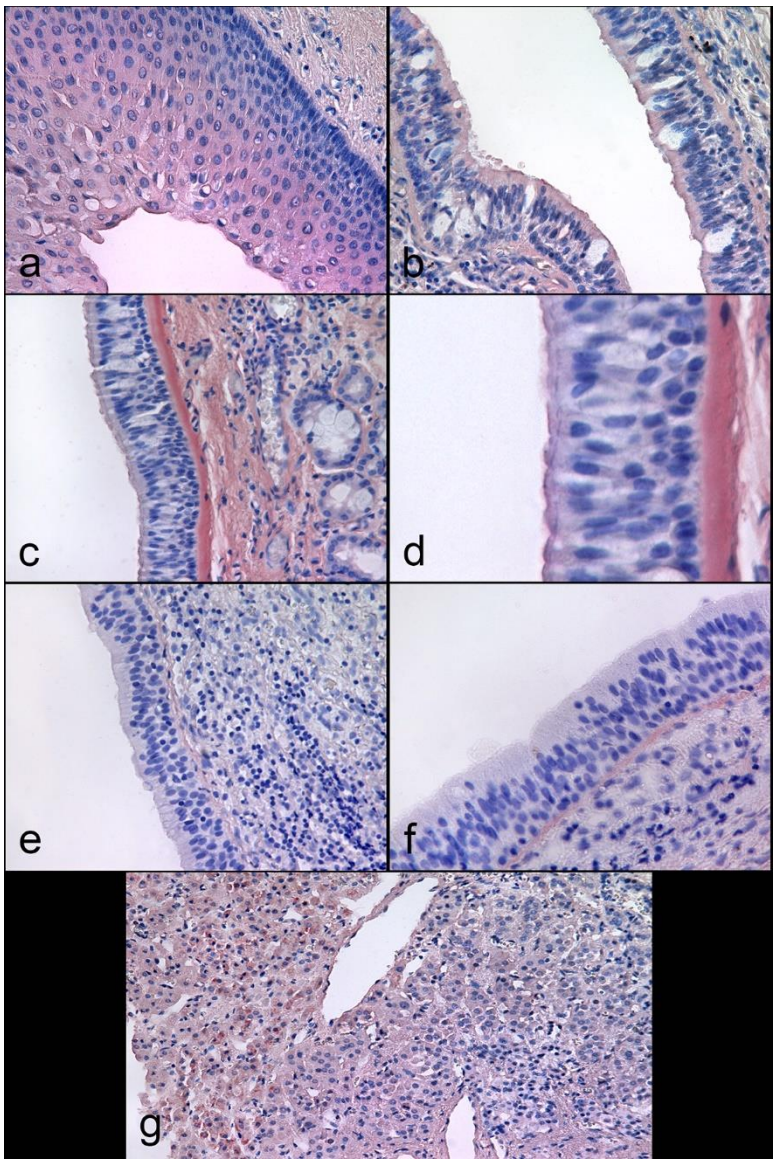


Fig 4.5: Testosterone receptor expression: Weak TSTR expression in the canaliculus (**Fig 5a, TSTR x400**). Compare it with Figs 1a and 2a. Good TSTR expression in the epithelial lining of a normal male lacrimal drainage system. Note the faint staining of the basement membrane (**Fig 5b, TSTR x400**). Good TSTR expression in the epithelial lining of a normal female lacrimal drainage system. Note the intense staining of the basement membrane and compare it to fig 5b (**Fig 5c, TSTR x400**). Magnified image from figure 5c clearly demonstrating the intensely stained basement membrane (**Fig 5d, TSTR original magnification (OM) x400**). Microphotograph of the diseased (PANDO) lacrimal sac (**Fig 5e, TSTR x400**) and magnified nasolacrimal duct (**Fig 5f, TSTR OM x400**) showing no expression of the lining epithelium and appreciable loss of basement membrane staining. Microphotograph of a positive control; adrenal gland (**Fig 5g, TSTR x200**).

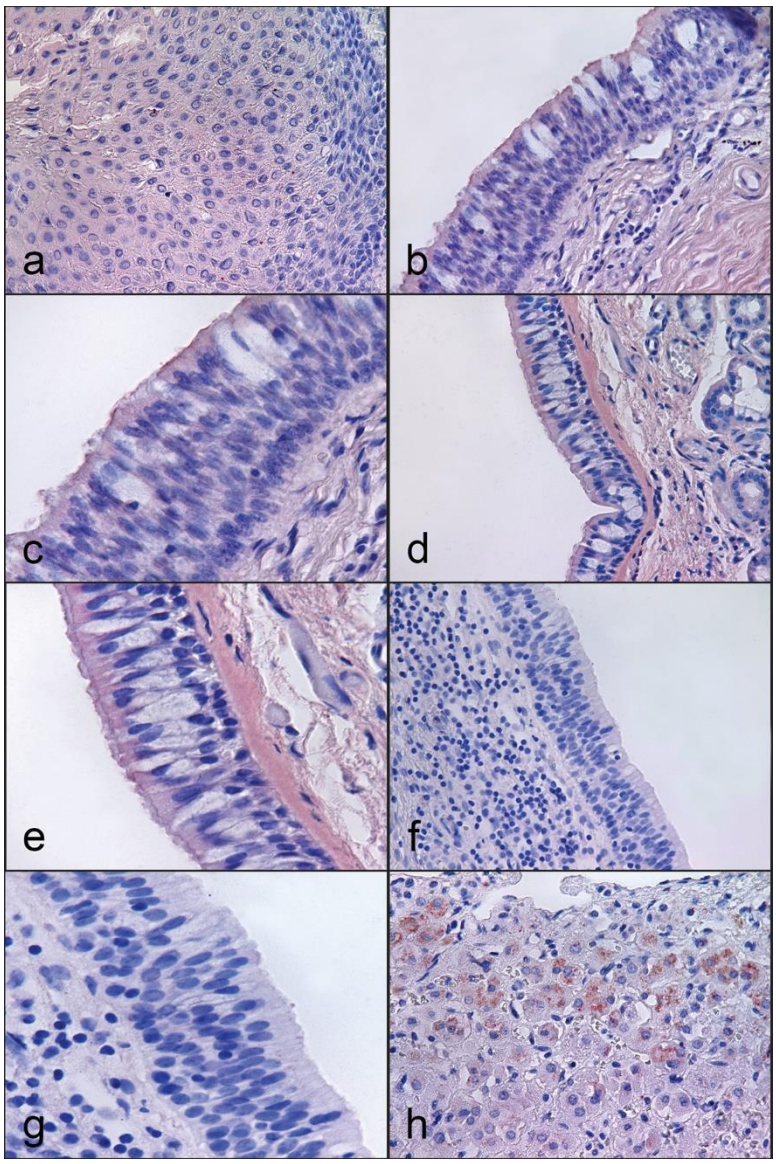


Fig 4.6: Progesterone receptor expression: Negative PGR expression in the canaliculus (**Fig 6a, PGR x400**). Good PGR expression in the adluminal epithelial lining of a normal male lacrimal drainage system. Note the poor staining of the basement membrane (**Fig 6b, PGR x400**). Magnified view of a portion of fig 6b (**Fig 6c, PGR OM x400**). Good PGR expression in the epithelial lining of a normal female lacrimal drainage system. Note the intense staining of the basement membrane and compare it to fig 6b (**Fig 6d, PGR x400**). Magnified image from figure 6d clearly demonstrating the intensely stained basement membrane (**Fig 6e, PGR, OM x400**). Microphotograph of the diseased (PANDO) lacrimal sac (**Fig 6f, PGR x400**) and magnified image of 6f **(Fig 6g, PGR OM x400**) showing no expression of the lining epithelium and basement membrane staining. Microphotograph of a positive control; adrenal gland (**Fig 6h, PGR x200**).

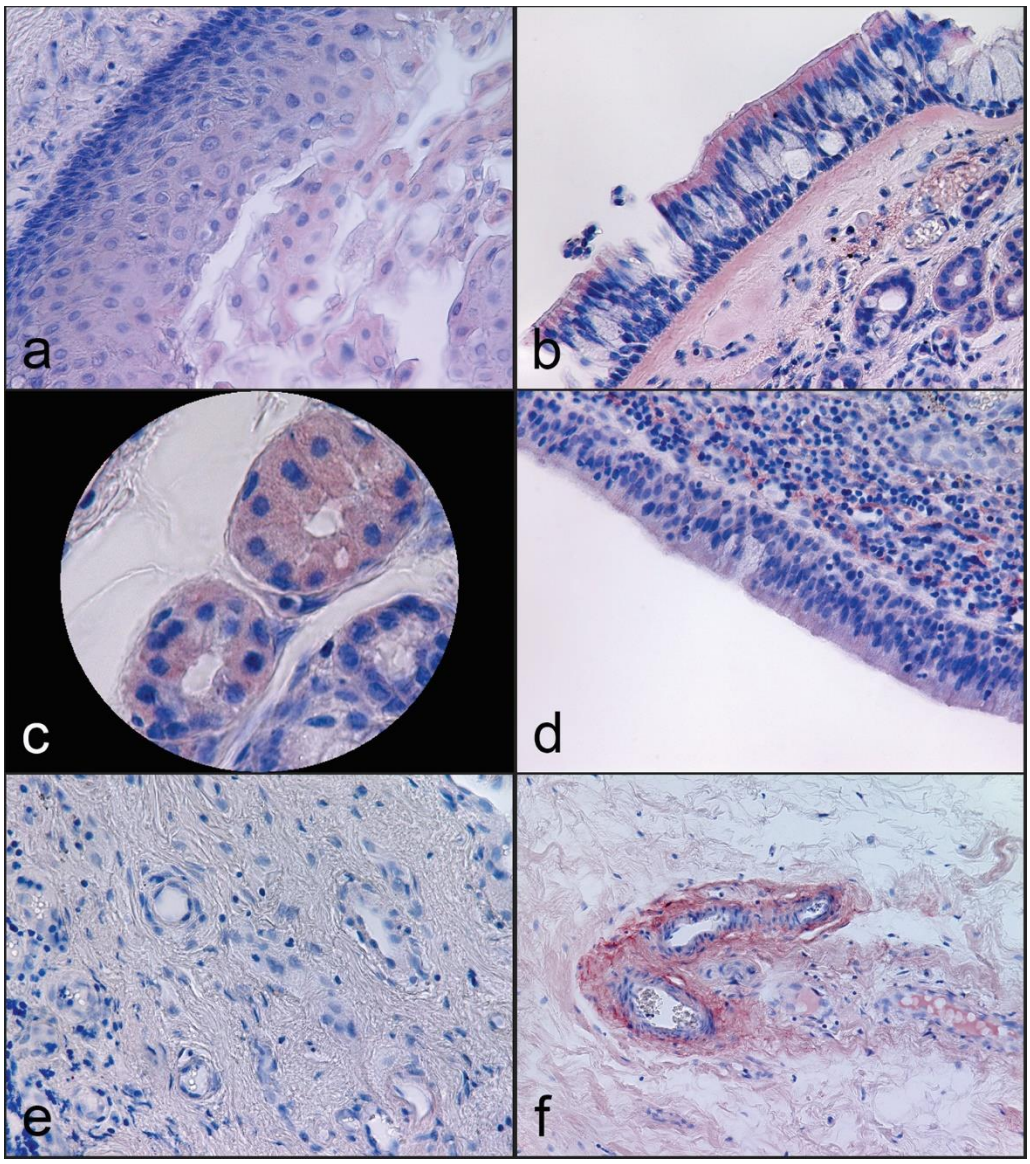


Fig 4.7: Prolactin receptor expression: Poor PRL expression in the canaliculus (**Fig 7a, PRL x400**). Good PRL expression in the adluminal epithelial lining of a normal lacrimal drainage system (**Fig 7b, PRL x400**). Magnified view of a portion of sub-mucosal glands showing the expression in the cytoplasm of glandular epithelium (**Fig 7c, PRL, OM x400**). Microphotograph of the diseased (PANDO) lacrimal sac (**Fig 7d, PRL x400**) showing weak expression in the lining epithelium and loss of expression in the glandular epithelium (**Fig 7e, PRL x400**). Microphotograph of a positive control; breast (**Fig 7f, PRL x200**).

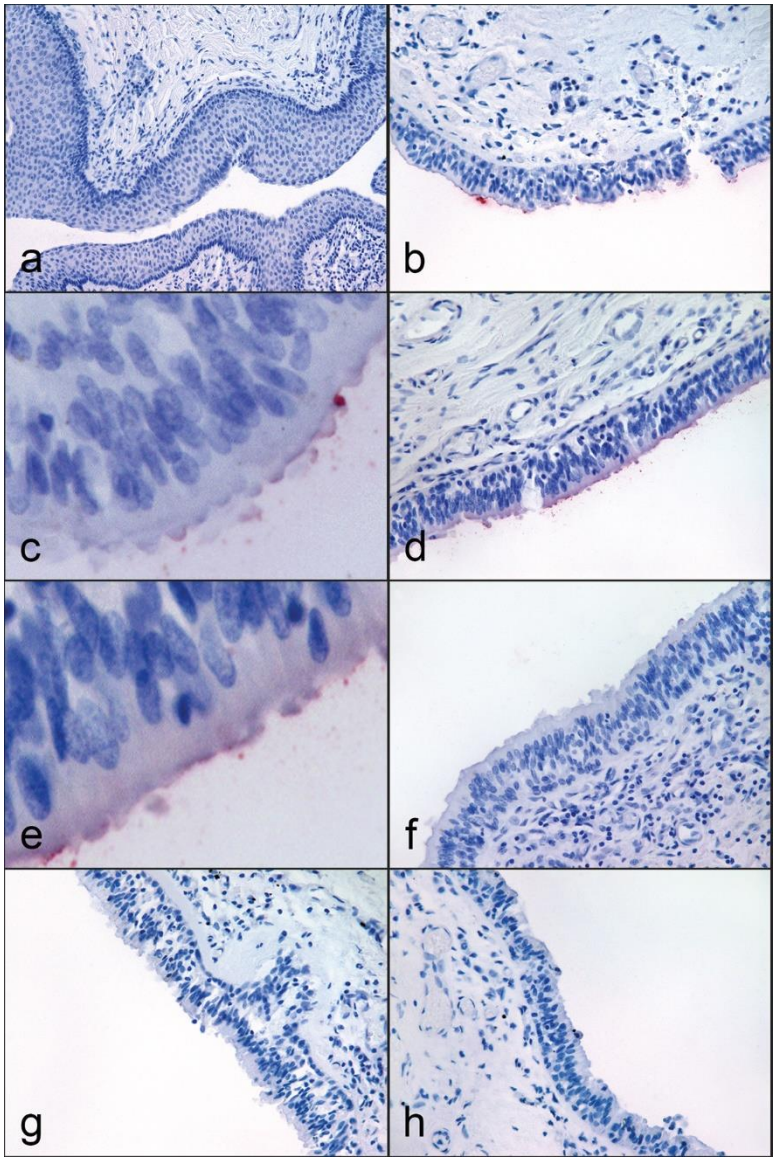


Fig 4.8: Somatostatin receptors 1-5 expression: Negative SSTR2 expression in the canaliculus (**Fig 8a, SSTR2 x400**), however the lacrimal sac showed positive immunoreactivity in the superficial most layer of the adluminal epithelium (**Fig 8b, SSTR2 x400**) and a magnified image of fig 8b, makes this clear (**Fig 8c, SSTR2, OM x400**). Good SSTR4 expression in the adluminal epithelial lining of a normal lacrimal sac (**Fig 8d, SSTR4 x400**). Magnified view of a portion of fig 8d, showing the clear and strong expression (**Fig 8e, SSTR4, OM x400**). Microphotograph of normal lacrimal sac lining showing no expression for SSTR1 (**Fig 8f, SSTR1 x400**), SSTR3 (**Fig 8g, SSTR3 x400**) and SSTR5 (**Fig 8h, SSTR5 x400**).

CHAPTER 5:
LACRIMAL SAC LYSOSOMAL ENZYME ASSAYS

5.1. Lysosomes

“Membrane-delimited structures containing characteristic hydrolytic enzymes, most of which have acidic pH optima”. This definition is the most accepted one regardless of the heterogeneity of the lysosomal forms and functions. Functionally the lysosomes are classified into three different forms.

Primary lysosomes: these are the structures containing hydrolases in an unused state and the enzymes are considered to be packaged for future use. Primary lysosomal forms are fully functional but have not encountered the materials destined to be digested.

Secondary lysosomes: these are the continuation of the primary lysosomal forms and these contain the materials that are ingested by any of the autophagic processes and the internalized materials are in an undigested, being digested or in already digested form.

Residual body: secondary lysosomes advance and form residual body in which much of the ingested content is in residual materials which are not susceptible to, or only slowly susceptible to further degradation (Duve and Wattiaux, 1966).

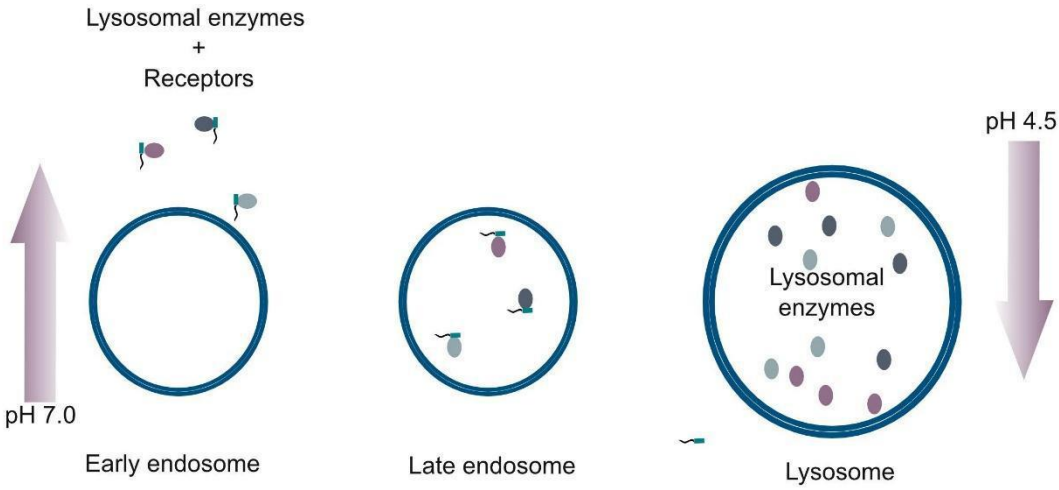


Figure 5.1. Receptor mediated transport of lysosomal enzymes and the maturation of early endosomes to late endosomes.

5.1A. Structure of lysosome

A typical lysosome is a phospholipid bi-layer membrane bound organelle which encloses a lumen of lower pH than the external cytosolic pH. The pH is maintained at 4.5 to 5 by the help of Hydrogen ion pumps working in the membrane of the organelle. The lower pH is essential for the action of a group of enzymes collectively called lysosomal hydrolases which help in the digestion or catabolism of macromolecules that are internalized in the lysosomes (Mellman et al., 1986).

There are multiple models for the origin of lysosomes depending on the cells and tissues. Formation of lysosomes can be associated with pinocytosis where pinocytic vacuoles form from plasma membrane with clathrin coated vesicles and fuse with endosomes to form lysosomes. In other instances, the lysosomes are formed by the fusion of transport vesicles originated from the Golgi complex as the particles get accumulated, which fuse with endosome and the endosomes further mature into lysosomes as lysosomal hydrolases are transported and the pH is lowered to 4.5 during the maturation phase (**Fig 5.1**).

The membrane of the lysosomes is equipped with proton pumps which are essential in maintaining the pH of the lysosomal lumen lower than cellular pH. The luminal pH is maintained at 4.5 to 5 when lysosomes mature from the late endosomes in the endocytic pathway. A V-type ATPase enzyme complex spanning the lysosomal membrane and the interior lumen acts as a pump which maintains the efflux of hydrogen ion from the exterior cytoplasm into the lysosomal lumen (**Fig 5.2**). The enzyme complex is similar to F0 F' enzyme complex found in the mitochondria but it does not generate ATP unlike the later. The V-type ATPase complex is formed of different subunit fractions that are both membrane bound and soluble proteins.

The soluble fraction reversibly detaches from the membrane bound counterpart as the lysosomal pH drops further due to high amount of H^+ ions in the lumen and hence regulates the V-type channel activity. The continuous flux of the protons into the lumen necessitates the requirement of a counter ion pathway to regulate the charge inside the lysosomes (Harikumar and Reeves, 1983). This is achieved mainly by two ways; by membrane permeability of the lysosomal membrane which allows the movement of a cation (K^+) out of the lumen and/or by operating another channel which helps in the inward movement of an anion into the lumen. Most studied counter ion pathway is CLC-7 channel which transport Cl^- ions into the lysosome as shown below (Mindell, 2012).

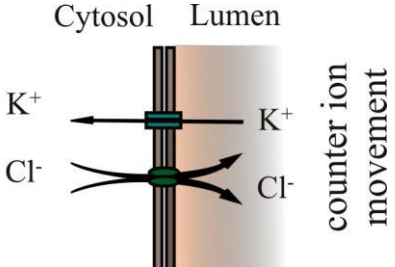


Figure 5.2: V- type ATPase helps in maintaining the lower pH inside the lysosome by fluxing hydronium ions into the lysosome. The increase in net positive charge is negated by the counter ion mechanism in which Cl^- ions are transported inwards or K^+ ions are transported out of the lysosome.

5.1B. Recognition marker for lysosomal transport

The low pH environment in the lysosomal lumen helps in the hydrolysis of internalized macromolecules for the degradation along with a group of enzymes called lysosomal hydrolases. The optimum pH of all lysosomal hydrolases are 4.5 to 5 and there are more than fifty different lysosomal enzymes reported in higher vertebrates. The enzymes are synthesized in the rough endoplasmic reticulum and are transported to the lysosomes by special receptor proteins which detect the enzymes by a special recognition marker on the glycan of the enzymes (von Figura and Hasilik, 1986). In the *trans* Golgi region, the mannose residues of the processed glycan in lysosomal enzymes forms a phosphodiester bond with UDP- N acetyl glucosamine in a reaction by UDP N- acetyl phosphotransferase (**Fig 5.3**). The N acetyl group is later removed by phosphodiester glycosidase and phosphate groups remain attached to the mannose sugar and act as a recognition marker. The sequence of events are shown belwo in Fig 5.3. While impaired mechanisms in this pathway can lead to severe abnormalities, most of lysosomal hydrolases are tagged with phosphosugar and the glycosylation process of these proteins is an essential requirement are essential for their successful transport to the lysosomes (von Figura and Hasilik, 1986).

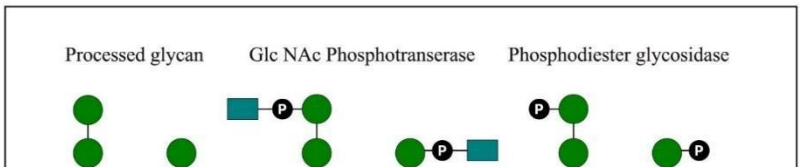


Figure 5.3. Post-translational modifications leading to the acquisition of mannose 6-phosphate recognition marker on the glycan structure of the lysosomal enzymes.

5.2. Lysosomal Enzymes

5.2A. Lysosomal hydrolases

These are the collection of different groups of enzymes present in the lysosomes and with optimum pH of 4 to 5 in the acidic lysosomal lumen (**Table 5.1**). Most of the enzymes are glycoproteins targeted into the lysosomes through the mannose 6-phosphate receptors mediated transport by virtue of mannose 6-phosphate recognition marker on the glycan structure of the enzyme (von Figura and Hasilik, 1986). Even though more than fifty of such enzymes are reported from various sources, only a few of them are characterized in the invertebrates and the lysosomal targeting of the invertebrate enzymes are poorly understood (Kumar and Bhamidimarri, 2015). Lysosomal hyrolases include proteases, glycosidases, lipases, nucleases and other lysosomal enzymes. Different lysosomal enzymes and their substrates are listed in table 2. A brief description of important classes of lysosomal enzymes is given below.

5.2B. Lysosomal proteases

These are different class of protease enzymes consisting of aspartic, cysteine and serine proteases. Their function may vary according to cell type and physiological conditions. Major function of these proteins are the degradation of bulk of the proteins in the lysosomes. Another major function is the antigen processing in the early endosome. They also function in pre-protein processing and pro hormone processing in vesicles and in extracellular matrix (Brix, 2013). A recently discovered function of the lysosomal protease is the initiation of apoptosis in the cytosol. A few of these proteases exhibit exoglycosidase function and many are

endopeptidases. The class of lysosomal endoproteases are collectively called cathepsins. Several cathepins have been identified in mammalian cells with varying active site motifs and substrate specificity.

5.2C. Lysosomal glycosidases

Glycosyl hydrolases in lysosomes function in the release of sugar moieties from the glycoproteins, glycans and polypeptide ingested by the lysosomes. Collective action of different glycosyl hydrolases releases the sugar monomers which are salvaged in later stages of the cells (Mahadevan et al., 1969). Mutations in these enzymes leads to lysosomal storage disorders, a rare collection of genetic abnormalities like Anderson-Fabry, Gaucher, Tay-Sachs, and Sandhoff's disease. Some of these diseases may arise due to the missense or change in the amino acid at the active site which can result in the loss of partial activity of the enzyme function. In addition to this the impaired trafficking of these enzymes to the lysosomes due to misfolding or the absence of lysosomal targeting marker can also lead to these disorders. Lysosomal glycosidases forms several categories and major ones are fucosidases, mannosidases, glucuronidases, galactosidase, hexosaminidase etc., (Futerman and van Meer, 2004; Vellodi, 2005).

5.2 D. Lipases

Lipid catabolism during fasting plays one of the key mechanism for the survival of organism. Lipophagy of lipid droplets, lipid vacuoles and adipose cells in the lysosome play an important role in the release of energy from liphophagic processes (Settembre et al., 2013).

5.2E. Other lysosomal enzymes

The nucleic acids transported inside the lysosomes are degraded by nucleases enzymes such as acid deoxyribonuclease and ribonuclease. Aryl sulfatases and other sulfatase enzymes found in the lysosomes removes sulfate groups from proteins and sugars. Similarly lysosomes host various phosphatases collectively called as acid phosphatases.

5.3. Evolution of lysosomal targeting

Both the receptors CIMPR and CDMPR are extensively characterized from human and bovine species and are also found in other mammals (**Fig 5.4**). Phosphomannan core affinity chromatography has proven to be an excellent technique for the purification of both these receptors under different buffer conditions (Siva Kumar, 1996). The initial identification of two distinct but homologous receptors performing same function raised a question at what stage in evolution it became necessary for both receptors to appear. This has enabled researchers to look for homologues of these receptors from other non-mammalian vertebrates like birds, reptiles, amphibians and fishes. Through the ability to complement the functions of wild type receptors in mutant cell lines it was confirmed that the homologous proteins purified by phosphomannan affinity chromatography were indeed CIMPR and CDMPR. Further research has proven that the same receptors are present in invertebrate animals such as echinoderms and molluscs (Nadimpalli and Amancha, 2010). In the case of insects, in *Drosophila*, the lysosomal enzyme targeting is achieved by another protein of 98 kDa molecular weight. This protein was termed Lysosomal Enzyme Receptor Protein (LERP). However the targeting is not through the mannose 6-phosphate residues as seen in other vertebrates and some invertebrates. Even though this protein binds to the lysosomal enzymes through a different mechanism, the transport of the receptor enzyme complex though GGA adaptor proteins in clathrin coated vesicles are found to be conserved (Dennes et al., 2005).

More recent studies have discovered the lysosomal enzymes and their sorting receptors in the Cnidarian *Hydra vulgaris* suggesting that the lysosomal biogenesis pathway has evolved several hundreds of millions of years ago (Bhamidimarri et al. 2018). Interestingly the lysosomal enzyme sorting receptors exhibited high degree of homology with the human counterparts.

Table 5.1. Lysosomal enzymes and their substrates classified according to their general biochemical characteristics

substrate	Enzyme
proteins	Acid carboxypeptidase Amino acid naphthalamide Cathepsin A Cathepsin B Cathepsin C Cathepsin D Dipeptidyl amino peptidase II
Polysacharides and glycan side chains of proteins	N-acetyl-α-galactosaminidase N-acetyl-α-glucosaminidase N-acetyl-β-hexosaminidase Fucosidases β-Galactosidases(both) Glucosidases Glucoronidases Mannosidases Beta Xylosidases
Nucleic acids	Acid deoxyribonuclease Acid ribonuclease
lipids	Acid lipase Ceramidase Phospholipase A1 Phospholipase A2 Spingomyelinase (Phospolipase C)
Complex lipids	Hyaluronidase Iduronidase Neuraminidase
Sulphates	Aryl sulfatase Chondrosulfatse Sulfamidase
Phosphate	Acid phosphatase Acid pyrophosphatase Phosphodiesterase

(Courtesy: Lysosomes in Biology and Pathology, J T Dingle, P J Jacques, I H Shaw, 1981**)**

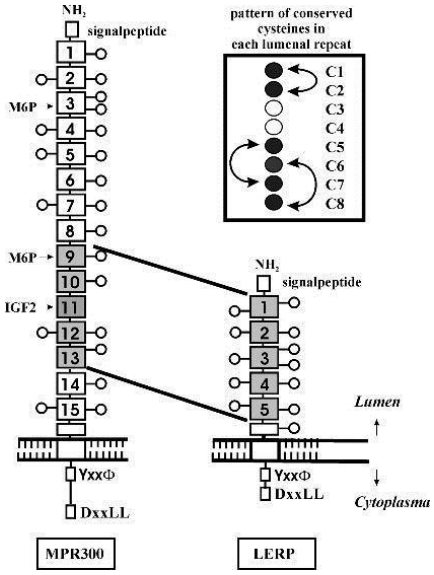


Figure 5.4. Structural representation of *Drosophila* LERP compared to mammalian CIMPR. (Dennes et al. J. Biol. Chem. 2005;280:12849-12857)

Even though the lysosomal transport of enzymes are present in animals like amoeba, protozoa and yeast, there is no evidence to indicate the presence of the putative MPR proteins. In the case of *Dictyostelium discoideum*, the phosphorylated sugar residues are present which indicates the machinery of the N-acetyl glucosamine phosphotransferase is intact as in the higher organisms (Couso et al., 1987). These finding suggests that the mannose 6-phosphate mediated lysosomal targeting is an evolutionarily conserved pathway among the vertebrates and exists among the invertebrates but limited to echinoderms and molluscs (Vegiraju et al., 2012). In lower organisms such as the insects and worms, no such mechanism has been identified (these organisms lack the receptors similar to those found in echinoderms and molluscs). This hypothesis is further verified by the genome data published in the invertebrate animals showing the absence of MPR receptors among these organisms.

5.4. Mannose 6-Phosphate receptors

5.4A. Cation independent mannose 6-phosphate receptors (CIMPR)

Mammalian CIMPR is a ~ 300kDa protein with 15 repetitive domains, a single transmembrane domain and a cytoplasmic tail (**Fig 5.5**). Each repetitive domain is made of approximately 147 amino acids and each domain has sequence similarity of 14-38 % and the distribution of cysteine is also conserved among the domains.

Additionally, they also share sequence similarity with cation dependent mannose 6-phosphate receptors (Bohnsack et al., 2009). Of the 15 domains, domain 3 and 9 have high affinity to the mannose 6-phosphate molecules and it has been proved that the intact tertiary structure and the presence of disulfide linkages are necessary for the adequate ligand binding for these domains. The carbohydrate recognition domain (CRD) is placed deep inside the protein and hydrophilic sugar binding structure interacts with the glycan even at the penultimate sugar in the glycan chain. The domain 11 shows a hydrophobic pocket instead of the hydrophilic carbohydrate binding region of 3^{rd} and 9^{th} domain. This enables the 11^{th} domain to bind to insulin like growth factor 2 (IGF-II), another major ligand for the CIMPR which helps in regulating the levels of circulating IGF-II (Kornfeld, 1992; Delaine et al., 2007) and therefore the CIMPR is also referred to as the IGF-II receptor.

In addition to the lysosomal hydrolases and the IGF-II, the CIMPR binds to a number of other ligands possibly at different binding sites on the protein. These molecules may either contain mannose 6-phosphate receptor like TGF-β or they may be non-mannose 6-phosphate containing ligands like IGF-II and retinoic acid (Ghosh et al., 2003).

5.4B.Cation dependent mannose 6-phosphate receptor

CDMPR is a 46 kDa protein and it exists as a dimer in membranes. Each polypeptide of the receptor has a single domain that is related to each of the 15 repetitive domains of CIMPR, a single transmembrane domain and a cytoplasmic tail. It has similar ligand binding residues as seen in domains 3 and 9 of the CIMPR. Compared to CIMPR the aspartic acid at 103 co-ordinates the binding to divalent cation and hence ligand binding is enhanced in the presence of divalent cations like Ca^{++} or Mg^{++} (Dahms et al., 1987). Other structural features are similar to CIMPR like the palmitoylation and phosphorylation of the transmembrane domain. CDMPR

transports solely the lysosomal hydrolases from the TGN to the early lysosomes and unlike the CIMPR, it does not bind to any other ligand other than the mannose 6-phosphate containing lysosomal enzymes (Schweizer et al., 1996) and has no endocytosis function like the CIMPR.

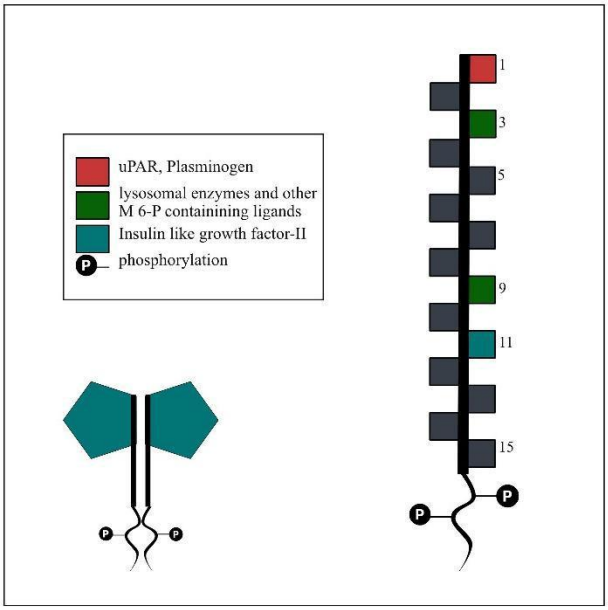


Figure 5.5. Schematic representation of mannose 6- phosphate receptor proteins showing the binding domains, cytoplasmic tail and phosphorylation.

5.4C. Mechanism of MPR mediated cargo transport

The mechanism of transport for both the receptors in the lysosomal enzyme sorting is the same where adaptin 1 complex (AP1) has a major role in the initiation of the transport. The cytoplasmic tail of both the receptors has multiple sites for binding to the AP1 and the AP1 complex forms the clathrin coated vesicles around them in a reaction facilitated by γ-ear-containing ADP ribosylation factor binding protein (GGA proteins) (Ghosh et al., 2003). The CCVs then exit through the *tras*- most stack (C7) of the TGN for its destination in endosomes. In addition to this, free molecules of the receptor may also reach the early or late endosomes and the ratio of free receptor to facilitated receptor may depend on the cell type and function. The clathrin coated vesicles then fuses with endosomes. SNARE and Rab proteins (Rab 5 or Rab 7 GTPase) help in fusion with late endosomes and phosphofurin acidic cluster sorting protein-1. AP 1 helps in fusion with early endosomes. In both the cases the proteins bind with the tail of both CIMPR and CDMPR. The pH dependent delivery of lysosomal enzymes take place in the early or late endosomes and never the receptor proteins are found in matured

lysosomes (**Fig 5.1**). The return and recycling of the receptor is mediated by TIP47 and Rab 9 GTPase which delivers the receptor enclosed vesicles back in TGN (Dennes et al., 2005). In the case of plasma membrane targeting of the CIMPR, the vesicles emerges from the C5 or C6 stack of the TGN with the help of AP 2 protein (Riederer et al., 1994).

5.5. Mannose 6-phosphate independent mechanisms for the lysosomal enzyme transport.

Even though the mannose 6-phosphate dependent lysosomal enzyme pathway is vital to the optimum functioning of the lysosomes, certain enzymes are transported even in the absence of the receptors or the lysosomal recognition markers. Sortilin, a 95 kDa protein also known as neurotensin receptor 3 transports lysosomal enzymes such as prosaposin and acid sphingomylinase to the lysosomes. Lysosomal integral membrane protein type 2 (LIMP-2) takes part in the lysosomal trafficking of β-glucocerebrosidase in a mannose 6- phosphate independent manner (Braulke and Bonifacino, 2009). In addition to this, receptor independent cell to cell transport of lysosomal enzymes have also been proposed under *in vivo* conditions for mice hepatocytes. Even though cathepsin D is transported in I-cell disease condition in the aforementioned manner in mice hepatocytes, such mechanisms are surprisingly absent in thymocytes and fibroblast cells for the same enzyme indicating that the mannose 6-phosphate independent mechanisms may vary according to the cell type (Dittmer et al., 1999).

5.6 Rationale of the study

Lysosomes are intracellular organelles composed of acidic compartments with more than 50 membrane proteins and 60 hydrolases (Xu H et al. 2015). They together play a major role in degradation of extracellular materials through endocytosis and intracellular wastes by autophagy (Luzio et al. 2007; Saftig et al. 2009). The hydrolases; glycosidases, proteases and lipases are involved in the catabolic degradation of polysaccharides, complex proteins and lipids and products are exported out of lysosomes for excretion or reutilization in the biological pathways. Lysosomes are hence energy and nutrient sensors and involved in intracellular ion conductances. Disturbances of these functions commonly lead to lysosomal storage disorders. However, lysosomes are also a focus of increasing attention because of their role in the

regulation of inflammatory gluco-corticoids pathways and other inflammatory signaling mechanisms (Ge et al. 2015; He et al. 2011; Blott et al. 2002; Andrei et al. 1999). Lysosomal enzymes are known to widely express in ocular tissues and lacrimal gland, with uvea and retina showing high concentrations of glycosidases, acid phosphatases and cathepsins (Hayasaka. 1983; Kitaoka et al. 1985, 1986; Hawkins et al. 1981; Bruner et al. 1981; Yamaguchi et al. 1989). These enzymes have been potentially implicated in the pathogenesis of ocular storage disorders, retinal degenerations, uveitis and glaucoma (Hayasaki. 1983). To the best of the authors knowledge, no exploration had been carried out for lysosomal enzymes and their receptors in the lacrimal drainage system. The current study explores the lysosomal enzymes and their mannose 6-phosphate transport receptors in the lacrimal sac and nasolacrimal ducts.

5.7. Methods

Institutional review board approval was obtained and the study complied with the Tenets of Declaration of Helsinki. The study was performed on healthy lacrimal sacs and nasolacrimal ducts obtained from exenteration samples (n=3, 2 females, 1 male, age range: 54-67 years) immediately after surgery and frozen at -80^{o}C for subsequent analysis. None of the exenteration patients had a history of lacrimal or nasal disorders, trauma or nasal surgery. Irrigation of the lacrimal drainage system prior to exenteration was patent. The substrates used for lysosomal enzyme activities and the sugars phenyl Sepharose CL-4B, BCIP/NBT and Con A-Sepharose gels were from Sigma Chemicals (St. Louis, MO, USA). 4-Methylumbelliferyl substrates namely 4-methylumbelliferyl -β-glucuronide, 4-methylumbelliferyl α-D- mannopyranoside and 4-methylumbelliferyl α-L-fucopyranoside (Carbosynth, Berkshire, UK) were used for activity staining. The details of each antibody used for western blot are listed in **Table 5.2**.

5.7A. Lysosomal Enzyme assays

Enzyme assays with soluble extracts of human lacrimal sac at pH 5.0 and pH 7.0 were carried out with techniques described previously (Venugopal et al. 2017). The substrates used for the assays were, *p*-nitrophenyl *N*-acetyl-*β*-D-glucosaminide for *β*- hexosaminidase; *p*-nitrophenyl α-L-fucopyranoside for α-fucosidase; *p*-nitrophenyl α-D-mannopyranoside for α-mannosidase; *p*-nitrophenyl α-D-galactopyranoside for α-galactosidase; *p*-nitrocatechol sulfate dipotassium salt for arylsulfatase A; *p*-nitrophenyl β-D-glucuronide for β-glucuronidase and *p*-nitrophenyl phosphate for acid phosphatase. The absorbance of the released *p*-nitrophenol was measured at 405 nm. One unit of enzyme activity was defined as the absorbance equivalent of 1 µmol *p*-nitrophenol released per min, per mL of the enzyme solution under the experimental conditions. Each enzyme assay was carried out in triplicate.

5.7B. Activity staining

Activity staining was performed in 10% Native PAGE as described previously (Venugopal et al. 2017) using the 4-methylumberlliferyl substrates and the active protein bands were visualized by illuminating the gel under UV light.

5.7C. Western blot analysis

Aliquots of the soluble extract and membrane extracts were subjected to Western blot analysis for each of the lysosomal enzymes and receptors (MPR 46) separately, with their respective antibodies (**Table 5.2**) The antibodies to enzymes, hexosaminidase, fucosidase and MPR receptors were raised in rabbits and affinity purified in the laboratory as per the supervisor of this thesis prior publications (Nadimapalli et al. 2004, Venugopal et al. 2013; Suresh et al. 2003; Visa et al. 2012). After SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), the proteins were transferred to a PVDF (polyvinylidene difluoride) membrane. Each membrane was incubated separately with each antibody (1:1,000 dilution). The membranes were subsequently washed and incubated separately with alkaline phosphatase conjugated anti-rabbit IgG for fucosidase, hexosaminidase, arylsulfatase, acid phosphatase, glucuronidase, MPR 46, and anti-goat IgG (1:1,000 dilutions in PBST) for mannosidase as secondary antibody. The membrane was finally developed using BCIP/NBT (5-bromo 4-

choloro 3-indolyl phosphate/Nitroblue tetrazolium reagents (Sigma chemicals, St. Louis, USA).

5.8. Results

5.8A. Lysosomal enzyme assays

The soluble extracts of the human lacrimal sac obtained by sodium acetate (pH 5.0) and Tris-HCl (pH 7.4) buffer extractions, exhibited several lysosomal enzyme activities (**Figs 5.6A and 5.6B**) and amongst them acid phosphatase and *β*- hexosaminidase activities were found to be high at both the pH values **(Fig. 5.6A & 5.6B).** When the pH 8.0 eluates were assayed, acid phosphatase activity was found to be high followed by hexosaminidase activity similar to the earlier assays. However, when pH 9.0 eluates were assayed, higher activity of glucosidase followed by hexosaminidase and mannosidase was found. And when pH 10.0 eluates were assayed, activity of glucosidase alone was observed to be very high **(Fig. 5.7)**. These results clearly demonstrate the strong binding of the enzymes indicating the highly hydrophobic nature of the lacrimal drainage lysosomal enzymes, and among the lysosomal enzymes assayed, glucosidase was found to be most hydrophobic.

5.8B. Activity staining

When the native gel containing the soluble extract of lacrimal sac was subjected to activity staining for moderately expressed lysosomal enzymes, with respective 4-methylumbelliferyl conjugated substrates separately, as described above, strong fluorescence was observed with 4-methylumbelliferyl -β-glucuronide, 4-methylumbelliferyl α-D- mannopyranoside and with 4-methylumbelliferyl α-L-fucopyranoside, confirming the presence of α-fucosidase **(Fig. 5.8A)**, β-glucuronidase **(Fig. 5.8B)** and α-mannosidase **(Fig. 5.8C)** in the human lacrimal drainage system.

5.8C. Western blot analysis

Western blot analysis confirmed the presence of lysosomal enzymes in the human lacrimal drainage system and showed strong signals for α-fucosidase **(Fig. 5.9A)** glucuronidase **(Fig. 5.9B)**, arylsulfatase A **(Fig. 5.9C)**, mannosidase **(Fig. 5.9D)**, β-hexosaminidase **(Fig. 5.9E)**, and

acid phosphatase **(Fig. 5.9F).** Membrane extract analysis with western blot using specific receptor antibodies (mammalian synthetic cytoplasmic tail 1 antibody, MSCI for MPR 46, **Table 5.3**) showed presence of MPR 46 receptors (**Fig 5.9G**), which indicate the possible roles of cation dependent lysosomal targeting in human lacrimal drainage system.

Table 5.2: Details of the antibodies used

	Antigen	Host	Clonality	Source
1.	Hexosaminidase	Rabbits	Polyclonal	In-house
2.	Fucosidase	Rabbits	Polyclonal	In-house
3.	Mannosidase II	Goat	Polyclonal	Santa Cruz
4.	Arylsulfatase A	Goat	Polyclonal	Sigma
5.	Acid phosphatase 2	Goat	Polyclonal	Abcam
6.	ß-glucuronidase	Rabbit	Polyclonal	Abcam
7.	MSC1 (for MPR46)	Rabbit	Polyclonal	In-house

MSC1 – mammalian synthetic cytoplasmic tail 1, MPR – mannose 6 phosphate receptor

5.9. Discussion and Hypothesis

This study has provided a proof of principle of the presence of several lysosomal enzymes and the mannose 6-phosphate ligand transport receptors in the lacrimal drainage system. Differential activity patterns of enzymes were observed. In the light of current evidence of

lysosomal involvement in several inflammatory pathways, lysosomal dysfunctions may add another potential dimension into the multifactorial etiopathogenesis of primary acquired nasolacrimal duct obstruction (PANDO), which needs to be further investigated in detail.

Lysosomes are degradation centers of the cell and are aided in this function by its numerous membrane proteins and hydrolase enzymes. They degrade extracellular molecules and phagocytosed pathogens and also aid in intracellular proteins turnover (Xu et al. 2015). In addition, lysosomes are also involved in immune functions, pigmentation, cell signaling pathways, cell adhesions and membrane repair mechanisms (Saftig et al. 2009). Mannose 6-phosophate receptors (MPR) are transmembrane glycoproteins that play a crucial role in the transport of newly synthesized lysosomal enzymes (Suresh et al. 2003; Nadimapalli et al. 2010; Koduru et al. 2006). Targeting of the lysosomal enzymes depend on the presence of mannose 6-phosphate moiety within them and their recognition by two specific MPR proteins; the cation-independent, MPR 300 and the cation dependent MPR 46. Their functions are to some extent distinct but mostly overlapping (Nadimpalli et al. 2010).

Lysosomes are known to negatively regulate the anti-inflammatory actions of gluco-corticoids. Cytoplasmic glucocorticoid receptors (GR) mediate anti-inflammatory effects via inhibiting the synthesis of cytokines, prostaglandins and prostacyclin's. Lysosomal autophagy degrades the cytoplasmic GR and enhances inflammation by negating their actions. The lysosomes can also positively or negatively regulate inflammatory pathways by secreting or degrading numerous cytokines like interleukins (IL-1 β, IL-6, IL-18, and IL-8), β-interferons (IFN-β). Tumor necrosis factor-alpha (TNF-α) and transforming growth factor-β (TGF-β) (Ge et al. 2015; He et al. 2011). A lysosomal membrane protein, TMEM9B (trans-membrane protein 9B) is also involved in activation of NF-κB (nuclear factor 'kappa-light-chain-enhancer' activated B cells) and MAPK (mitogen-activated protein kinase) pathways, the central signaling pathways of inflammation and hence reflects on the potent regulatory role of lysosomes in inflammation (Dodeller et al. 2008).

The hypothetical role of lysosomal dysfunctions in PANDO should be assessed in the light of their major influences in core inflammatory cascades. Since lysosomes can negatively or

positively regulate inflammation, a critical pro and anti-inflammatory balance is maintained. Disturbance of this equilibrium can lead to chronic inflammation and lysosomes have been implicated in the pathogenesis of autoimmune, metabolic, cardiovascular and neurodegenerative disorders. The widespread presence of lysosomal hydrolases and their transport receptors within the healthy lacrimal drainage system points towards optimal functions with pro and anti-inflammatory equilibrium. Hypothetically, it is possible that numerous exogenous or endogenous triggering agents within the lacrimal drainage system can create lysosomal instability and lysosomes may respond to them. Prolong presence of triggering agents or repeated assaults may lead to severe instability of lysosomal functions and these dysfunctions may have a potential role in mediating, coordinating and enhancing the inflammatory pathways leading to chronic inflammation of the lacrimal sac and nasolacrimal duct, followed by the response of fibrosis and subsequent acquired obstructions (PANDO).

Selective autophagic clearance of bacteria is an important host defense mechanism where lysosomes play an important role and dysfunctions in this regard have been implicated in the pathogenesis of sepsis and inflammation (Mizumura et al. 2016; Ryter et al. 2015). Severe inflammation and tissue destruction in experimental toxoplasma infestation, in part, has been attributed to inability of the lysosome to fuse with phagosome (*Toxoplasma gondii*), resulting in the abundant lysosomal enzymes intended against the organism, instead, destroying the tissues in the vicinity (Yoshizumi et al. 1977). Similarly lysosomal dysregulation of selective autophagy of mitochondria and cilia have been implicated in chronic obstructive pulmonary disease and lung fibrosis (Mizumura et al 2016). The lacrimal sac and the nasolacrimal ducts also have numerous cilia on their luminal surfaces and the adluminal mucosa acts as an important first line defense against sustained bacterial and exogenous triggers that attack the ocular surface and are subsequently washed into the lacrimal drainage through tears. Hypothetically, lysosomal autophagic dysfunctions in the lacrimal drainage system have the potential to trigger chronic inflammation, tissue destruction and subsequent fibrosis and finally resulting in acquired obstructions (PANDO).

Lysosomal hydrolases are widely distributed in ocular tissues with higher concentrations in the retina and uvea (Hayasaka. 1983). Every tissue has its own differential expression levels of various enzymes, for example ciliary body shows higher expression of fucosidase, mannosidase and cathepsin B; retinal pigment epithelium shows higher concentration of cathepsins, tear analysis shows higher concentration of acid phosphatase, fucosidase and glucosaminidase. Heterogeneity in regional and differential distribution of these enzymes could potentially be involved in various tissue-specific disease involvement and their variable clinical presentations (Hayasaka. 1983). In concurrence with this, it has also been found that the receptor proteins involved in the targeting of lysosomal enzymes are also differentially expressed in different tissues in humans as well as other vertebrates (Suresh et al. 2002). High activity of acid phosphatase and β-glucuronidases were found in Behcet's disease (Hayasaka et al. 1977) and similarly release of lysosomal enzymes from macrophages have been implicated in chronic inflammation in sarcoidosis (Carr and Norris. 1977). Glucocorticoids have been demonstrated to suppress the acid phosphatase activity (Tanaka et al. 1997). The assessment of various lysosomal enzymes in diseased lacrimal sacs and nasolacrimal duct would be useful in nailing the over-expressed hydrolases with possible therapeutic solutions.

The limitations of the current study includes lack of assays of other lysosomal enzymes and the current speculative nature of the hypotheses that needs further validation. However, the strengths of the study include wide assessment of common lysosomal enzymes within a focused area of lacrimal drainage system and providing the proof of principle of presence of their ligand transport receptors.

In conclusion, the hypothesis suggested in the current study needs further investigations, including the identification of MPR 300 proteins. It is also important to understand that such lysosomal pathways of inflammation are one amongst the many that could be involved in the etiopathogenesis of PANDO. The widespread presence of lysosomal enzymes, particularly glucosidases and their MPR receptors within the lacrimal drainage system opens up exciting newer avenues for further exploration to demystify the etiopathogenesis of PANDO.

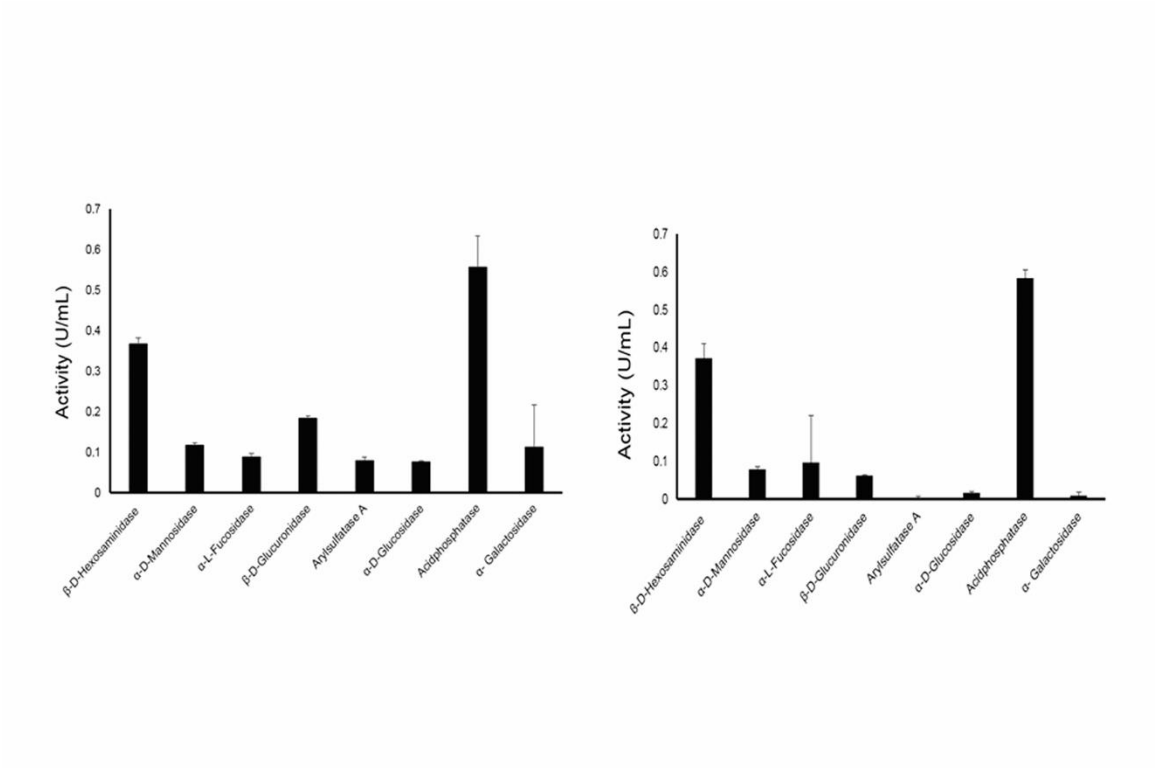


Figure 5.6: Lysosomal enzyme activities of human lacrimal sac soluble extract. Lysosomal enzyme activities of 25 mM Tris-HCl buffer pH 7.4 extract **(left panel**), Lysosomal enzyme activities of 50 mM sodium acetate buffer pH 5.0 extract **(right panel).**

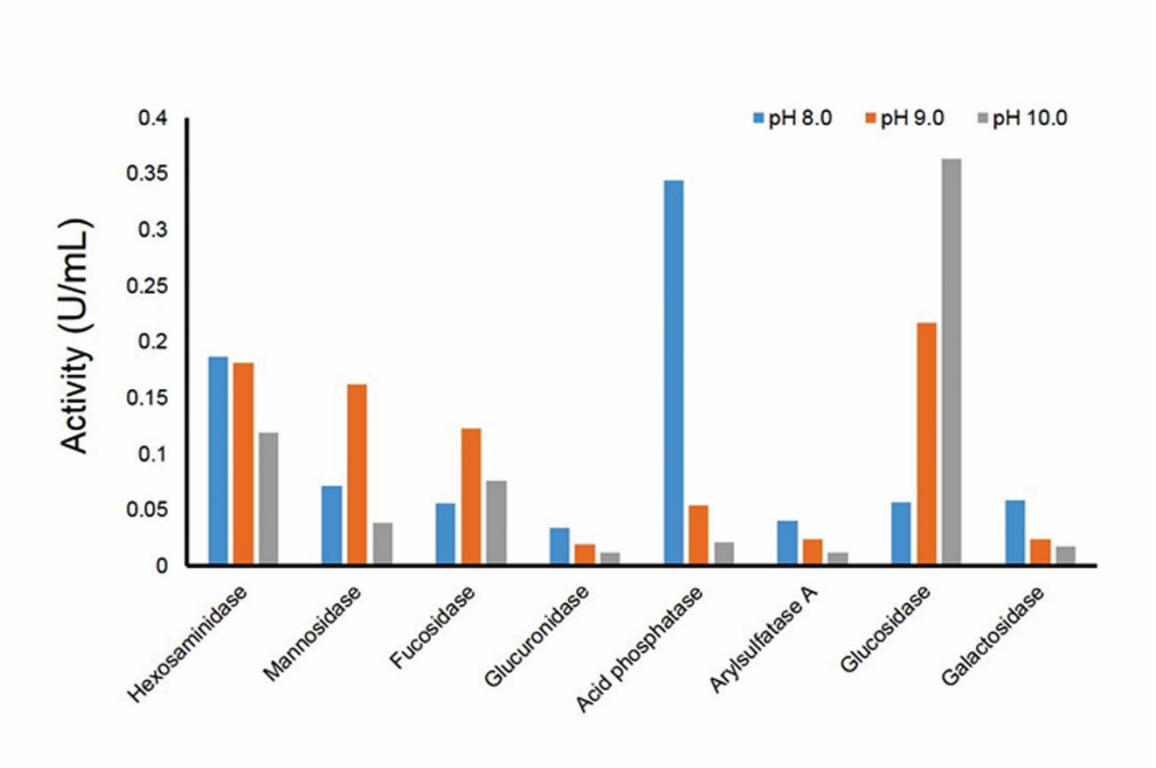


Figure 5.7: Chromatography on phenyl Sepharose matrix.

Phenyl sepharose elution was carried out at pH 8.0, pH 9.0 and pH 10.0 without salt. The sample TRIS HCl pH 7.4 extract in 1 molar ammonium sulphate was applies on phenyl Sepharose gel, equilibrated with same buffer. After washing elutions were done at different pH values. Note the differential activities of various lysosomal enzymes at various pH elution.

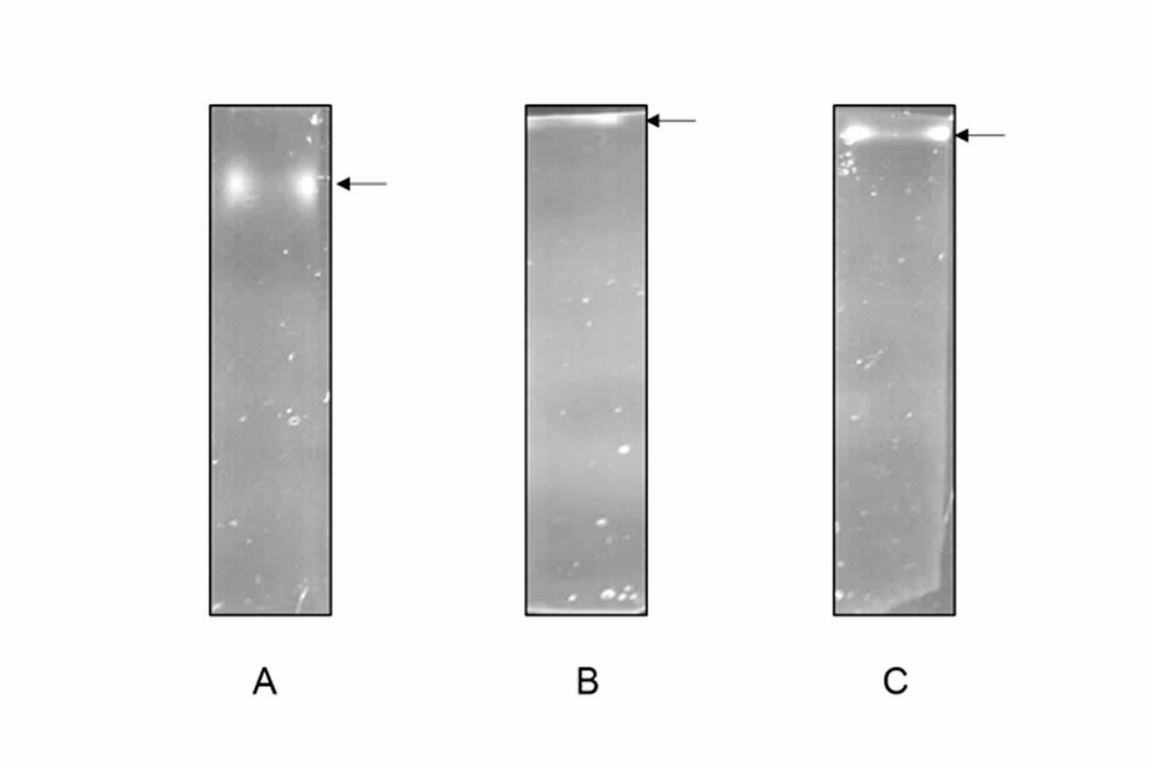


Figure 5.8: Activity staining of human lacrimal sac soluble extract.

Enzymes were detected using respective 4-methylumbelliferyl substrates. A). Fucosidase (Panel A), Glucuronidase (Panel B), and Mannosidase (Panel C). Arrows indicates the position of the enzyme.

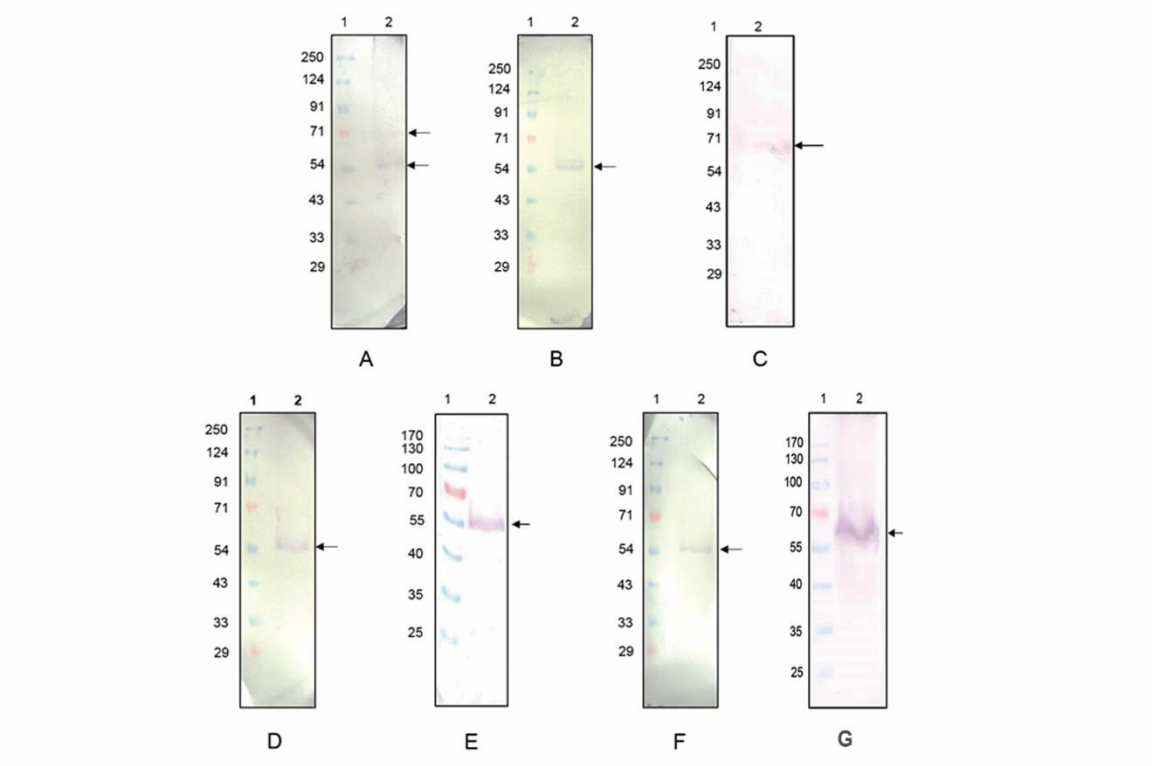


Figure 5.9: Western blot analysis of human lacrimal sac soluble extract.

Strong signals were noted for Fucosidase (Fig 4A), Glucuronidase (Fig 4B), Arylsulfatase (Fig 4C), Mannosidase (Fig 4D), Hexosaminidase (Fig 4E) and Acid phosphatase (Fig 4F). Membrane extracts showed strong signal for MPR 46 (Fig 4G). Lane 1: Molecular weight marker, Lane 2: crude soluble extract. Arrows indicate the signals of respective enzyme in the blot.

CHAPTER 6:

LECTIN PURIFICATION AND LACRIMAL SAC GLYCOPROTEIN PROFILING

6.1 Lectins and their Types

Lectins are a class of proteins of non-immune origin which carry at least one non-enzymatic carbohydrate recognition domain (CRD) (**Fig 6.1**). First report of a lectin dates back to more than 100 years where Peter Hermann Stillmark isolated highly toxic hemagglutinin Ricin from the seeds of the castor plant (*Ricinus communis*) (Barondes, 1988). Although, lectins were first identified and purified in large scale from plant seeds, they were later found in animals, microorganisms (Sharon and Lis, 2004). Lectin activity of a protein is monitored by checking its ability to agglutinate erythrocytes in a saline medium. The specificity of a carbohydrate recognition domain is then determined by the loss of agglutination activity in the presence of a particular carbohydrate. The activity of the lectin is the minimum amount of lectin required to exhibit agglutination activity and specific activity is the activity per milligram of the protein (Miller et al., 1982).

6.1A. Animal lectins

In animals, lectins play a diverse role in immunity, signaling and transport. Based on the nature and amino acid sequence similarity of the carbohydrate recognition domain (CRD), lectins are classified into several categories. Major lectin families found in the animal kingdom and a brief description about their characteristics are described below.

6.1B. C-type lectins

C-type lectins contain CRD which require Ca^{+2} ion for their optimum binding. They are important in the recognition of oligosaccharides attached to cell surface. Binding of these lectins to different sugar molecules are associated with various cellular events such as cell-cell adhesion, antigen presenting and apoptosis. Microbial recognition by C-type lectins involves the networking between these lectins and other

innate immune response molecules. Specificity and sugar binding may vary according to the species of the lectin present and genetic studies have shown that these lectins are one of the most diverse lectins in the animal kingdom (Drickamer and Taylor, 1993; Cummings and McEver, 2009). The CRD in CTLD (C- type lectin domain super family) changes in number and orientation in different directions and the subunit composition among different types may also vary according to the organism. More than a dozen functions of CTLDs have been reported and the major cellular functions of the lectin may not involve the calcium dependent sugar binding. In addition to several carbohydrate ligands, CTLD exhibits affinity towards proteins and inorganic molecules (Cummings and McEver, 2009).

6.1C. S-type lectins

S- type lectins are also called galectins. These lectins depend on the disulfide linkge for the optimal binding of CRD which is specific for the β- galactoside sugar and hence are named as S-type lectins and galectins respectively. Although specific to galactose and all its derivatives, galactins may bind strongly to lactose and to N-acetylglucosmine under physiological conditions. The galectins can bind to galactose sugar in both N-linked and O- linked glycans. 15 different galectins are reported in mammals and are encoded by LGALS genes. The galectins are distributed across the animal kingdom including rodents, birds, amphibians, fishes and in invertebrates (Barondes et al., 1994). Galectins are involved in a wide range functions such as apoptosis, suppression of T cell receptor activation, cell adhesion and splicing of mRNA. Roles of these molecules in association with various disease progression are also studied in recent years. Cancer, HIV, allergic reactions, autoimmune diseases and Chagas disease are some notable examples (Liu and Rabinovich, 2005; de Oliveira et al., 2015; Abdel-Mohsen et al., 2016).

6.1D. I-type lectins

I-type lectins are glycan binding proteins belonging to immunoglobulin super families. An exclusion of these are the T- cell receptors and antigen presenting cells. More than 500 proteins

in the mammalian genome belong to I-type lectins. Among these, Siglec family or sialic acid binding lectins are studied in detail (Varki and Crocker, 2009). Siglec family of proteins include more than 15 different proteins functioning in the cell-cell interaction, signaling of nervous system, immune cells and in hematopoetic cells. Other than Siglec families L1 cell adhesion molecules, neutral cell adhesion molecules, hyaluran and insect hemolins are also examples of I type lectins (Powell and Varki, 1995).

6.1E. P-type lectins

P-type lectins bind to the phosphorylated sugar molecules and plays an important role in the lysosomal transport. Two P-type lectins found in the vertebrates and some invertebrates. P-type lectins play an important role in the lysosomal transport of newly synthesized enzymes from the rough endoplasmic reticulum to the lysosomes. In addition to the lysosomal enzymes, CIMPR binds to numerous other ligands based on mannose 6-phosphate mediated interaction and protein-protein interaction (Dahms et al., 1989; Bohnsack et al., 2009). Receptor mediated endocytosis and lysosomal degradation of pro hormones via CIMPR is also important in the cell growth and disease progression (Schellens et al., 2003). CIMPR is a type 1 integral membrane glycoprotein and its domain 11 has high affinity towards Insulin like Growth Factor 2 (IGF-II) (Brown, 2002). pH dependent binding of the receptor to the peptide ligand helps in the clearance of excess IGF-II from the embryonic blood thereby maintaining the optimum growth of fetus in placental mammals (Wang et al., 1994). Binding of the IGF-II and its resultant lysosomal clearance is found to be associated significantly to the evolution of the placental and metatherial mammals. The affinity of the receptor towards IGF-II is believed to be increased when the low affinity amino acids were deleted from the CD loop of the CIMPR-domain 11 by an exon splicing enhancer around 150 million years ago (Williams et al., 2012).

The presence of lysosomal enzymes and their transport mediated by two MPR proteins have been confirmed in the mammals and in non-mammalian vertebrates and similar transport processes might occur in the starfish and fresh water mussel which are also known to contain the MPR proteins. Interaction of the purified lysosomal enzymes from these organisms with

the lysosomal enzyme receptors undoubtedly proves the receptors are responsible for the transport of these enzymes (Nadimpalli and Amancha 2010).

6.1F. Pentraxins

These are calcium dependent lectins with beta jelly roll structure of five non-covalently bound identical subunits arranged in a pentameric disc structure similar to legume lectins. Pentraxins are plasma proteins and are essential components of innate immunity of both vertebrate and invertebrate animals. Soluble pentraxins act as pattern recognition molecules and help in identification of foreign microbes as a part of adaptive immunity (Gupta, 2012). These lectins are further classified into short and long pentraxins based on the primary structure of the subunit. C reactive proteins (CRP) and serum amyloid proteins (SAP) are most studied pentraxins. In addition to polysaccharide ligands, these lectins can bind to nuclear components such as the amino terminal of lysine rich histone and small nuclear ribonuclear protiens. Role of CRP and SAP in various disease conditions such as Alzheimer's disease, pulmonary alveolitis and tumor are being studied in mammalian systems. Further these proteins were characterized from mouse, gunea pig, amphibians, *Limulus* and from many other invertebrates (Gewurz et al., 1995).

6.1G. Calnexin and calreticulin

Calnexin and calreticulin are chaperon proteins associated with endoplasmic reticulum and are essential for the folding and assembly of glycoproteins. Calnexin is a luminal protein present in the endoplasmic reticulum whereas the calretciulin is a type 1 transmembrane protein found attached to the interior phase of membrane of the endoplasmic reticulum where most of the protein mass is found in the lumen (Leach and Williams, 2013). Both the proteins share similarity in carbohydrate binding. The sugar specificity of these lectins may vary from monosaccharide glucose to β-1-3 linked glucose attached to the mannose of a pausimannosidic glycan chain. Binding to oligosaccharide is enhanced by ATP and calcium ions are essential for successful ligand binding of these proteins (Vassilakos et al., 1998). In addition to sugar molecules calnexin and calreticulin binds to a number of other ligands such as non-glycosylated phosphatidyl inositol anchored MHC

class one molecules and other luminal proteins such as influenza hemeagglutinin (Cannon et al., 1996; Hebert et al., 1996).

6.1H. Discoidins

Discoidins are blood coagulating proteins found in soil amoeba *Dictyostelium discoideum.* Discoidin 1 and Discoidin 2 are two different discoidins produced by the organism upon starvation and both the proteins bind to galactose and N acetylgalactosmine. The N-terminal domain of the lectins show sequence similarity to lectins from eukaryotes and C-terminal shares structural similarity to invertebrate lectins (Aragão et al., 2008; Springer et al., 1984). They are found in large quantities in newly developed spores and help in adhesion of cells to the substratum (Springer et al., 1984).

6.1I. F-type lectins

F-type lectins shows specificity for fucose sugar. Also known as fucolectins or eel agglutinin as they were first identified in the European eel (*Anguilla anguilla*) with a single CRD. Later, they were found to be an important part of adaptive immunity in several other fishes and invertebrates and were identified with multiple CRDs (Ogawa et al., 2011). These domains help in binding of fucose sugar in the prokaryotic glycan sequence resulting in the opsonisation of microbial pathogens by cross-linking them to the surface of phagocytic cells. Even though this lectin is identified in many teleost fishes of marine and fresh water origin, they are not found in mammals and other higher vertebrates.

6.1J. Annexins

Annexins are family of proteins found in all eukaryotic cells. They are phospholipid binding proteins playing an important role in the cell cycle, exocytosis and apoptosis. Specific function of different annexins are dependent on the type and location of the cells in which they are present. They play a major role in vesicular transport during exocytosis and endocytosis (Moss and Morgan, 2004). Single nucleotide polymorphism (SNP) in annexin genes have been associated with many diseases. SNP in annexin A2 gene may cause sickle cell anemia in Indian patients and another mutation in annexin A5 gene is found to be responsible for recurrent loss

of pregnancy in Japanese population. High levels of annexin is found in autoimmune responses such as rheumatoid arthritis and role of some annexins in relation to cancer and diabetes has also been observed. Certain annexins can be used as a biomarker in lung cancer and apoptosis (Mirsaeidi et al., 2016).

6.1K. Rhamnose binding lectins (RBL's)

These lectins are present in ovaries, eggs, skin and in mucus of fish and other invertebrate animals. They were first identified in the eggs of sea urchin (SEUL) and specifically bind to rhamnose, L- arabinose and D- galactose. They are often found in homodimer configuration and disulfide linkages between the dimers are important for the successful binding (Vasta et al., 2004). Except for the organs related to reproduction system, RBLs are abundantly seen in the systems related to immunity such as mucus cells, goblet cells of intestine, gills, spleen, thrombocytes, lymphocytes and monocytes. Crystal structure of the RBL from snake head fish (*Channa argus*) indicates that the expression of these protein may be induced in response to the inflammatory stimuli like LPS, IL-6 and IFN-γ (Vasta et al., 2011).

6.2. Applications of lectins

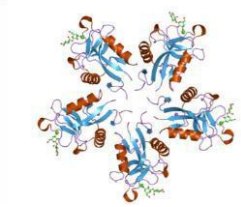
Lectins are promising candidates for therapeutic drug discovery from natural sources. Most of the lectins from plants and lower invertebrates function as a part of their non-specific defense mechanism and this property can be exploited for medical or industrial applications. Some lectins are used for blood typing and fucolectins are widely used as immune recognition molecules in studying the physiology of many invertebrate animals (Khan et al., 2002). Griffithsin provides an excellent example for the therapeutic application of an invertebrate lectin where the ability of this protein to bind to the glycoproteins in the HIV can make this lectin to be used as a potential therapeutic agent to develop anti-HIV drugs (Mori et al., 2005). Cyanovirin is another lectin isolated from cyanobacteria which reportedly exhibited anti-HIV properties (O'Keefe et al., 1997). Certain plant lectins have insecticidal activity, most of them can arrest the growth of the larvae and some can affect the metabolism when supplied with an artificial diet. This properties enable application of lectins as organic pesticides or insecticides in agriculture. Certain chitin binding

lectins can bind to the fungal cell wall and prevent biosynthesis or deposition of chitin in the cell wall. This property can be used for preventing fungal growth on plants. Anti-apoptotic properties of certain animal lectins enable them to exploit the lectins as anti-cancer agents (Lam and Ng, 2011). Other than these, lectins are widely used in the detection and isolation of glycoproteins and other glycoconjugates. Con A lectin coupled to affinity matrix is commonly used in the glycoprotein purification (Iscove et al., 1974). Lectin micro arrays are earning popularity in the detection and characterization of glycans from various sources. Lectin based nanoparticles are being tested abundantly for their potential to be used as specific agents for targeted drug delivery (Yin et al., 2007, Tao et al., 2008).

6.2A. P-type lectins in lysosomal trafficking

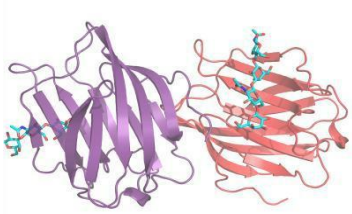
P-type lectins are type 1 integral membrane glycoproteins with one or more domains specific for the detection of phosphosugar molecules. The P-type lectins were first discovered when their absence was associated with a set of rare genetic disorders called lysosomal storage disorders. These lectins bind to the lysosomal enzymes by virtue of the mannose 6-phosphate recognition marker and transport them to the late endosomal compartment (Dahms and Hancock, 2002). Two distinct P-type lectins have been identified in mammals with different molecular weight but more or less with complimentary functions. These are cation independent mannose 6-phosphate receptor or IGF-II receptor (CI MPR Mr 300 kDa) and cation dependent mannose 6-phosphate receptor (CD MPR, Mr 46 kDa). Both these receptors are structurally similar in the amino acid composition of the CRDs that bind and deliver the mannose 6-phosphate containing lysosomal enzymes to lysosomes in a pH dependent manner. Both the receptors can be tracked in TGN and late endosomes as well as in the plasma membrane but are absent in the mature lysosome indicating that the receptors are recycled several times immediately after the delivery of their ligand molecules (von Figura and Hasilik, 1986). These P-type lectins have been demonstrated in the lacrimal drainage system as discussed in chapter 5 with regards to the MRP46 receptor.

C-type lectin



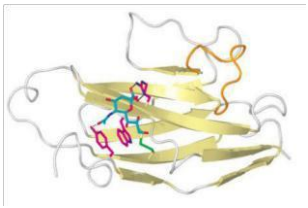
Galactose binding C-type lectin found in snake venum

S-type lectin



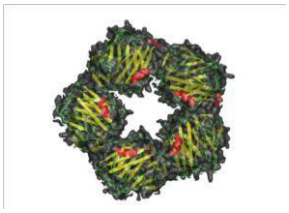
Human galectin 3 showing dimeric structure and bound carbohydrate

I-type lectin



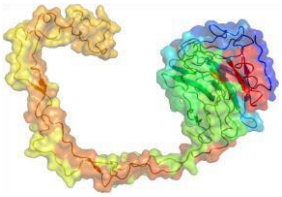
Crystal structure of human siglec

Pentraxins



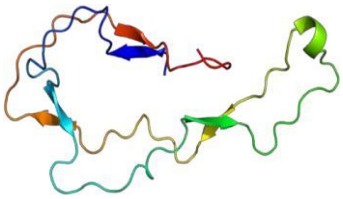
Structure of human C-reactive protein

Calnexin



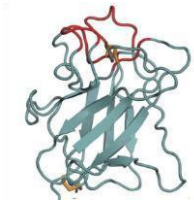
Predicted structure of human calnexin

Calreticulin



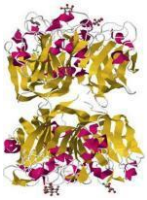
Predicted structure of human calrteticulin

Discoidin



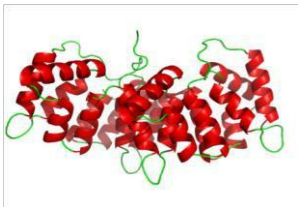
Structure of discoidin domain 1 receptor

F-type lectin



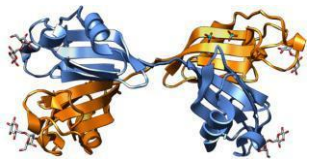
Crystal structure of the dimeric complex of eel agglutinin

Annexin



Predicted structure of human annexin III

Rhamnose Biniding lectin(RBL)



Structure of rhamnose binding lectin CSL-3 from the egg of chum salmon

Figure 6.1 Structural representation of different lectins described in the chapter. Source of the lectin is given in description below each figure.

Methods used in this study

The various methods used in the coupling of sugars to the Seralose gels, affinity purification of the lectins on the sugar specific gels, elution and confirmation of the homogeneity and subunit composition of the purified lectins have been well characterized in the laboratory. The details are given below together with the results

6.3. Lectin Purification and Coupling

Lectins are glycoproteins which have been affinity purified from numerous sources. The following procedures were adopted from the supervisor and the senior author's (NSK) work (Venugopal et al (2016) (**Table 6.1**)

Dolichos lablab seeds (KR-307) were purchased from Wipro seeds (India); Wheat germ, DVS, Sephacryl S-200 were from Sigma-Aldrich; Seralose 6B was from SRL, India; carbon-coated grids and uranyl acetate from M/s Icon analytical equipment Pvt LTD, Mumbai. All other chemicals and reagents used in this study were of high purity and procured from standards firms.

6.3A. Preparation of sugar-specific affinity matrices

Coupling of specific sugars (galactose, lactose, mannose, and *N*-acetyl glucosamine) to Seralose gel was carried out as described earlier (Latha, Rao, and Nadimpalli, 2006) and the gels were stored in equilibration buffer Tris-buffered saline (TBS) until use.

6.3B. Extraction of Dolichos lablab–I (DLL-I), Dolichos lablab-II (DLL-II) from Dolichos lablab seeds and affinity purification

About 50 g of the seed powder was homogenized and extracted overnight with 1 L (20 vol) of 25 mM Tris-HCl buffer pH 7.4 (TBS). The suspension was centrifuged at 16260 × g for 20 min at 4°C. The clear supernatant was saturated to 60% with solid ammonium sulfate and stirred for 3 h. The supernatant and pellet were separated by centrifugation and the clear supernatant was subjected to 60–80% saturation with ammonium sulfate, stirred for 2 h, and centrifuged as described above and the pellet obtained was used to purify the DLL-I.

The glucose/mannose-specific lectin (DLL-I) was purified as described earlier (Gnanesh Kumar, Pohlentz, Schulte, Mormann, & Nadimpalli, 2014). Briefly, the pellet obtained after 60% ammonium sulfate saturation was dialyzed extensively against sodium acetate buffer pH 5.0. The precipitate obtained after dialysis was discarded and clear supernatant was dialyzed

against TBS and was applied on Seralose–mannose affinity gel equilibrated with TBS. After extensive washing with column buffer, bound protein was eluted with 0.25 M glucose in TBS. The protein-containing fractions were pooled, concentrated, and applied on Sephacryl S-200 gel filtration column to remove any possible aggregates or contaminating proteins. The DLL-II was purified as follows. The pellet obtained after 80% saturation was dissolved in TBS and applied onto pre-equilibrated Seralose–galactose matrix. After washing, the bound galactose specific lectin was eluted with 300 mM galactose in TBS, fractions were collected, purified lectin was analysed by 10% SDS PAGE and this protein sample was used subsequently (**Fig 6.2 and Table 6.2**).

6.3C. Extraction and purification of WGA

Wheat germ powder was ground to a fine powder in a blender, defatted using acetone, and the powder air-dried. Forty-six grams of the defatted powder was extracted with 460 mL (10 vol) of 50 mM sodium acetate buffer pH 4.5, kept under stirring for overnight at 4°C. The homogenate was centrifuged at 16260 × g for 25 min at 4°C; the clear supernatant obtained was brought to 70% saturation with ammonium sulfate and kept for stirring for 3 h at 4°C. The pellet was collected by centrifugation at 16260 × g for 20 min, dissolved in 50 mM sodium acetate buffer pH 4.5 and dialyzed against the same for overnight. The dialyzed sample was loaded on the Seralose-N-acetyl glucosamine affinity matrix. After washing, the bound lectin was desorbed with 0.5 M N-acetylglucosamine in PBS and 4-mL fractions were collected. The fractions with maximum A280 were pooled and dialyzed extensively against PBS to remove the free sugar, and was used for SDS-PAGE.

6.3D. SDS-PAGE

The purity of each lectin was analyzed by 10 and 12% SDS-PAGE under reducing conditions (Laemmli, 1970). An example of SDS-PAGE for DLL-II showing two subunits at 29 and 31 KDa (**Fig 6.2**).

6.3E. Purification of DLL-I, DLL-II, MCL, and WGA

The affinity-purified DLL-I from lablab beans was resolved into five subunits on 12% SDS-PAGE with molecular masses in the range of 12–20 kDa and the subunits were designated as alpha α-1, α-2, α-3, α-4, and β (Gnanesh Kumar et al., 2014). The affinity-purified galactose-specific lectin was resolved in to two distinct subunits with molecular masses corresponding to 31 kDa (α) and 29 kDa (β) (Latha et al., 2006), consistent with our earlier results. Lactose-specific lectin was purified by employing the combination of affinity and gel filtration chromatographic methods. The purified lactose-specific lectin migrated as three subunit protein bands in 10% SDS-PAGE under reducing conditions with molecular masses of approximately 29 kDa, 28 kDa, and 26 kDa . The affinity purified WGA migrated as a single subunit in 10% SDS PAGE under reducing conditions. For the MCL, the exact process was followed but the proteins were passed through the Seralose-galactose gel.

Lectin	Starting material	Specificity and affinity matrix used	Extraction Buffer	Ammonium sulphate precipitation	Column Buffer & Wash Buffer	Elution Buffer
Concanavalin A (Con A)	*Canavalia ensiformis* / Jackbean meal (100g)	Mannose/Glucose specific Seralose- Mannose gel	1 M NaCl + 5 mM $CaCl_2$ & $MnCl_2$	80% (51.6g for 100mL)	1 M NaCl + 5 mM $CaCl_2$ & $MnCl_2$	0.25 M Glucose + column buffer
Dolichos lablab lectin I (DLL I)	*Dolichos lablab* /Indian bean (100g)	Mannose/Glucose specific Seralose- Mannose gel	Tris buffered saline (TBS – 25mM Tris + 150 mM NaCl)	60% (36.1g for 100mL)	Tris buffered saline (TBS – 25mM Tris + 150 mM NaCl)	0.25 M Glucose + column buffer
Dolichos lablab lectin II (DLL II)	*Dolichos lablab* /Indian bean (100g)	Galactose specific Seralose- Galactose gel	Tris buffered saline + 1.5 M Ammonium sulphate	80% (51.6g for 100mL)	Tris buffered saline + 1.5 M Ammonium sulphate	0.3 M Galactose + column buffer
Momordica charantia lectin (MCL)	*Momordica charantia* / Bittergourd (60g)	Galactose specific Seralose- Galactose gel	Tris buffered saline + 1 mM $CaCl_2$ & $MnCl_2$	60% (36.1g for 100mL)	Tris buffered saline + 1 mM $CaCl_2$ & $MnCl_2$	0.15 M Galactose + column buffer
Wheat germ agglutinin (WGA)	*Triticum aestivum* / Wheat (100g)	N-Acetyl-D-Glucosamine specific Seralose - N-Acetyl-D-Glucosamine	0.05 M Sodium acetate buffer pH 4.5	40% (22.6g for 100mL)	0.05 M Sodium acetate buffer pH 4.5 Wash Buffer: Distilled water & Phosphate Buffered Saline (PBS)	0.5 M N-Acetyl-D-Glucosamine in PBS

Table 6.1: Affinity purification protocols used for lectin purifications

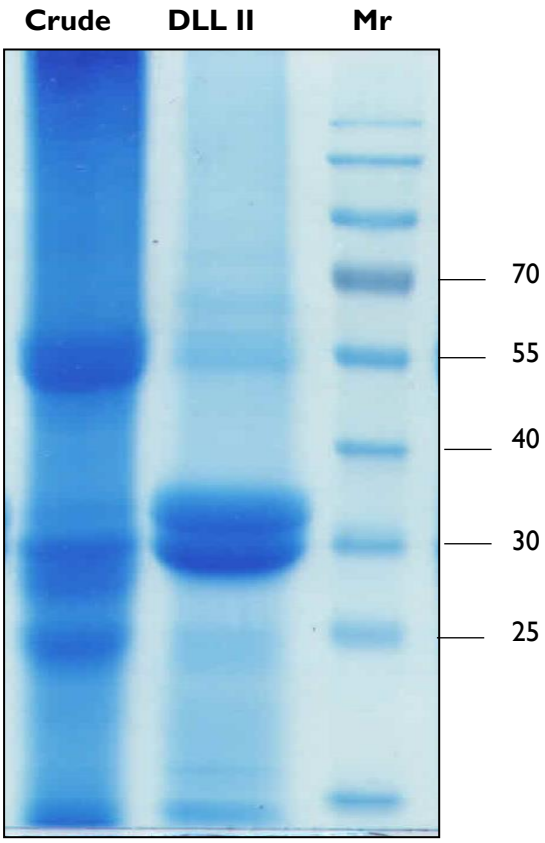


Fig 6.2A: 10%SDS-PAGE ANALYSIS SHOWING PURIFICATION OF DLL-II

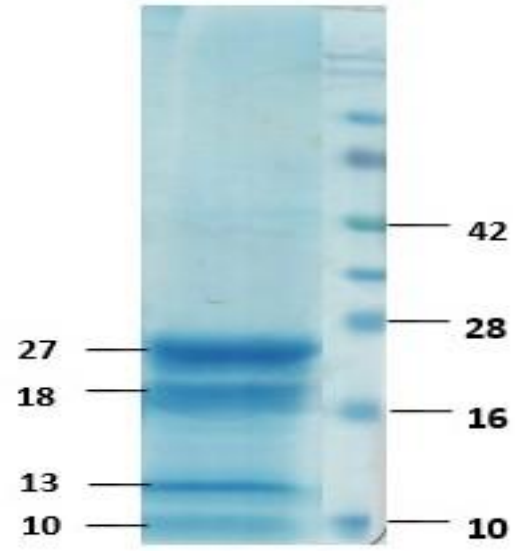


Fig 6.2B: 10%SDS-PAGE ANALYSIS SHOWING PURIFICATION OF CON-A

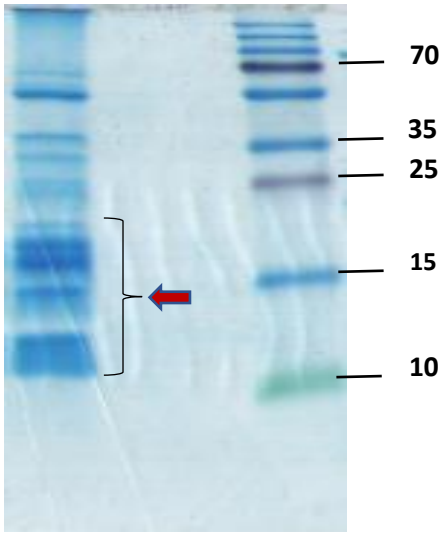


Fig 6.2C: 10%SDS-PAGE ANALYSIS SHOWING PURIFICATION OF DLL-I

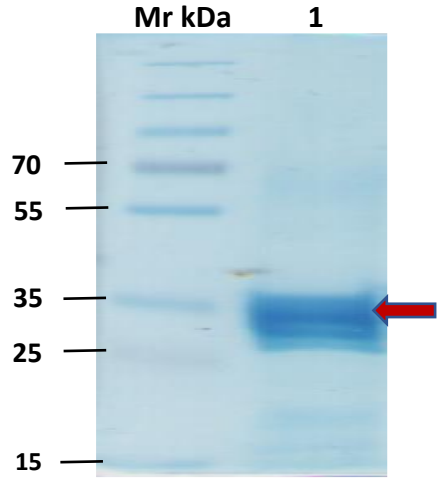


Fig 6.2D: 10%SDS-PAGE ANALYSIS SHOWING PURIFICATION OF MCL

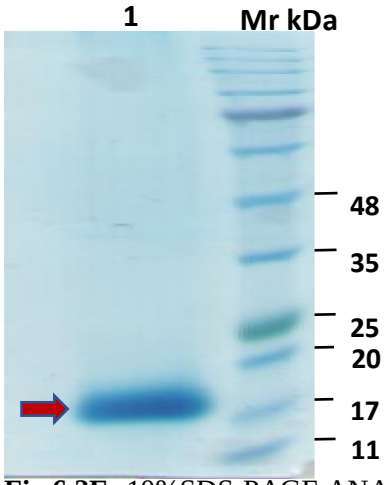


Fig 6.2E: 10%SDS-PAGE ANALYSIS SHOWING PURIFICATION OF WGA

Lectin	Source	Molecular weight (kDa)	No. of subunits	Molecular weight of subunits (kDa)
Concanavalin A (Con A)	*Canavalia ensiformis* / Jackbean meal	112	4	27, 18, 13 and 10
***Dolichos lablab* lectin I (DLL I)**	*Dolichos lablab* /Indian bean	67	5	12 - 20
***Dolichos lablab* lectin II (DLL II)**	*Dolichos lablab* /Indian bean	120	4	31 and 29
***Momordica charantia* lectin (MCL)**	*Momordica charantia* / Bittergourd	120	4	28 and 30
Wheat germ agglutinin (WGA)	*Triticum aestivum*/ Wheat	35	2	17

TABLE 6.2: Lectins purified for lacrimal sac protein profiling and their molecular details

6.4. Protein Profiling of the Lacrimal Sac

6.4A. Extraction of soluble proteins

About 1 g of the human lacrimal sac was excised in to small pieces with a sterile scalpel blade, washed with saline and homogenized with 20 mL of PBS containing 1 mM PMSF, at 4°C. The soluble proteins were extracted overnight at 4°C and the homogenate was centrifuged at 26,892 X g for 30 min, supernatant was collected, dialyzed against 25 mM Tris-HCl buffer, pH 7.4 and subjected to affinity chromatography on lectin-affigels.

6.4B. Chromatography on lectin-affigels

About 5 mg of each of the purified lectins (Glucose-specific DLL-I and galactose-specific DLL-II from *Dolichos lablab*, N-acetyl glucosamine-specific WGA from wheat germ, galactose-specific MCL from *Momordica charantia*, and mannose-specific Con A) were coupled separately to affigel-10, according to manufacturer's instructions (Bio-Rad). About 500 µL of each of the lectin-coupled affigel was packed separately into a column and equilibrated with 25 mM Tris-HCl pH 7.4 containing 150 mM NaCl (TBS). Soluble extract

obtained above was dialyzed extensively against TBS, and mixed separately with each of the lectin-affigel and kept for rotation at 4C for overnight. Unbound proteins were washed off from each of the matrix separately, and the bound proteins were eluted using specific sugar in TBS (0.25 M glucose was u6sed to elute proteins from DLL-I coupled-affigel, 0.3 M galactose was used to elute proteins from DLL-II coupled-affigel, 0.5 M N-acetyl glucosamine was used to elute proteins from WGA coupled-affigel, 0.2 M galactose was used to elute proteins from MCL coupled-affigel and 0.25 M glucose was used to elute proteins from Con A coupled-affigel). The eluted protein from lectin-affigels were precipitated by TCA and analyzed by 10 % SDS-PAGE.

6.4C. SDS-PAGE analysis

Eluted samples from each of the lectin coupled-affigels were analyzed by 10% SDS-PAGE, reducing conditions, according to *Laemmli* (Laemmli 1970). The protein bands were visualized by Coomassie blue staining (**Fig 6.3**).

6.4E. Electrophoresis

The human proteins from Lacrimal Sac were separated on 1D SDS-PAGE and stained with coomassie blue. Four gel pieces of Mol.wt ranging from 43 to 54 kDa were cut, reduced-alkylated and digested with trypsin with for 18 hrs as describe earlier (Jagannadham 2008). The trypsinized peptides were desalted and submitted to Q-Exactive HF (Thermo fisher corporation) attached with an Easy- nLC 1200 and an Easy spray column PepMap RSLC-C18, with a particle size of 3µM, pore size of 100 Å and a column diameter and length 75 µM X 15cm. The instrument was run with solvent A and B containing 95% H2O 0.1% Formic acid and 95% ACN 0.1% formic acid respectively. The normalised collision energy of 28, flow rate of 250nl/min having a gradient of 35 mins was set in which 3% of solvent B was used at 0 mins to 70% at 31 mins followed by a wash till 35 mins.

6.4F. Identification of proteins using Database

The MS/MS spectra containing in the raw files were analysed by the database search software Thermo Proteome discoverer version 1.4 supplied by the manufacturer. Human database from NCBI was downloaded in FASTA format, which were uploaded into the software. The raw files were run against the database using the Sequest HT algorithm in the software. The algorithm was run by applying the following conditions- trypsin as the enzyme with two missed cleavages, dynamic modification – oxidation of Methionine and Static modification- carbamidomethylation. The proteins were identified with atleast two unique peptides.

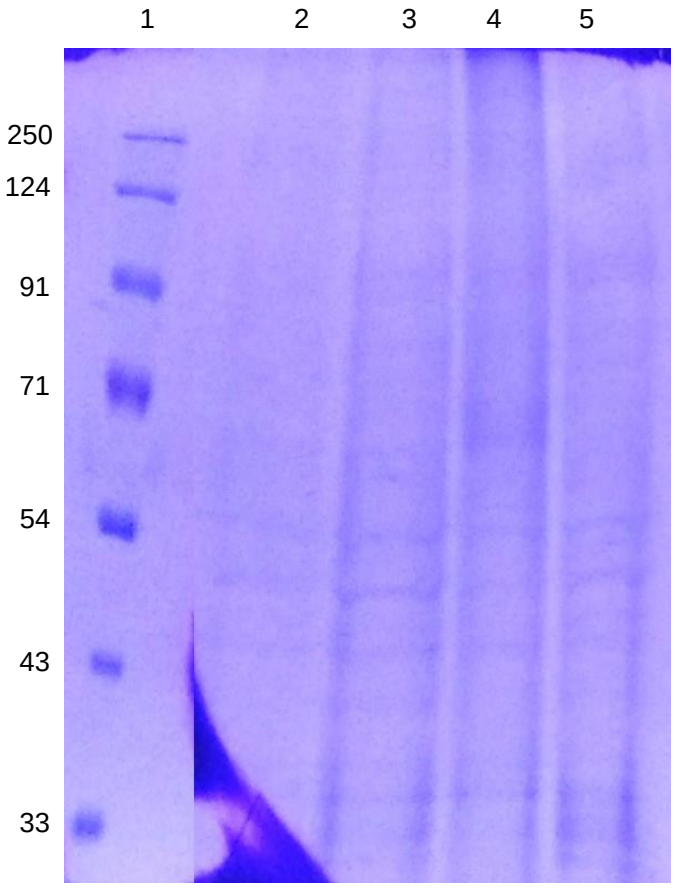


Figure 6.3. 10% SDS-PAGE analysis of the lectin-affigel eluted lacrimal sac soluble extract.

Lane 1: Marker (kDa); lane 2: Con A –Affigel eluted sample; lane 3: DLL-I –Affigel eluted sample; lane 4: WGA-Affigel eluted sample; and lane 5: MCL-Affigel eluted sample.

6.5. Discussion

Numerous proteins were identified by the proteome discoverer software in the lacrimal sac elute. 9-10 specific glycoproteins were commonly noted in all the affi gels studies namely; Con A affi-eluted gel, DLL-1 affi-eluted gel, WGA affi-eluted gel and MCL affi eluted gels. The common proteins identified are listed in the tables presented below and the entire sequence of Mass spectroscopy analysis is presented at the end of this thesis. Although a score of 2 and above was flagged as significant, we used more stringent criteria and took into considerations only those glycoproteins whose score came beyond 4. On the other hand, we took into considerations only those glycoproteins where a minimum of 2 unique peptides could be identified by mass spectroscopy. Most of the common glycoproteins identified were related to immune functions. Maximum number of glycoproteins were identified with the WGA affi-eluted gel band. Of Interest also was the presence of Prolactin inducible protein with an accession number of P12273 which was flagged in all the affi-gels analysed by mass spectroscopy. This is an important supplementary evidence to the finding of prolactin receptors that was performed in the hormonal study part of this thesis and this needs to be explored further as a lead in etiopathogenesis of primary acquired nasolacrimal duct obstructions. **Tables 6.3 to 6.5** details the mass spectroscopy Results of Common Glycoprotiens. **Tables 6.6 to 6.9** are large excel sheets which demonstrates the raw mass spectroscopy datas with different affigel columns.

In conclusion, this is the first step towards cataloguing the glycoproteins isolated from the lacrimal sac. This could be the starting point to assess the changes in glycoproteins in different disease conditions. The lead of prolactin inducible protien in correlation with the prolactin receptor expression demonstrated in chapter 4 signals towards a possible significant role in the inflammation scenarios of the lacrimal drainage milleiu and this needs to be followed up by studies in diseased models.

SUMMARY OF THE THESIS

SUMMARY OF THE THESIS

INTRODUCTION

The lacrimal drainage apparatus begins on the eyelid from a small opening called as lacrimal punctum and courses into the canaliculus, lacrimal sac and nasolacrimal ducts to open into the inferior meatus of the nose. This drainage pathway carries tears from the ocular surface in a unidirectional flow into the nasal cavity.

There are numerous diseases of the lacrimal drainage system and this thesis was done by a Clinician who wanted to understand the basic sciences of three major disorders namely; Primary acquired nasolacrimal duct obstructions, Functional nasolacrimal duct obstructions and Dacryolithiasis. To do this, the core aim of the present thesis was to explore the Glycobiology of the human lacrimal drainage system. In this context the following is the outline of our research work into five parts as follows:

A. Exploring the Surfactant proteins in the human lacrimal canaliculus.
B. Ultrastructural features of lacrimal sac and nasolacrimal duct mucopeptide concretions.
C. Qualitative hormonal receptors analysis of the human lacrimal drainage.
D. Exploring the presence of Mannose 6-phosphate receptors and lysosomal enzyme assay within a non-diseased lacrimal drainage pathway.
E. Purification of plant lectins and using lectin-affinity chromatography, exploring the various major glycoproteins in the lacrimal drainage tissues.

The thesis is divided into six chapters.

Chapter 1

This chapter is the general introduction of the human lacrimal drainage system, its anatomy, physiology and known biochemical functions other than tear drainage. Important principles for the understanding of the punctum, canaliculi, lacrimal sac and nasolacrimal ducts along with their structural-functional correlations and clinic-embryological profiles are briefly described. Various mechanisms of tear flow through each of the parts along with immunological characteristics of each and concepts of recent significance are discussed. Further the scope of the present investigation is also mentioned.

The subsequent chapters of the thesis 2, 3 and 4 are divided into following subheadings.

1. Introduction; 2. Materials and methods; 3. Results; and 4. Discussion.

Chapter 2

Exploring Human Lung Surfactants within the Lacrimal Canaliculus

2.1. Surfactant is a complex mixture of dipalmitoylphosphatidylcholine (DPCC), other lipids and proteins produced by type II alveolar epithelial cells and stored in specific intracellular lamellar bodies. Extensively studied in the lungs, the surfactants are crucial for pulmonary physiology and mainly function to reduce the surface tension and prevent alveolar collapse. Four major surfactant proteins, SP-A, SP-B, SP-C and SP-D are well studied with SP-A and SP-D demonstrating additional immunological functions. Recently whole genome sequencing and bioinformatics sequence analysis have revealed the presence of additional two novel putative surfactant proteins; SP-G or surfactant associated protein 2 (SFTA2) and SP-H or surfactant associated protein 3 (SFTA3). Although the specific roles of these are yet unclear, SFTA3 is known to mediate defense mechanisms like phagocytosis. The current chapter discusses the study protocols that looks into the expressions and distribution patterns of

surfactant proteins in the canalicular system and attempts to hypothesize their potential roles in tear flow and lacrimal drainage defense mechanisms.

2.2. The study was performed on fresh frozen cadaveric samples of canaliculi. Immunohistochemical labeling was performed for assessing the presence and distribution of surfactant proteins (SP); SP-A, SP-B, SP-C, SP-D, SP-G/SFTA2 and SP-H/SFTA3. Immunofluorescence double staining was performed using the respective fluorescein conjugated antibodies and the results were scored as positive or negative and the distribution pattern within the canalicular system was assessed. Western blot analysis was performed on the protein content which were resolved by reducing 15% SDS polyacrylamide electrophoresis and bands were studied following staining with primary and secondary antibodies. Human lung tissues were used as controls. The different standard methods used are also described.

2.3. This section describes the results obtained from the above experiments. Fluorescence double staining with DAPI and surfactant proteins showed strong immunostaining for SP-A, SP-B, SP-C, SP-D and SP-H/SFTA3. The positive immunofluorescence was noticed across all the layers of the epithelium but not the sub-epithelial structures. The expression was noted on the surfaces and superficial cytoplasm of the superficial and deep epithelial cells. There was no expression of SP-G/SFTA2 across the canalicular system. Western blot analysis of the proteins confirmed and concurred with the immunofluorescence findings.

2.4. The results obtained in the above section are discussed in detail with the available literature. This study provides a proof of principle for the presence of surfactant proteins known from lungs in the canalicular system and hypothesizes their possible functions and also their potential role in the tear flow dynamics between the ocular surface and the lacrimal drainage system.

Chapter 3

Ultrastructural features of the mucopeptide concretions to understand the pathogenesis of Dacryolithiasis.

3. 1. This section gives a brief introduction about dacryolithiasis in general, which is a condition of having stone like structures within the lacrimal drainage system which could cause obstructions and disease manifestations subsequently. Since the dacryoliths are predominantly composed of mucopeptides, recently, they are being called as mucopeptide concretions. The aim of this study was to examine the ultrastructural features of the mucopeptide concretions obtained from the lacrimal sac.

3. 2. This section gives the details of the materials and methods employed for assessing the ultrastructural features of the mucopeptide concretions. The concretions obtained from the lacrimal sacs of 10 patients during a dacryocystorhinostomy were immediately fixed for electron microscopic analysis. The surfaces were studied separately and longitudinal and transverse ultra-thin sections were obtained at different levels and all were studied using the standard protocols of scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

3. 3. Mucopeptide concretions based on their extent takes the shape of the lacrimal sac and nasolacrimal duct. The external surfaces and cut sections show mostly areas of homogenous deposits with occasional intervening heterogenic areas. Two distinct types of craters were noted, mostly in the heterogeneous areas. The core of the concretions is made up of extensive networks of fibril like tangles filled predominantly with granular material and red blood cells with occasional presence of granulocytes and epithelial cells. Numerous vacuoles and fissures appear to be more of artifacts than any metabolic process. No organic fibers of fungal filaments were noted within the concretions. There was no evidence of any bacterial biofilms other than few focal areas of scattered bacteria.

3. 4. The results from the previous section are discussed with current literature in this section. Ultrastructural features of mucopeptide concretions from the lacrimal sac help in better understanding of their etiopathogenesis and tissue interactions. Further exploration of different stages of a concretion is needed to understand the potential factors that trigger its genesis and evolution.

Chapter 4

Qualitative Hormonal Profiling of the Human Lacrimal Drainage System

4. 1. This section deals with a general introduction with regards to the most common disorder of the lacrimal drainage system called as primary acquired nasolacrimal duct obstruction. This disorder manifests in the form of watery eye followed by serious infections which need a surgical bypass of the lacrimal sac into the nasal cavity, a procedure called as dacryocystorhinostomy. It is a well-known fact the post-menopausal females are most commonly affected and to explain this gender predilection, the aim of the present part of the study was to investigate the presence and distribution patterns of hormones and their receptors in the lacrimal drainage system in normal and diseased states.

4. 2. This section describes the materials and methods used in the study. The study was performed on cadaveric and clinical samples of lacrimal drainage system. Immunohistochemical labeling was performed for assessing the presence and distribution of receptors of estrogen alpha (ERα), estrogen beta (ERβ), aromatase (CYP19), testosterone (TSTR), progesterone (PGR), oxytocin (OXTR), prolactin (PRL) and somatostatins 1 to 5 (SSTR1, SSTR2, SSTR3, SSTR4, SSTR5). The immunohistochemistry stains were scored as positive or negative and the distribution patterns in the canaliculus, lacrimal sac and nasolacrimal duct were assessed.

4. 3. Results obtained with various experimentations are detailed in this section. There was heavy expression of ERα, ERβ and OXTR but this showed variations in distribution patterns. TSTR and PGR expressions were more localized to the basement membrane of the epithelium in post-menopausal females. While SSTR2 and SSTR4 expressed only on the villus surfaces of superficial epithelial cells; OXTR, aromatase and PRL additionally expressed in the sub epithelial lamina propria and sub-mucosal glands. Diseased samples from PANDO showed dramatic reduction or absence of the receptor expression patterns of all the hormones with the exception of epithelial immunoreactivity with prolactin.

4. 4. The results obtained in the previous section are discussed in this section along with literature review. This study provides a proof of principle for the presence of multiple hormone receptors and hypothesizes their possible links in the etiopathogenesis of primary acquired nasolacrimal duct obstructions.

Chapter 5

Lacrimal Sac Lysosomal Enzyme assays and identification of their transport receptor

5. 1. Lysosomes are intracellular organelles composed of acidic compartments with more than 50 membrane proteins and 60 hydrolases. They together play a major role in degradation of extracellular materials through endocytosis and intracellular wastes by autophagy. The hydrolases; glycosidases, proteases and lipases are involved in the catabolic degradation of polysaccharides, complex proteins and lipids and products are exported out of lysosomes for excretion or reutilization in the biological pathways. Lysosomes are hence energy and nutrient sensors and involved in intracellular ion conductance. Disturbances of these functions commonly lead to lysosomal storage disorders. However, lysosomes are also a focus of increasing attention because of their role in the regulation of inflammatory gluco-corticoids pathways and other inflammatory signaling mechanisms. Lysosomal enzymes are known to

widely express in ocular tissues and lacrimal gland, with uvea and retina showing high concentrations of glycosidases, acid phosphatases and cathepsins. These enzymes have been potentially implicated in the pathogenesis of ocular storage disorders, retinal degenerations, uveitis and glaucoma. To the best of the authors knowledge, no exploration had been carried out for lysosomal enzymes and their receptors in the lacrimal drainage system. The current study explores the lysosomal enzymes and their mannose 6-phosphate (MPR) transport receptors in the lacrimal sac and nasolacrimal ducts.

5.2. This section details the methodology used for the lysosomal enzymes assays. The study was performed on healthy lacrimal sacs and nasolacrimal ducts obtained from exenteration samples immediately after surgery and frozen at -80 degrees for subsequent analysis. Soluble proteins extract was used for enzyme assays, SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), Native PAGE, activity staining and western blot analysis. Membrane proteins were separately assessed for detection of mannose 6-phosphate receptors; MPR 46. 4-methylumbelliferyl substrates and antibodies (some in house and some commercial) against common lysosomal enzymes and MPR receptor were used. Enzyme assays were carried out in triplicate to ascertain the results.

5. 3. This section details the results of the experiments mentioned above. Several lysosomal enzyme activities were documented and amongst them acid phosphatase and β-hexosaminidase were found to be high. Western blot analysis using enzyme antibodies and subsequent activity staining confirmed strong signals for moderately expressed enzymes like fucosidase, glucuronidase and mannosidase. Membrane extracts demonstrated the presence of MPR 46 receptor, which indicate the possible roles of cation dependent MPR's in lysosomal targeting in human lacrimal drainage system.

5. 4. This section discusses in details the findings and proposes possible hypothesis which may link the disequilibrium of lysosomal enzymes to the etiopathogenesis of primary acquired

nasolacrimal duct obstructions. This study provides a proof of principle for the presence of differential lysosomal activity and mannose 6-phosphate ligand transport receptors in the human lacrimal drainage system and hypothesizes the potential implications of their dysfunctions.

Chapter 6: Lectin Purifications, Lectin affinity chromatography and Lacrimal Sac glycoprotein profiling

6. 1. This section details the different types of lectins, their uses and the protocols followed for their purifications. The section details how each of the lectins were purified using standard protocols published by the supervisors laboratory. In total, five lectins with different sugar specificities were affinity purified (affinity gels contained different sugars immobilized to Sepharose via divinylsulfone) from different seed extracts namely, Concanavalin A, *Dolichos lab lab* I *(glucose/mannose specific)* and *Dolichos lab lab* II (galactose specific), Wheat Germ Agglutinin (N-acetylglucosamine specific) and *Momordica Charantia* (galactose specific) lectin. Each of the purified lectins were then separately immobilized to affigel-10.

6. 2. This section details the methodologies used for glycoprotein profiling. The soluble extracts from the lacrimal sac were passed separately through the different lectin-affigel columns equilibrated with column buffer (TBS), washed with column buffer and eluted with the specific sugar dissolved in column buffer. Aliquots of the eluates were then analyzed by SDS-PAGE analysis and the protein bands visualized. Three distinct bands were obtained in each of the eluates and the selected bands were cut and subjected to mass spectroscopy. The trypsinized peptides were desalted and submitted to Q-Exactive HF. The MS/MS spectra containing in the raw files were analysed by the database search software Thermo Proteome discoverer version 1.4 supplied by the manufacturer. Human database from NCBI was downloaded in FASTA format, which were uploaded into the software.

6. 3. This section details the results obtained from the above experiments. Numerous proteins were identified by the proteome discoverer software in the lacrimal sac elute. 9-10 specific glycoproteins were commonly noted in all the affi gels studies namely; Con A affi-eluted gel, DLL-1 affi-eluted gel, WGA affi-eluted gel and MCL affi eluted gels. The common proteins identified were listed in the tables presented in this section and the entire sequence of Mass spectroscopy analysis is presented at the end of this thesis. Although a score of 2 and above was flagged as significant, we used more stringent criteria and took into considerations only those glycoproteins whose score came beyond 4. On the other hand, we took into considerations only those glycoproteins where a minimum of 2 unique peptides could be identified by mass spectroscopy. Most of the common glycoproteins identified were related to immune functions. Maximum number of glycoproteins were identified with the WGA affi-eluted gel bands. Of interest also was the presence of Prolactin inducible protein with an accession number of P12273 which was flagged in all the affi-gel eluates analysed by mass spectroscopy.

6. 4. This section discusses the details of the finding in view of current literature. Other than the defense glycoproteins, the most important finding was the presence of prolactin inducible protein. This is an important supplementary evidence to the finding of prolactin receptors that was performed in the hormonal study part of this thesis and this needs to be explored further as a lead in etiopathogenesis of primary acquired nasolacrimal duct obstructions

Conclusions

- Canalicular epithelium expresses human lung surfactant proteins and they have a potential role in tear flow dynamics and lacrimal mucosal defenses.
- The core of the mucopeptide concretions is composed of dense fibrillary tangles intermixed with numerous red blood cells and this could imply that a epithelial breach

followed by a small bleed and clot can be the nidus to initiate the mucopeptide layering around them and subsequent formation of dacryoliths.

- Numerous hormonal receptors were demonstrated across the lacrimal drainage system. There were specific in distribution with distinct expressions between the healthy and diseased individuals and their dysfunctions could potentially play a role in the lacrimal obstructions.
- Lysosomal enzyme assays from the lacrimal sac showed differential expressions of these enzymes. The distinct enzymatic patterns and the presence of their transport receptors indicate their possible potential functions.
- Lacrimal sac protein profiling shows common glycoproteins on different affigels, most of which have role in mucosal defenses. In addition, the finding of prolactin inducible protein on all affigel and this when seen in the light of prolactin receptors found in the earlier parts of this thesis can provide a major lead to further study the etiopathogenesis of primary acquired nasolacrimal duct obstructions.
- The present exploratory work allowed to catalogue the various proteins/glycoproteins in the human lacrimal sac and has laid sufficient foundation to extend these studies in future in diseased models.

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Papers Published and Conference Presentations

PUBLISHED PAPERS OUT OF THIS PhD WORK

1. Ali MJ, Schicht M, Paulsen F. Qualitative hormonal analysis of the lacrimal drainage system: insights into the etiopathogenesis of primary acquired nasolacrimal duct obstruction. Ophthal Plast Reconstr Surg 2017;33(5):381-388.

2. Ali MJ, Schicht M, Paulsen F, Brauer L, Kumar NS. Electron microscopic features of mucopeptide concretions. Graefes Archives Clinical and Experimental Ophthalmology (Decision – Minor revisions, Revision Submitted).

3. Ali MJ, Schicht M, Brauer L, Paulsen F, Kumar NS. Surfactant proteins in the human lacrimal canaliculus. Ophthal Plast Reconstr Surg (Revies complete, Decision awaited).

4. Ali MJ, Venugopal A, Kavyashree P, Kumar NS. Lysosomal enzymatic assay and M6P receptors of the human lacrimal sacs. Indian Journal of Ophthalmology (Current statuts – Under Review).

5. Ali MJ, Venugopoal A, Kavyashree P, Kumar NS. Glycoprotein analysis of lacrimal sac using mass spectroscopy. (Manuscript Under Preparation)

B. Conference Presentations

1. American Academy of Ophthalmology, Annual meet, Nov 2017, New Orleans.

2. 12th International Society of Dacryology and Dry Eyes, Oct 2017, Athens, Greece

3. Mid-Term Oculoplastic association of India, May 2017, Varanasi

4. Otolaryngology workshop, Schroff Hospital, May 2017, New Delhi

5. Fantastic Breakthroughs in understanding lacrimal disorders', KKESH 2018, Riyadh.

6. Lacrimal Disorders- Updates 2017, Shangri La, Dec 2017, Abu Dhabi.

7. Pathogenesis of PANDO', 45th Anatomy Colloquium, Dec 2017, Nuremberg.