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INTERRELATIONSHIP BETWEEN DRY EYE SYNDROME
AND TEAR FLUID PHOSPHOLIPID TRANSFER PROTEIN

Niko Setälä

Academic Dissertation

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To my family
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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications, which are referred to in the text by Roman numerals I–IV:


IV. Setälä NL, Metso J, Jauhiainen M, Sajantila A, Holopainen JM. Dry eye symptoms are increased in mice deficient in phospholipid transfer protein. *In press Am J Pathol*.

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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>apoA, apoE</td>
<td>Apolipoproteins A and E</td>
</tr>
<tr>
<td>AsFIFFF</td>
<td>Asymmetrical flow field-flow fractionation</td>
</tr>
<tr>
<td>BSM</td>
<td>Bovine submaxillary mucin</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesteryl ester transfer protein</td>
</tr>
<tr>
<td>CF</td>
<td>Carboxyfluorescein</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>DES</td>
<td>Dry eye syndrome</td>
</tr>
<tr>
<td>DPPC</td>
<td>Dipalmitoylphosphatidylcholine</td>
</tr>
<tr>
<td>EIDE</td>
<td>Experimentally induced dry eye</td>
</tr>
<tr>
<td>HA-PLTP</td>
<td>High-activity phospholipid transfer protein</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>KCS</td>
<td>Keratoconjunctivitis sicca</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LA-PLTP</td>
<td>Low-activity phospholipid transfer protein</td>
</tr>
<tr>
<td>MGD</td>
<td>Meibomian gland dysfunction</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MUC</td>
<td>Mucin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipid</td>
</tr>
<tr>
<td>PLTP</td>
<td>Phospholipid transfer protein</td>
</tr>
<tr>
<td>POPC</td>
<td>Palmitoyl-oleoyl phosphatidylcholine</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>SEC</td>
<td>Size-exclusion chromatography</td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicle</td>
</tr>
<tr>
<td>TBUT</td>
<td>Tear break-up time</td>
</tr>
<tr>
<td>TF</td>
<td>Tear Fluid</td>
</tr>
<tr>
<td>Tlc</td>
<td>Human tear lipocalin</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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<tr>
<td>π</td>
<td>Surface pressure</td>
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ABSTRACT

Background and aims: The simplified model of human tear fluid (TF) is a three-layered structure composed of a homogenous gel-like layer of hydrated mucins, an aqueous phase, and a lipid-rich outermost layer found in the tear-air interface. The precise composition of the superficial lipid layer remains unknown. It is assumed that amphiphilic phospholipids are found adjacent to the aqueous-mucin layer and externally to this a layer composed of non-polar lipids, such as cholesteryl esters and triglycerides, face the tear-air interface. The lipid layer prevents evaporation of the TF and protects the eye, but excess accumulation of lipids may lead to drying of the corneal epithelium. Thus the lipid layer must be controlled and maintained by some molecular mechanisms. In the circulation, phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP) mediate lipid transfers. The aim of this thesis was to investigate the presence, molecular mechanisms and interactions of lipid transfer proteins in human TF. The purpose was also to study the role of these proteins in the development of dry eye syndrome.

Methods: TF samples were collected with microcapillaries. The presence of TF PLTP and CETP was studied by western blotting and mass spectrometry. The concentration of these proteins was determined by ELISA. The activities of the enzymes were determined by specific lipid transfer assays. To study the molecular mechanisms involved in PLTP mediated lipid transfer Langmuir monolayers and asymmetrical flow field-flow fractionation (AsFIFFF) was used. The AsFIFFF method was also used to determine the sizes of these molecular assemblies. Ocular tissue samples were stained with monoclonal antibodies against PLTP to study the secretion route of PLTP. Heparin-Sepharose affinity chromatography was used for PLTP pull-down experiments and co-eluted proteins were identified with MALDI-TOF mass spectrometry or Western blot analysis. To study whether PLTP plays any functional role in TF PLTP-deficient and experimentally induced dry eye (EIDE) mice were examined. The activity of PLTP was studied in dry eye patients.

Results: PLTP is a component of normal human TF, whereas CETP is not. Also the TF of wild-type mice displayed immunoreactivity against PLTP. In human TF PLTP concentration was about 2-fold higher than that in human plasma. PLTP-facilitated phospholipid transfer activity in TF was also significantly higher than that measured in plasma. Inactivation of PLTP by heat treatment or immunoinhibition abolished the phospholipid transfer activity in tear fluid. PLTP was found to be secreted from lacrimal glands. PLTP seems to be surface active and is capable of accepting lipid molecules without the presence of lipid-protein complexes. The active movement of radioactively labeled lipids and high activity form of PLTP to acceptor particles suggested a shuttle model of PLTP-mediated lipid transfer. In this model, PLTP physically transports lipids between the donor and acceptor. The particle diameter of PLTP was 6 nm as determined by AsFIFFF analysis. MALDI-TOF analysis of Heparin-Sepharose fractions identified several candidate proteins, but protein-protein interaction assays revealed only ocular mucins as PLTP interaction partners in TF.
mice with a full deficiency of functional PLTP enhanced corneal epithelial damage, increased corneal permeability to carboxyfluorescein, and decreased corneal epithelial occludin expression was demonstrated. These pathological signs were worsened by EIDE both in wild-type and PLTP knock-out mice. Increased tear fluid PLTP activity was observed among human DES patients.

**Conclusions:** These results together suggest a scavenger property of TF PLTP: if the corneal epithelium is contaminated by hydrophobic material, PLTP could remove them and transport them to the superficial layer of the TF or, alternatively, abolish lipophilic substances by binding them and transporting them through the naso-lacrimal duct. Thus, PLTP might play an integral role in tear lipid trafficking and in the protection of the corneal epithelium. The increased PLTP activity in human DES patients suggests an ocular surface protective role for this lipid transfer protein.
1 INTRODUCTION

Dry eye syndrome (DES) is a common external eye disease that arises from a wide variety of etiologies. The prevalence of dry eye ranges from 5% to 30% in people aged over 50 years. Dryness, redness, foreign body sensation, and burning and itching of the eyes are typical symptoms of dry eye. Somewhat controversially, intermittent excessive tearing can also be the main symptom of irritated eye. These symptoms are often unpleasant; moderate cases may affect quality of sight and life, but severe cases may lead to the damage of the ocular surface, resulting in impaired vision or even perforation of the eye.

The underlying biochemical mechanisms in the development of DES and the precise composition of human tear fluid are only partially known. The tear film is a complex three-dimensional gel-like aqueous fluid composed of a complex mixture of hydrocarbons, proteins, and lipids. The outermost structure of the tear fluid, the lipid layer, is composed of wax esters, sterol esters, and polar lipids. Based on the hydrophobic effect previously identified, it has been suggested that in the tear fluid, the polar phospholipids are disposed adjacent to the aqueous-mucin layer and externally to this is a layer composed of non-polar lipids, such as cholesteryl esters and triglycerides, facing the tear-air interface. This type of organization of lipids opposes evaporation, but the ocular surface epithelium and mucin components become vulnerable to lipid contamination. Excess lipidation of the ocular surface would lead to increased dewetting of the corneal epithelium and the development of DES. A mechanism is needed to organize and maintain the homeostasis of the lipid layer and to prevent epithelial or mucin contamination.

Phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP) have an important role in plasma lipoprotein metabolism. PLTP is a glycoprotein that was originally identified from plasma based on its capability to transfer phospholipids between phospholipid vesicles and high-density lipoprotein (HDL). In this study the possible role of PLTP in maintaining the delicate lipid homeostasis in the tear fluid is explored.


2 REVIEW OF THE LITERATURE

2.1 Tear fluid

Tear fluid (TF) covers the entire ocular surface and serves multiple purposes, including a barrier function, lubrication, nutrition, and antimicrobial protection. Good visual acuity is also dependent on normal TF because it provides a smoother ocular surface, which is important for the optical properties of the eye (Bron et al., 2004; Goto et al., 2006).

The “three layer theory” proposed by Wolff (1946) has stood as a standard model for the composition of tear fluid for a long time. This model describes the tear fluid as three separate layers: the superficial lipid layer from Meibomian secretion; the middle aqueous layer, which is the main component, secreted by the lacrimal gland and the accessory glands of Krause and Wolfring; and the deepest mucin layer secreted by goblet cells of the conjunctiva. The trilayered model is based on observations of ocular surface sections and findings from slitlamp attached to a microscope. However, recent studies with more highly developed equipment have questioned this model and described TF as having a more homogeneous structure, without separate aqueous and mucin layers (Chen et al., 1997; Butovich, 2008). It is easy to imagine that TF does not stand as a stable structure. Continuous movement of the eyelid and blinking, secretion of new meibum, aqueous, and mucin, tear evaporation and drainage to the nasal ducts keep these dynamic structures in constant movement. The structure of TF lipids and mucins are reviewed below in detail (see 2.2.1 and 2.2.2).

The production of tears is divided into basal, reflex, and emotional tearing. The volume of basal TF is estimated to be around 7 µl (Scherz et al., 1974; Tiffany, 1994), where around 1 µl covers the ocular surface and the rest is situated on the marginal meniscuses. The turnover rate of TF is 24.0 +/- 14.2 %/min (Shimizu et al., 1993), and basal tear secretion produces 2–3 ml in a day, calculated from dye dilution tests and from corneal epithelial cell desquamation (Ehlers et al., 1972). The thickness of TF ranges from 3 to 45 µm depending on the investigative method, while the most novel interferometry and optical coherence tomography measurements indicate that values around 3–4 µm seem to be more accurate (Prydal et al., 1992; King-Smith et al., 2000; Bron et al., 2004). Thickness of TF is highest after each blink, after which it starts to decrease. The tear evaporation rate is estimated to be 15.6 +/- 3.8 x 10(-7) g/cm² per second (Tsubota and Yamada, 1992). TF stability has been associated with a high surface pressure (low surface tension), which is due to both TF lipids and proteins. The average TF surface tension is around 70 mN/m (Glasgow et al., 2010). During sleep, secretion of all major proteins and water is inhibited, but the release of immunoglobulin A (IgA) continues (Tan et al., 1993).

In the basal TF, the principal electrolytes are Na⁺, K⁺, Cl⁻, and HCO₃⁻, with lower levels of Mg²⁺ and Ca²⁺. Tears are isotonic with serum, although the proportions of ions are slightly different and especially the concentration of tear potassium (K⁺) is higher (Gilbard, 1994). Small molecules that occur in serum are found in TF; these
include glucose, lactate, urea, etc., but at different levels. The mean pH of basal TF is 7.0, ranging from 6.5 to 7.6 (Abelson et al., 1981), and the TF’s osmolarity is 303 to 349 mOsm (Tomlinson et al., 2006). Many conditions, such as infection, dryness, and the neural control of lacrimal glands, change these proportions of the TF components. Typically, this is seen in rapid reflex tearing, where a substance irritating the cornea launches water secretion and dilution, or in slower changes like in dry eye syndrome, where the rise of TF osmolarity is a key element.

Proteins in TF have numerous functions, including bacterial defense, wetting by lowering surface tension, transport, wound healing, and the regulation of inflammatory processes. In basal TF, total protein quantity is around 20 g/L (Dohlman et al., 1976). The protein composition of tear film has been recently studied using mass spectrometric techniques. Li et al. (2005) identified 54 proteins and Zhou et al. (2009) counted 93 proteins, while 491 proteins were identified by de Souza et al. (2006) and our unpublished data identified around 250 proteins (Rantamäki et al., unpublished data). These last two results seem to be overestimates, as the TF proteome contains several intracellular proteins. The four major proteins—lysozyme, lactoferrin, lipocalin, and IgA—are secreted into TF from the ductal epithelium of the lacrimal gland and associated plasma cells.

Lysozyme damages bacterial cell walls by hydrolyzing 1,4-beta-linkages between N-acetylmuramic acid and N-acetylglucosamine (Van Haeringen, 1981); lactoferrin binds ion iron (Fe III) away from microbial metabolism (Broekhuyse, 1975); and IgA has antibody specificity to various pathogens (Franklin, 1989). These are all part of ocular immune defense.

Tear lipocalin (Tlc), firstly called tear prealbumin by Erickson due to its properties in electrophoresis, was characterized from TF in 1956. Tlc comprises 15–33% of all proteins in TF. It is supposed to act as a general scavenger of TF because it has endogenous ligands for lipophilic substances from different chemical classes, including phospholipids, fatty acids, fatty alcohols, and glycolipids (Glasgow et al., 1995). Recently, Tlc’s ability to remove fluorescently labeled lipids from the surface of the cornea has been shown (Glasgow et al., 2010), even though it seems that Tlc does not possess any neutral or polar lipid transfer activity between lipid vesicles (Saaren-Seppala et al., 2005). Tlc also binds microbial siderophores with high affinities. The role of Tlc in innate immunity is discussed, while the addition of exogenous Tlc could inhibit bacterial and fungal growth under iron-limiting conditions (Fluckinger et al., 2004). In addition to TF, Tlc is also expressed in von Ebner’s gland, the prostate, trachea, and nasal mucosa (Redl, 2000).

Various other substances like inflammatory mediators (interleukin-1 (IL-1), tissue necrosis factor alpha (TNF-α), matrix metalloproteinase-9 (MMP-9), growth factors (epidermal growth factor (EGF), transforming growth factor beta (TGF-β), hepatocyte growth factor (HGF), and remodeling enzymes (collagenase, plasmin, plasminogen activator) have been detected in tears in various disorders relative to normal controls. TF also contains corneal and conjunctival epithelial cells and white blood cells, which increase in number during inflammation (Tiffany, 2003).
Irritation of the ocular surface, and especially the cornea, results in reflex tearing, which is under the control of the parasympathetic nervous system. The mechanical corneal stimulus causes tearing more easily than chemical stimuli, and the central cornea is more sensitive than the periphery (Situ and Simpson, 2010). Ocular stimulus that causes discomfort and/or pain stimulates the sensorial endings of the nervus trigeminus, which results in a signal being given to the lacrimal glands, leading to their reflex hypersecretion. In addition, an irritation of the non-ocular area of the first branch of the trigeminus that innervates the nasal mucosa leads to reflex tearing. The Schirmer II test is used to measure reflex tearing related to nasal irritation. The purpose of reflex hypersecretion is to wash out foreign material and post-irritative secretions. Damage to nerves or the chemical blockade of the parasympathetic stimulus with cocaine or atropine diminishes reflex tearing.

Emotional tears are secreted due to emotional stimulus like sorrow, suffering, physical pain, and sometimes even happiness. Emotional tears contain more protein-based hormones such as prolactin, adrenocorticotropic hormone, and leucine enkephalin, than do basal or reflex tears (Sullivan, 2004). The limbic system, specifically the hypothalamus, which is involved in emotional control, also has a degree of control over the autonomic system. The parasympathetic branch of the autonomic system controls the lacrimal glands via the neurotransmitter acetylcholine through nicotinic and muscarinic receptors. When these receptors are activated, the lacrimal gland is stimulated to produce tears. Thus, as basal tears are the result of a spontaneous neuroglandular activity, and reflex tears are the result of an external sensorial stimulation, emotional tears are the result of a cognitive and emotional brain process. Most mammals produce tears in response to severe pain stimulus, but crying as an emotional phenomenon is considered to be uniquely human, possibly due to advanced self-awareness. However, some studies suggest that elephants, gorillas, and camels may cry.

2.1.1 Lipids of tear fluid

Meibomian glands, described in 1666 by the German physician Heinrich Meibom, are sebaceous glands that are located at the margins of the upper and lower eyelids of humans and mammals. These glands produce a lipid-rich secretion, also called meibum (Nicolaides et al., 1981), which is constantly released from the orifices of the glands. As lipids are less dense than water and due to the hydrophobic effect, they rise on the surface of the aqueous subphase, forming the lipid-enriched outermost layer of TF (McCulley and Shine, 1997). The lipid layer plays an important role in reducing the evaporation rate of the tear film and stabilizing it (Holly, 1973). It is also noted that protein adsorption at the air-water interface could be associated with structural changes. In the experimental tear model presented by Miano et al. (2005), the enzymatic activity of lysozyme is maintained in the presence of an outermost Meibomian lipid layer that prevents denaturation, while exposure at the air-aqueous interface induces significant lysozyme degradation. Thus, the TF lipid layer seems to be important in maintaining tear enzyme activity.
The Meibomian gland is a prominent source of lipids for the tear film, and originally the samples to study TF lipids were collected directly from Meibomian gland secretions (Nicolaides et al., 1981). However, it would have been a mistake to exclude other likely sources of lipids, such as the conjunctiva, cornea, and lipids secreted from palpebral skin (Butovich, 2008). Another problem in TF lipid research has been that former experimental techniques include sample hydrolysis and transesterification to achieve simpler structures before analysis. With this technique, the lipid compounds have been characterized only in lipid classes, and it has been difficult to resolve the correct complex lipid composition from the mosaic fragments. This is important, as even a little variation in a chemical structure of a lipid compound leads to changes in the properties and physiological activity of the molecule (Butovich, 2009).

At present, the proposed arrangement of the TF lipid layer is described as a bilayered structure and, based on its hydrophobic effect, it has been suggested that the polar phospholipids are disposed adjacent to the aqueous-mucin layer. Externally to this, a layer composed of non-polar lipids such as cholesteryl esters and triglycerides faces the tear-air interface (McCulley and Shine, 1997; Greiner et al., 1996; Kulovesi et al., 2010).

The mechanism that controls and organizes the tear lipid layer is important. If aqueous-mucin layer becomes thin enough, the lipids can contaminate the ocular surface. This would lead to dewetting and drying of the corneal epithelium. Furthermore, some lipids, including fatty acids, are known to induce apoptosis in a variety of epithelia (Malhi et al., 2006; Said et al., 2007).
Figure 1. Proposed model of tear film (Bron et al., 2004) and tear film lipid layer (McCulley and Ward, 2001).

2.1.2 Mucins of tear fluid

Mucins are a family of high molecular weight (0.5 to 20 MDa) and heavily O-glycosylated proteins produced by epithelial cells. In addition to the human ocular surface, mucins are found in the respiratory, gastrointestinal, and urogenital tracts, and the middle ear epithelium. Mucin molecules can be divided into two subfamilies: the secreted mucins and membrane-bound mucins. Characteristic of the mucin molecules are serine, threonine, and proline–rich tandem repeats (STP-repeats) of amino acids in their protein backbone (Bansil and Turner, 2006). These STP-repeats are the sites for O-glycosylation, where branched oligosaccharide chains consisting of 5–15 monomers are attached to the protein core and arranged in a “bottle brush” configuration (Gendler and Spicer, 1995). Mucins have a considerable capacity to tie up water and the high glycosylation makes them resistant to proteolysis. Between the STP-repeats of secreted mucins lie regions with a high proportion of cysteine. These sites have been shown to be involved in the dimerization of two mucin molecules via disulfide bond formation, as well as the subsequent polymerization of the dimers to form multimers (Sheehan et al., 2004). Through polymerization, secreted mucin molecules form a complex, gel-like adherent mucus that lubricates, hydrates, and serves as a barrier to pathogens. Atomic force microscopy studies of ocular mucin show that most
of the mucin fibers are between 200 and 600 nm long (Round et al., 2002), while fibers up to 2 µm have been observed in pig gastric mucin (Deacon et al., 2000).

At least 19 human mucin genes have been distinguished by cDNA cloning. The mucin genes show a high variation. While each mucin gene has its own tandem repeat amino acid sequence and its alleles vary in number, wide polymorphisms are found within and among individuals (Perez-Vilar and Hill, 1999). In the literature, the abbreviation for human mucins is MUC, while Muc refers to mice mucins and rMuc to rat-derived mucins. Several analogs to human mucins are found in animals, such as in Bovine submaxillary mucin (BSM), which is widely used in experimental procedures.

The mucins of the ocular surface are secreted by conjunctival goblet cells and the apical cells of the stratified conjunctival and corneal epithelia (Gipson et al., 2003). Like all mucins, the ocular mucins can be divided into secreted mucins (MUCs 2, 5AC, and 7) and membrane-bound mucins (MUCs 1, 4, 15, 16, and 20) (Gipson et al., 2010). The secreted mucins of TF are stored as polymers but secreted as monomers. The lower detected molecular weight of TF mucins indicates that the mucins do not form large polymers on the TF, perhaps preventing the formation of viscous mucus that could induce light scatter within the tear film (Spurr-Michaud et al., 2007).

MUC5AC is the major secreted mucin in human TF. In human TF, MUC2 is only detected at low levels by polymerase chain reaction (PCR) and the immunoblotting of tears. MUC7 is a small soluble mucin, which does not have cysteine-rich domains and is found as a monomer in TF. MUC7 has been shown to have antifungal and antimicrobial activity (Situ et al., 2003).

The epithelial surfaces of the cornea and conjunctiva serve as a border between the epithelial layer and TF. The surfaces of these tissues consist of many tiny membrane folds called microplicae. Membrane-bound mucins, which are smaller in molecular weight than the polymeric secreted mucins, are situated at the apices of the microplicae to form a structure known as the glycocalyx (Gipson, 2004). MUC1 and MUC16 are present on both corneal and conjunctival epithelia, while MUC4 is most prevalent in the conjunctival epithelium. These membrane-bound mucins are transmembrane proteins that have a short cytoplasmic tail, a transmembrane domain, an autoproteolytic domain called the SEA region, and an extracellular region that forms the glycocalyx. The glycocalyx layer can extend up to 500 nm from the epithelial surface (Hilkens et al., 1992). Data from freeze-fracture experiments of gut epithelium and mouse TF indicate that these mucins form rod-like extensions from the surface membrane (Chen et al., 1997). The extracellular parts of membrane-bound mucins are released from the SEA regions into the tear film and these ectodomains constitute a component of aqueous tear fluid. Recent data from yeast studies have shown that the mucin-like transmembrane proteins can activate a signaling pathway in response to increased osmolarity (Tatebayashi et al., 2007). Thus, it is hypothesized that membrane-bound mucins could have a sensory function in relation to the extracellular environment.
2.2 Dry eye syndrome

DES is the most common ocular problem. It was defined by the International Dry Eye WorkShop (2007) as a: *multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface.*

In population studies (i.e., the Salisbury Eye Evaluation, the Beaver Dam Eye Study, the Melbourne Visual Impairment Project, Blue Mountains, and the Women’s Health Study) the prevalence of dry eye ranges from 5% to 30% in people aged over 50 years (Schein et al., 1997; Moss et al., 2000; McCarty et al., 1998; Chia et al., 2003; Schaumberg et al., 2003). The prevalence of moderate-to-severe dry eye is closer to the lower estimate of the range, whereas the prevalence of mild dry eyes lies closer to the higher estimate of the range. The requirement of symptoms in the definition of dry eye is noteworthy, as tens of millions of people have mild dry eyes that may be notable only when some adverse extrinsic factor is present, such as low humidity or contact lens (CL) wear. Thus, most people with mild dry eye are unaware that their ocular symptoms belong to a dry eye syndrome. Dryness, redness, foreign body sensation, burning and itching of the eyes are the most common symptoms of dry eye. Usually, both eyes are affected and unpleasant sensations seem to get worse as the day goes on, during work, and in dry air conditions.

TF instability and hyperosmolarity are core mechanisms of dry eye (Figure 2). Tear film hyperosmolarity causes hyperosmolarity of the ocular surface epithelial cells and stimulates a cascade of inflammatory events involving mitogen-activated protein (MAP) kinases and nuclear factor kappa B (NFkB) signaling pathways (Li et al., 2004) and the generation of inflammatory cytokines (interleukin-1), tumor necrosis factor alpha (TNF-α), and matrix metalloproteinases (MMP-9). In addition, altered concentrations of lysozyme and lactoferrin have been detected in the tears of DES patients (Stuchell et al., 1981).
DES has several different etiologies and several forms of appearance; thus, it has been described with several different terms. Keratoconjunctivitis sicca (KCS), a Latin term referring to dryness and inflammation of the cornea and conjunctiva, was first described by Swedish ophthalmologist Henrik Sjögren (1933). Nowadays, KCS is commonly used interchangeably with DES, whereas an autoimmune-mediated disease of the lacrimal and salivary glands, Sjögren syndrome, is named after Doctor Sjögren. Xerophthalmia (the Greek term Xeros meaning dry) is a bilateral ocular disease caused by Vitamin A deficiency. It is characterized by night blindness, dryness of the ocular surface, and keratomalacia, softening and malformation of the cornea. The Delphi group (Behrens et al., 2006) has proposed to use the term dysfunctional tear syndrome (DTS) as a replacement for the term dry eye, to better describe the underlying failure in wetting of the ocular surface.

Figure 2. Tear Hyperosmolarity in the development of dry eye syndrome.
2.2.1 Classification of dry eye syndrome

Over time, new knowledge and diagnostic possibilities have changed the picture, as well as the classification, of DES. First, only the most severe cases of dry eye were documented. Thereafter, the Sjögren syndrome type of dry eye was described. The contemporary high requirements of sight and the knowledge of the pathogenesis of dry eye have led to the establishment of several classifications of DES for different diagnostic, clinical, or treatment purposes.

The National Eye Institute/Industry Workshop on Clinical Trials in Dry Eye (Lemp et al., 1995) was the first major consensus committee to describe DES. However, the classification in its report does not reflect newer knowledge about pathophysiological mechanisms, effects on vision, or the utility of an assessment of the severity of the disease.

The triple classification of dry eye for practical clinical use (Murube et al., 2005) is combined from reports presented at the 14th Congress of the European Society of Ophthalmology. It represents dry eye in relation to three separate schemes: one based on etiopathogenesis; one based on the glands and tissues targeted in dry eye; and one based on disease severity. However, the evidence-based referencing of this classification has been found to be limited (Lemp et al., 2007).

The Delphi panel (Behrens et al., 2006) proposed to change the name dry eye syndrome, although embedded in the literature, to dysfunctional tear syndrome, which more accurately describes the pathophysiological events in dry eye. The Delphi panel neglected to state whether lid disease is present or absent in particular cases of dry eye, as it is frequently difficult to identify these two forms of ocular surface disease.

DEWS (Lemp et al., 2007) presented the most recent classification of the epidemiology, pathogenesis, clinical manifestation, and possible therapy of DES. It gathered knowledge from previous classifications and research knowledge existing at the time. The two major classes of DES, aqueous tear-deficient dry eye (ADDE) and evaporative dry eye (EDE), were introduced. ADDE refers to a failure of lacrimal tear secretion, while EDE is constituted of intrinsic or extrinsic causes that lead to the evaporation of the tear film. The DEWS type classification of DES is presented in Figure 3 and below in detail.
2.2.2 Aqueous Tear Deficient Dry Eye

ADDE entails that the eye is dry due to diminished lacrimal tear secretion. The different forms of aqueous DES are listed here according to the DEWS recommendation.

I. Sjögren syndrome dry eye

*Primary Sjögren syndrome* is defined as an autoimmune disease of the exocrine glands, characterized by lymphocytic infiltration to the lacrimal and salivary glands and multiple sites of the epithelia. Inflammatory activation within the glands leads to the expression of alpha-fodrin and Ro/La ribonucleoprotein complex autoantigens at the surface of epithelial cells (Nakamura et al., 2006). The lacrimal glands are infiltrated by activated CD4\(^+\) and CD8\(^+\) T-cells (Hayashi et al., 2003). Inflammation in the lacrimal glands causes acinar and ductular cell death and the hyposecretion of tears. Hyposecretion of TF is enhanced by a neurosecretory block, due to the effects of locally released and circulating inflammatory antimuscarinic antibodies (anti-3 antibody) directed against muscarinic receptors within the glands (Dawson et al., 2005). In Sjögren syndrome, the antibodies to nuclear Ro(SSA) or La(SSB) antigens are present in the serum. The total prevalence of Sjögren syndrome is estimated to range between 3 to 4%, while the manifestation of Sjögren syndrome varies from very mild irritation to severe dry eye; furthermore, no all of the patients are aware of their diagnosis.

*Secondary Sjögren syndrome* consists of the features of primary Sjögren syndrome together with the features of another autoimmune disease, such as rheumatoid arthritis, systemic lupus erythematosus, polyarteritis nodosa, Wegener's granulomatosis,
systemic sclerosis, primary biliary sclerosis, or mixed connective tissue disease (Fries et al., 1994). The precise origin of autoimmune damage to acinar cells is not known, but risk factors including genetic profile (Moutsopoulos and Manoussakis, 1989), low androgen hormone status (Porola et al., 2007), and a nutritional deficiency in omega-3 fatty acids and vitamin C has been reported (Cermak et al., 2003). One proposed mechanism for the accumulation of autoimmune cells in the lacrimal glands is an interferon system activation launched by a previous viral infection. In addition, environmental factors leading to increased evaporation may act as a trigger by invoking inflammatory events at the ocular surface through a hyperosmolar mechanism (Figure 2).

II. Non-Sjögren syndrome dry eye
Non-Sjögren syndrome dry eye is a form of tear-deficient dry eye due to lacrimal dysfunction, where a systemic autoimmune features characteristic of Sjögren syndrome has been excluded. Causes of lacrimal dysfunction are divided into primary, congenital, secondary obstructive, and neurological forms.

IIa. Primary lacrimal gland deficiencies
Aging is the main reason for lacrimal gland dysfunction. According to population studies, the incidence of DES increases with age, although in the literature different aspects of normal tear secretion volume in elderly people have been postulated (Mathers et al., 1996 and Damato et al., 1984). In a study of lacrimal gland histology from elderly people, the majority of samples showed an increase in periductal fibrosis, interacinar fibrosis, paraductal blood vessel loss, and acinar cell atrophy (Obata et al., 1995). The aging of the population in Western countries will increase the prevalence of DES in the near future.

Alacrima is a rare congenital cause of lacrimal secretory disorders (Davidoff and Friedman, 1977). It is caused by mutations in the AAAS gene on chromosome band 12q13. The affected gene encodes the alacrima-achalasia-adrenal insufficiency-neurologic (ALADIN) protein disorder, which plays a role in RNA and/or protein trafficking to regulate intracellular protein transport (Sarathi and Shah, 2010). Symptoms include a selective absence of tearing in response to emotional stimulation, but a normal secretory response to mechanical stimulation.

Familial dysautonomia (Riley Day syndrome) is an autosomal recessive disorder where lacrimal dysfunction is a major feature (Riley et al., 1949). It is caused by a splicing mutations on chromosome 9(q31) in the gene encoding an IkappaB kinase-associated protein (Slaugenhaupt et al., 2001). A developmental and progressive neuronal abnormality of the parasympathetic innervations of the lacrimal gland and a defective sensory innervation of the ocular surface, which affects both small myelinated and unmyelinated trigeminal neurons, has been found. A generalized insensitivity to pain is accompanied by a lack of both emotional and reflex tearing. There is a unique pattern of serum catechols with high norepinephrine and low dopamine levels, and during physical and emotional stress, autonomic crises may develop.
IIb. Secondary lacrimal gland deficiencies

*Lacrimal gland infiltration* due to systemic disease can reduce the secretion of tear fluid. In sarcoidosis, infiltration of the lacrimal gland by sarcoid granulomata (Prabhakaran et al., 2007); in lymphoma, the lymphomatous cells (McKelvie, 2010); and in AIDS, the infiltration of T-cells may cause the development of DES. In AIDS-related dry eye, there seems to be a predominance of CD8 suppressor cells, while CD4 helper cells are dominant in Sjögren syndrome (Itescu et al., 1989). In graft versus host disease, a colocalization of transplanted periductal T-lymphocytes (CD4 and CD8) with antigen-presenting fibroblasts cause fibrosis of the lacrimal glands and dry eye symptoms that typically occur six months after hematopoietic stem cell transplantation (Tabbara et al., 2009).

*Lacrimal gland ablation* can occur due to trauma, or sometimes is unavoidable in tumor surgery. Structurally, the ducts from the main lacrimal gland pass through the palpebral part; thus, failed lid surgery can be expected to have the same effect as excision of the main gland. DES can be caused by partial or complete ablation of the lacrimal gland, but is not an obligatory consequence of this, presumably because accessory gland and conjunctival secretion may be enough to compensate for the secretion (Scherz and Dohlman, 1975).

*Lacrimal gland denervation* can cause DES, particularly when the compensative accessory glands are innervated and under similar reflex to the main lacrimal gland (Seifert et al., 1997).

IIc. Obstruction of the lacrimal gland ducts

Obstruction of the ducts of the main and accessory lacrimal glands, caused by different forms of cicatrizing conjunctivitis, leads to aqueous-deficient dry eye. The picture of obstructive Meibomian gland dysfunction (MGD) is common, and deformation of the palpebral aperture hinders the spreading of the tear fluid. Trachoma caused by Chlamydia trachomatis is a globally vast cause of blindness, where corneal opacity is caused by a trichiasis and a cicatrizing Meibomian gland obstruction. Dry eye is very common, resulting from lacrimal duct obstruction and lid malapposition (Guzey et al., 2000).

Dry eye due to lacrimal obstruction, cicatricial MGD, poor lid apposition, and notable conjunctival scarring, is seen in cicatrical and mucous membrane pemphigoid (a mucocutaneous disorder characterized by blistering of the skin and mucous membranes), Stevens-Johnson syndrome (an acute mucocutaneous disorder usually precipitated by drugs or infection), and after chemical and thermal burns. (Dart. 2005; Power et al., 1995; Kruse and Cursiefen, 2008).

IIId. Reflex hyposecretion

In an open eye, the lacrimal secretion is stimulated by the trigeminal sensory inputs arising from the naso-lacrimal channels and the surface of the cornea. Reduction of this stimulus decreases both tear secretion and the blink rate, which increases the evaporative loss of TF (Bourcier et al., 2005).
Bilateral sensory loss reduces both tear secretion and the blink rate. Topical anesthesia decreases the blink rate by about 30% and tear secretion by 60–75% (Nakamori et al., 1997). Contact lens wear (especially hard lenses or extended wear) reduces corneal sensitivity, and increased tear osmolarity is measured among CL wearers (Nichols and Sinnott, 2006). Decreased corneal sensitivity is supposed to correspond to dry eye symptoms after corneal LASIK surgery, although neurotrophic deficiency or neuralgic disorder are other possibilities reasons for this (Nettune and Pflugfelder, 2010; Tuisku et al., 2007). Diabetes mellitus has been noted as a risk factor for dry eye in several population studies (Moss et al., 2000; Moss et al., 2008). Diabetic sensory or autonomic neuropathy and the occurrence of microvascular changes in the lacrimal gland are supposed mechanisms of DES (Kaiserman et al., 2005).

Neurotrophic keratitis is characterized by extensive sensory denervation of the cornea. It is typically seen after herpes zoster ophthalmicus, or can be induced by trigeminal nerve section, injection, compression, or toxicity. Dry eye, diffuse punctuate keratitis, goblet cell loss, and peripheral or central ulcerative keratitis, which may even lead to perforation of the eye, are typical findings for neurotrophic keratitis (Bonini et al., 2003).

III. Reflex Motor Block

The nervus intermedius, a part of the facial nerve (cranial nerve VII), carries postganglionic parasympathetic nerve fibers to the lacrimal gland. Central damage to this nerve decreases lacrimal secretomotor function and leads to incomplete lid closure (lagophthalmos).

Several systemic drugs can decrease lacrimal secretion. In population studies, antihistamines, beta blockers, antispasmodics, diuretics, tricyclic antidepressants, selective serotonin reuptake inhibitors (SSRIs), and other psychotropic drugs were independently and significantly associated with dry eye (Moss et al., 2000).

2.2.3 Evaporative dry eye syndrome

Evaporative dry entails that the surface of the eye is dry due to increased vapor of tear fluid in the presence of normal lacrimal secretory function. The excessive fade of tear fluid can occur due to intrinsic factors affecting the composition of TF or ocular structures, or due to extrinsic exposure to the ocular surface. The boundary between these two categories is not always clear.

I. Intrinsic causes of dry eye

Ia. Meibomian gland dysfunction

MGD is the most common cause of evaporative dry eye (Foulks and Borchman, 2010). Meibomian gland obstruction, seen in MGD, typically arises from acne rosacea, seborrheic dermatitis, or atopic dermatitis.
MGD can be simple, where the gland orifices remain in the normal position, or cicatricial, in which the secretion of lipids is hindered due to posteriorly drawn orifices. An insufficient or dysfunctional tear fluid lipid layer is associated with an increase in tear evaporation and the development of dry eye.

**Ib. Disorders of the lid aperture**
Increased ocular surface area and wider palpebral fissure, seen in endocrine exophthalmos, proptosis and in high myopia, correlates with increased TF evaporation (Rolando et al., 1985). The same effect is seen if poor lid apposition or lid deformity exposes the ocular surface to extensive evaporation. In addition, activities that induce upgaze are noted to induce the evaporation of TF (Tsubota and Nakamori, 1995).

**Ic. Low blink rate**
Reduced blink rate, which may occur physiologically during intensive gaze while reading or watching television, or due to a neurological disorder like Parkinson’s syndrome, exposes the surface of the eye to evaporation (Abelson et al., 2002).

**II. Extrinsic causes**

**IIa. Ocular surface disorders**
Topically administrated drugs and preservatives can induce toxic damage to the ocular surface. Benzalkonium chloride (BAC), which causes surface epithelial cell damage and punctate epithelial keratitis, is the most common factor within this group (Uusitalo et al., 2010). Topical anesthesia can cause ocular surface damage through reducing the blink rate and deceasing sensory feedback to the lacrimal gland.

The normal development of goblet cells and expression of glycocalyx mucins are dependent of vitamin A (Tei et al., 2000). Thus, a deficiency of this vitamin can cause xerophthalmia, a potentially severe form of dry eye, which is still common in developing countries. Vitamin A deficiency can also cause damage to the structure of lacrimal acini, and thus patients with xerophthalmia may also have aqueous tear-deficient dry eye.

**IIb. Contact lens wear**
Around half of CL wearers report dryness of the eyes, and this is by far the primary reason for CL intolerance. The increased evaporation, reduced feedback to stimulate lacrimal secretion, and raised temperature of TF are the main reasons for dry eye among CL wearers.

**IIc. Allergic conjunctivitis**
Ocular allergy has been noted to increase the prevalence of dry eye in population studies (Moss et al., 2008). Allergic Th2 type reactions to external antigens with a release of inflammatory cytokines leads to submucosal changes in the conjunctival and corneal epithelium. Surface damage and irregularities can lead to TF instability
and a local drying component. Lid swelling can cause lid apposition and hamper TF spreading, thus exacerbating the dry eye.

2.2.4 Clinical examination of dry eye

Symptom questionnaires
Ocular irritation is a common complaint of patients presenting to an ophthalmologist. Anamnesis and symptom assessment are among most important “ophthalmic tools” for dry eye diagnosis. Often, only a few correct questions about ocular symptoms leads a clinician toward the diagnosis of dry eye. Several formal questionnaires have been developed to determine a score that would indicate dry eye diagnosis. These questionnaires, like the McMonnies (McMonnies and Ho, 1986) and Ocular Surface Disease Index (OSDI; Schiffman et al., 2000) are also used to determine the possible effect of dry eye treatments between repeated eye examinations.

Staining of the ocular surface
In dry eye, the corneal surface cells begin to disrupt; these small ruptures can be visualized with dyes. Lissamine green (triarylmethane) and more commonly used fluorescein dye diffuse in the fluid spaces between the cells; this staining can be seen when viewed with cobalt blue light. Rose Bengal (4,5,6,7-tetrachloro-2`,4`,5`,7`-tetraiodofluorescein), a dye that was previously thought to stain dead cells, stains epithelial cells that are not covered by mucin and TF; thus, it can possibly visualize even milder dry eye changes (Doreen et al., 2004).

To quantify and record the staining pattern of the cornea, different grading scales are used. In the literature, the van Bijsterveld system (1969), the Oxford system (Bron et al., 2003), and a standardized version of the NEI/Industry Workshop system (Lemp, 1995) are typically mentioned, although no studies have been published that indicate that one grading system is innately better than another.

Tear film break-up time
Normal tear film continuously covers the surface of the eye. If an eye is kept open long enough, without blinking, the tear film will start to evaporate. The tear film break-up time (TFBUT) is used to measure the time in seconds that it takes for a tear film to evaporate, deteriorate, and finally break when the eye is open (Holly, 1993). To better visualize the tear film, fluorescein dye is applied to the lower eyelid fornix and the patient is asked to blink and then look straight ahead without blinking. The tear film is observed under cobalt-blue filtered light using a slit lamp microscope and the time that elapses between the last blink and the appearance of the first break in the tear film is recorded. According to a report of Lemp and Hamill (1973), a fluorescein TFBUT of 10 seconds or less is consistent with dry eyes diagnosis, although a correlation between a smaller fluorescein volume and shorter TFBUT times has been proposed (Abelson et al., 2002). Non-invasive break up time (NIBUT) refers to a technique/instrumentation (i.e., keratometer, hand-held keratoscope, or tearscope) where a reflection of an image on the surface of the eye without dye is monitored and
the appearance of distortion of the image is recorded (Mengher et al., 1985). NIBUT measurements are typically longer than TFBUT ones.

**Schirmer test**

The Schirmer test is used to determine the quantity of tear production. A filter paper strip, 35 mm x 5 mm size, is placed at the junction of the middle and lateral thirds of the lower eye lid for 5 minutes; the wetting of the paper is measured as the nearest integer of millimeters. Despite wide day-to-day and visit-to-visit variation, the Schirmer test is valuable in determining tear-deficient dry eye. The diagnostic cut-off for tear-deficient dry eye suggested by the van Bijsterveld study (1969) was ≤ 5.5 mm, though more recent recommendations have shifted the limit to < 5.0 mm of wetting (Pflugfelder et al., 1998; Vitali et al., 2002). Generally, wetting of more than 10 mm is considered to be a negative test result.

The National Eye Institute workshop on dry eyes (2007) recommend performing the test with the eyes closed and refraining from the use of anesthetic eye drops before performing the test. In this way, the test is thought to measure both basal and reflex tear secretion; in contrast, when using anesthetic eye drops, the test is thought to measure only basic tear secretion. To measure the reflex tear secretion, the Schirmer II test may be performed by irritating the nasal mucosa with a cotton-tipped applicator before measuring tear production. For this test, wetting under 15 mm after five minutes is considered abnormal.

**Osmolarity**

The role of tear fluid osmolarity is well described in the pathogenesis of DES (Farris et al., 1986; Tomlinson et al., 2006). In both tear-deficient and evaporative dry eye the elevated tear osmolarity is regarded as a key feature that characterizes the condition of the ocular surface (DEWS, 2007). The recommended cut-off value for dry eye is >316 mOsm/l (Tomlinson et al., 2006). Traditional methods to measure tear osmolarity were laborious, and high volumes of tears were needed. The equipment setup and maintenance, required specialist craft, length of the procedure, and potential errors due to evaporation of the test sample have hindered its acceptance and general application in clinical practice.

Recently, special miniaturized devices, i.e., the Tearlab (OcuSense, Inc, San Diego, CA) and Advanced Instruments Model 3100 Tear Osmometer (Norwood, Massachusetts) have been introduced that measure osmolarity at a nanoliter scale (Tomlinson et al., 2010; Yildiz et al., 2009). These devices can easily be used in daily practice, as well as in dry eye research. Presumably, the measurement of tear osmolarity in general practice will notably increase in the near future.

**Cytology**

Differentiating between the symptoms and signs of ocular irritation originating from DES, allergy, or infection is sometimes challenging. In selected cases for sampling of the cells, taking mucin and/or ocular cytokines using impression or brush cytology can be helpful. In both, techniques a sample from the ocular surface is fixed and stained, and the profile of the cells is examined under the microscope. Additional staining with specific antigens may be used (Lopin et al., 2009).
None of these tests alone can offer a straight and absolute diagnosis of dry eye. Concerning an individual patient, a pattern of clinical tests and a classification scheme can provide a guide, but an expert clinician applying appropriate diagnostic criteria is needed to establish a diagnosis.

2.2.5 Animal models of dry eye syndrome

Some animal strains develop DES spontaneously. Quimby (1979) recognized similarities between dogs with severe DES and human Sjögren’s syndrome patients. This similarity has been confirmed by a study of dog lacrimal glands and conjunctiva, revealing a decreased apoptosis in the lymphocytes infiltrating the lacrimal gland and increased apoptosis in lacrimal acinar and conjunctival epithelial cells (Gao et al., 1998). The incidence of spontaneous DES in the general canine population is unknown, although the prevalence is estimated to be highest for American cocker spaniels (20.6%), whereas mixed-breed and beagle dogs have lower prevalence rates of 11.5% and 1.2%, respectively (Kaswan et al., 1998). Spontaneous DES with the natural course of the disease in dogs provides a useful tool for dry eye studies. The large size of the dog’s globe compared with that of rodents provides benefits in ocular surface diagnostic tests and the collection of tears and mucin. The negative aspects of dog models include long and expensive maintenance, ethical questions, and difficulties in obtaining approval for experimental procedures on this species. In addition, the signs of DES tend to be considerably more severe in dogs than in humans.

Beside studies one spontaneous canine DES, several experimental animal models have been generated to define the mechanisms of human DES; these are important tools for investigating the multiple factors that have been implicated in the pathogenesis of DES. The National Eye Institute Industry Workshop on Clinical Trials in Dry Eyes has produced a classification that separates DES models into two major types: tear-deficient forms (including Sjögren’s syndrome and non-Sjögren’s tear-deficiency) and evaporative forms (Lemp, 1995). Several of the present DES models resemble tear deficiency or Sjögren syndrome-like autoimmune disease, since they diminish aqueous tear production via mechanical, autoimmune, or chemical effects on the lacrimal gland.

The mechanical removal of the main lacrimal gland decreases basal tear production, measured by the Schirmer test, in dogs, cats, monkeys, and mice, but it does not cause significant changes in the ocular surface (Maitchouk et al., 2000; Barabino and Dana, 2004). In rabbits, closure of the lacrimal gland excretory duct, together with removal of the nictitating membrane and Harderian glands, increases tear osmolarity; moreover, decreased conjunctival goblet cell density is seen (Gilbard et al., 1989). Thus, it seems that compensatory tear production by the accessory lacrimal glands alone may be sufficient to maintain a stable tear layer.

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Human autoimmune Sjögren syndrome is characterized by a chronic inflammation of the lacrimal gland. The histological picture of affected glands shows infiltration of CD4+ T-cells that have a defect in Fas-mediated apoptosis (Van Blokland and Versnel, 2002). In Sjögren syndrome, the aberrant presentation of autoantigens and class II major histocompatibility complex (MHC) molecules (cathepsins B and D, and ribonucleoprotein particle La/SSB proteins) by lacrimal gland acinar cells is capable of priming autoreactive T-cells (Yang et al., 1999). Several different animals are proposed to mimic the effect of abnormal CD4+ T-cell accumulation in the lacrimal glands. The non-obese diabetic (NOD) mouse model shows a lymphocyte infiltration of predominantly CD4+ Th1 cells in the lacrimal gland, as well as other organs, including the pancreas. TF secretion in these mice is reduced by 33% to 36% compared with wild-type (WT) animals (Humphreys-Beher et al., 1994). In IQI/Jic mice, the lymphocyte infiltration is restricted to salivary and lacrimal glands, making it more similar to the Sjögren-type model (Konno et al., 2003). In these mice, the development of complete dacryoadenitis takes around 9 months, which makes their use laborious. TGF-β1 knockout (KO) mice show significant inflammatory cell infiltration in the lacrimal gland (McCartney-Francis et al., 1997). Unfortunately, these mice die between 3 and 4 weeks, just when they start to show increasing symptoms of a dry eye. The shortened life of the TGF-β1 KO mouse complicates their use as a dry eye model, particularly for studies involving the testing of medical approaches to dry eye. The autoimmune mediated models of DES also include the direct injection of cultured and activated T-cells into the rabbit lacrimal gland (Zhu et al., 2003) and activation of the rat immune system through the intradermal administration of a lacrimal gland extract in complete Freund’s adjuvant (Liu et al., 1987).

The lacrimal secretion of TF is controlled through a neural reflex from ocular surface to maintain adequate wetting of eye. Human neurogenic dry eye rises from a traumatic or disease-originated defect in this system. A milder neural feedback blockade is found among CL wearers, especially when hard contact lenses are used. Like in human neurogenic DES, animal models also show that a surgical removal of the parasympathetic nervation of the lacrimal gland leads to the development of DES (Salvatore et al., 1999; Toshida et al., 2007) A KO mice model of protein kinase C (PKC)-α, which plays a major role in the parasympathetic neural stimulation of lacrimal gland secretion, significantly decreases the production of TF (Hodges et al., 2004). Clinical symptoms of DES are also found in neurturin-deficient mice, in which a defect of this neurotrophic factor for parasympathetic neurons and decreased parasympathetic innervation of lacrimal glands is presented (Song et al., 2003).

To mimic neural defect, pharmacological models of anticholinergic (antimuscarinic) blockade have been engendered. The anticholinergic effect of topical 1% atropine sulfate has been shown to reduce lacrimal secretion significantly (Burgalassi et al., 1999). Dursun et al. (2002) have presented a mouse model of DES in which transdermal scopolamine application is used to induce a pharmacologic blockade of cholinergic receptors in the lacrimal gland. In this model, significant DES changes were seen when neural blockade was combined with a continuous air flow that mimics environmental stressing conditions.
Animal models of evaporative DES are more infrequently studied than tear-deficient models. As tear lipid film imbalance and evaporative DES are common diseases of the eye, especially among elderly citizens, an experimental model that carefully mimics this condition is of utmost importance. The forced opening of the eyes with sutures or lid specula certainly leads to dry eye (Fujihara et al., 1995), but this procedure is far from ideal for studying the pathogenesis of DES, which is a chronic event. However, in the open eye, corneal epitheliopathy develops within a few hours; hence, this model can be used to evaluate the effect of artificial tears or other therapies. The mechanical cauterization of Meibomian gland orifices presented by Gilbard et al. (1989) and other lid surgery techniques definitely affect tear lipid film composition, but the normal appearance of palpebral aperture is easily hampered. Different mouse models of MGD (including aplasia, hypoplasia, and atrophy) have been created through mutant and transgenic techniques (Barabino and Dana, 2004). These models definitely have their advantages, even though a total absence of tear lipids is rare in human DES and the tear lipid film composition seems to differ from direct Meibomian secretion (Butovich, 2008). Animal models introduced for blepharitis show reduction in tear fluid lipid production associated with dry eye symptoms (Chan et al., 1995), but in these models the normal anatomical appearance of either lid or eye surface is also typically altered. However, the blepharitis itself induces ocular surface irritation and alters the immunological profile of the ocular surface, which is an important factor in the development of DES.

The existing animal models of DES mimic pathological mechanisms underlying DES, but at the moment none of them seems to mimic precisely the complexity and chronicity of human dry eye. The bigger animals, like rabbits and dogs, have their advantages since they have bigger eyes; still, despite the small size of mice, the fairly well-known murine immunogenetics and the good availability of reagents and KO and transgenic strains make the mouse model very useful. Concerning the use of laboratory animals, it is important to remember that immortalized human corneal and conjunctival epithelial cell lines are also available (Gipson et al., 2003; Robertson et al., 2005). These models are useful in studying the effects of tear osmolarity, inflammatory mediators, and artificial tears on surface epithelia. To better understand the molecular mechanisms underlying in the pathogenesis of human DES, these models must be further studied and new models should be developed to describe evaporative DES in more detail.

### 2.2.6 Treatment of dry eye

Unfortunately, there is no curative treatment for DES because the pathogenesis of this disease remains unknown. Several treatment options are, however, available to aid in managing its symptoms. Current treatment recommendations are based on the severity of the disease (Lemp et al., 2007; Jackson, 2009). Lifestyle and nutrition modifications (Larmo et al., 2010), regular lid hygiene, and artificial tear supplements are typically sufficient against mild DES. Numerous artificial lubricant drops, gels and ointments are introduced to moisture the surface of the eye and to compensate reduced tear
production. These products also dilute tear osmolarity and wash out inflammation-inducing agents from the ocular surface. Patients with moderate-to-severe symptoms are recommended to use additional anti-inflammatory medications. Pulse therapy with topical steroids can be applied while adverse side-effects limit their use. Topical cyclosporine seems to be well tolerated and effective in mild-to-moderate DES (Foulks, 2006). The muscarinic agonist pilocarpine administered orally has been used as a secretagogue to stimulate production of tear. It also temporarily increases the number of goblet cells (Aragona et al., 2006). In addition to medical therapy contact lenses, punctal plugs (Erwin et al., 2008) and moisture-retaining eyeglasses are used. In severe sight threatening situations autologous serum drops and surgery are recommended. Short term closure of lids, tarsorrhaphy, amnion-membrane transplantation and limbal transplantations are used when satisfactory response can not be achieved with other medications or when severe DES has already damaged ocular stem cells.

2.3 Classification of lipids

International Lipid Classification and Nomenclature Committee defines lipids as a broad group of hydrophobic or amphipathic molecules that may originate entirely or in part by carbanion-based condensations of thioesters and/or by carbocation-based condensations of isoprene unit (Fahy et al., 2005). Lipids are generally considered to be molecules that are insoluble in water and soluble in organic solvents (Smith, 2000). Biological lipids refer to a broad group of molecules which includes fatty acids, waxes, eicosanoids, glycerolipids, phospholipids, sphingolipids, sterols, prenols, and fat-soluble vitamins A, D, E and K (Fahy et al., 2009). The main biological functions of lipids include major roles in energy storage, as structural components of the cell membranes, and as important signaling molecules.
Figure 4. A schematic representation of a lipid molecule. The hydrophilic headgroup is pointed towards the aqueous, whereas the hydrocarbon chains are embedded into hydrophobic membrane interior. Due to this in aqueous solutions lipid molecules are aggregated into 3-dimensional aggregates, such as micelles, bilayers, or tube-like hexagonal phases (adapted from www.schoolworkhelper.net).

Fatty acids
The fatty acids (FA) are the major lipid building blocks of complex lipids and therefore they are the most fundamental categories of biological lipids. FA molecule is constructed by chain elongation of a repeating series of methylene groups that leads to the hydrophobic character to this category of lipids. The simplest subclass molecule is a straight chain of saturated fatty acids containing a hydrophilic carboxylic acid terminus. The core hydrocarbon chain is typically between 4 to 24 carbons long, and it may be saturated or unsaturated with one or more double or even triple bonds between the carbon atoms. Double bonds cause the fatty acid chain to bend, which is important in the formation and function of cell membranes. Cyclic fatty acids containing three to six carbon atoms as well as heterocyclic rings containing oxygen, nitrogen and sulfur are found in specific subclasses. Examples of biologically important fatty acid derivatives are the eicosanoids, derived from arachidonic acid and eicosapentaenoic acid, which include prostaglandins, leukotrienes and thromboxanes.
Other major classes of FA derivatives are the fatty esters and fatty amides. Fatty esters include important biochemical intermediates such as wax esters, fatty acid thioester coenzyme A derivatives, fatty acid thioester ACP derivatives and fatty acid carnitines.

**Glycerolipids**
Glycerolipids (GL) are composed mainly of mono-, di- and tri-substituted glycerols. GLs serve as a main fat storage fat in animal tissues and the most well-known lipid of these fatty acid esters of glycerol is triglyceride (TG). In GL compounds the three hydroxyl groups of glycerol are usually esterified by different fatty acids. Additional subclasses of glycerolipids are glycosylglycerols, which are characterized by the presence of one or more sugar residues attached to glycerol (Påhlsson et al., 2001). Of these digalactosyldiacylglycerols are found in plant membranes and seminolipid from mammalian sperm (Hölzl and Dörmann, 2007).

**Glycerophospholipids**
Glycerophospholipids, also known as phospholipids (PL), are abundant in nature. PLs are main structures of the biological cell lipid bilayers. The most common PLs found in these membranes are phosphatidylcholine (PC or lecithin) phosphatidylethanolamine (PE) and phosphatidylserine (PS). PLs are also involved in metabolism and cell signaling, while PLs serve as binding sites for intra- and intercellular proteins and some PLs are either precursors or are themselves membrane-derived second messengers. Glycerophospholipids are divided into different classes, based on the form of the polar headgroup at the sn-3 position of the glycerol backbone in eukaryotes and eubacteria, or the sn-1 position in the case of archebacteria.

**Sphingolipids**
Sphingolipids (SP) are a complex group of molecules that have a common sphingoid backbone synthesized from serine and a long-chain fatty acyl-coenzyme A, which is converted into ceramides, phosphosphingolipids, glycosphingolipids or other compounds. The major sphingoid base of mammals is commonly referred to as sphingosine. Ceramides (N-acyl-sphingoid bases) are a major subclass of sphingoid base derivatives. The major phosphosphingolipids of mammals are sphingomyelins. The glycosphingolipids are a versatile family of molecules composed of one or more sugar residues linked via a glycosidic bond to the sphingoid base. Sphingolipids protect the cell surface against harmful environmental factors by forming a mechanically stable and chemically resistant outer segment of lipid bilayer. Certain complex glycosphingolipids are involved in specific functions, such as cell recognition and signaling (Gratschev et al., 2009).

**Sterol lipids**
Sterol lipids (SL) are characterized by fused four-ring core structure. SLs are important components of membrane lipids such as the most well-known member, cholesterol, and its derivatives. The steroids, which contain the same core structure, have different biological roles as hormones and signaling molecules: The C18 steroids include the estrogen family whereas the C19 steroids include the androgens. The C21 subclass includes the progestogens, glucocorticoids and mineralocorticoids. The secosteroids, like different forms of vitamin D, are characterized by cleavage of the B
ring of the core structure. Phytosterols commonly known as plant sterols can block cholesterol absorption in intestine and thus reduce cholesterol levels.

**Prenol lipids**

Prenol lipids are synthesized from the 5-carbon precursors isopentenyl diphosphate and dimethylallyl diphosphate. The simple isoprenoids (linear alcohols, diphosphates) are formed by the consecutive addition of similar core C5 structures called terpene units. Structures containing greater than 40 carbons are known as polyterpenes. Carotenoids are important simple isoprenoids that function as antioxidants and as precursors of vitamin A. Another biologically important class of prenol lipids is quinines, which contain an isoprenoid tail attached to a quinonoid core of non-isoprenoid origin. Vitamin E and vitamin K are examples of these molecules.

**Saccharolipids**

Saccharolipids are molecules in which fatty acids are linked directly to a sugar backbone. This kind of structure matches to the membrane bilayers. The most familiar saccharolipids are the acylated glucosamine precursors of the Lipid A component of the lipopolysaccharides in Gram-negative bacteria. Typical lipid A molecules are disaccharides of glucosamine, which are derivatized with seven fatty-acyl chains.

**Polyketides**

Polyketides are synthesized as metabolites by polymerization of acetyl and propionyl subunits. Several polyketides are cyclic molecules whose backbones are often further modified by glycosylation, methylation, hydroxylation, oxidation or other processes. Many commonly used anti-microbial, anti-parasitic, and anti-cancer agents, like erythromycins, tetracyclines and antitumor epothilones are polyketides or polyketide derivatives.

### 2.3.1 Lipoproteins and lipid transfer processes in human circulation

Phospholipids, cholesterol, and triglycerides are the main lipids in human circulation. Many of these lipids have long hydrophobic hydrocarbon tails and are thus relatively insoluble in water. To transport lipids through the circulation, lipids are packed into water-soluble complexes referred to as lipoproteins. Most lipoproteins have a spherical structure that consists of a hydrophobic core of neutral lipids (mainly triacylglycerols and cholesteryl esters), surrounded by a polar surface monolayer of phospholipids, unesterified cholesterol, and apolipoproteins. The apolipoproteins solubilize the insoluble lipids and can act as cofactors for plasma enzymes and as ligands for cell surface receptors in tissue uptake. Six major classes of apolipoproteins (A, B, C, D, E, and H) and several subclasses are introduced. Lipoproteins constitute a heterogeneous population of particles that can be classified according to their densities. Traditionally, plasma lipoproteins have been separated from serum by sequential ultracentrifugation (Havel et al., 1955). Five major human plasma lipoproteins are chylomicrons (CM), very low-density lipoproteins (VLDL),
intermediate-density lipoprotein (IDL), low-density lipoproteins (LDL), and HDLs (Gotto et al., 1986).

Lipoprotein metabolism can be divided into two major pathways: the transport of dietary lipids taken up from the intestine, referred to as the exogenous pathway; and the transport of lipids synthesized in the liver, the endogenous pathway. The transport of cholesterol from peripheral tissues to the liver, where it is converted into bile acids and excreted, is referred to as reverse cholesterol transport.

**Figure 5.** PLTP catalyses HDL conversion generating large fused HDL particles and preβ-HDL that participate in the reverse cholesterol transport from peripheral cells (Courtesy of Riikka Vikstedt).

### 2.3.2 Lipid transfer proteins in lipoprotein metabolism

The exchange of lipids between lipoprotein subclasses is performed by two closely related proteins: CETP and PLTP. PLTP and CETP belong to the lipid transfer/lipopolysaccharide binding protein family (LT/LBP), which also includes the lipopolysaccharide binding protein (LBP) and bactericidal/permeability increasing protein (BPI) (Day et al., 1994). LBP and BPI are able to bind and transfer bacterial endotoxins and lipopolysaccharide (LPS), thereby modulating the response to bacterial infection (Schumann et al., 1990). CETP is able to transfer neural lipids, triglycerides,
and cholesteryl esters between lipoprotein particles. PLTP transfer phospholipids, diacylglycerol (Rao et al., 1997), free cholesterol (Nishida and Nishida, 1997), R-tocopherol (vitamin E) (Kostner et al., 1995), and lipopolysaccharide between lipoproteins and cells (Hailman et al., 1996; Levels et al., 2005).

2.3.3 Characteristics of cholesterol ester transfer protein

CETP, originally called lipid transfer protein I (LTP-I), was isolated from human plasma in the 1980s (Tall et al., 1983a; Albers et al., 1984). The human CETP protein contains 476 amino acids. The translated molecular mass of CETP is 53 kDa, but the mass estimated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) is 74 kDa (Drayna et al., 1987). The crystal structure of CETP was revealed in 2007 (Qiu et al., 2007). Based on this analysis, CETP is an elongated boomerang-shaped molecule. The protein contains four potential asparagine-linked glycosylation sites and a hydrophilic tunnel apparently mediating the lipid transfer. The human CETP gene is located in chromosome 16q12-16q21 (Lusis et al., 1987). The CETP gene is expressed at high levels in adipose tissue, the heart, and the skeletal muscle, and at lower levels in many other tissues in several mammalian species. In humans, high levels of CETP expression are found in adipose tissue and the liver (Jiang et al., 1991), and CETP deficiency results in elevated plasma HDL levels (Inazu et al., 1990), which is associated with increased cardiovascular risk (Dullaart et al., 2007).

2.3.4 Characteristics of PLTP

This protein was firstly called lipid transfer protein II (LTP-II). It was found to directly facilitate the transfer of phospholipids from apoB-containing lipoproteins to HDL, but it was incapable of transferring cholesteryl esters or triglycerides (Albers et al., 1984; Tollefson et al., 1988). LTP-II was later called phospholipid transfer protein (PLTP; van Tol, 2002).

Human PLTP contains 467 amino acids and is highly glycosylated (Day et al., 1994; Huuskonen et al., 1998). The computed molecular mass of mature human PLTP protein is 55 kDa, although the mass of human PLTP estimated by SDS-PAGE is 81 kDa. The difference between the computed mass and the mass estimated by SDS-PAGE may be due to N-glycosylation, since human PLTP contains six potential N-glycosylation sites (Pussinen et al., 1997b). The human PLTP gene is located in chromosome 20q12-q13.1 (Day et al., 1994; Whitmore et al., 1995), and the mouse PLTP gene is localized in chromosome 2, which corresponds to human chromosome 20 (LeBoeuf et al., 1996).

In the molecular model of PLTP, it is constructed of a two-domain architecture with conserved N- and C-terminal lipid-binding pockets consisting of hydrophobic residues in each domain. In BPI, these pockets bind phospholipid molecules, whereas LBP can bind both LPS and phospholipids. Although only two specific lipid-binding pockets have been identified in PLTP, it can accommodate up to 43 mol of phosphatidylcholine and 13 mol of cholesterol/mol of PLTP. The lipid-binding pockets of the PLTP molecule might be needed for specific lipid transfer of this
protein, while the rest of the associated phospholipids may be needed for proper folding of the protein and stabilizing the enzymatic activity.

In humans, PLTP expression is highest in the placenta, pancreas, and adipose tissue, and it is found at moderate levels found in the liver, kidney, and heart (Day et al., 1994; Huuskonen et al., 1998). PLTP mRNA is also expressed in alveolar type II epithelial cells of the lungs, and its expression is induced during hypoxia and in emphysema (Jiang et al., 1998).

In vitro studies have identified a number of functions for PLTP in lipoprotein metabolism (Albers and Cheung, 2004). PLTP displays two major functions in circulation: i) it transforms HDL particles in a conversion process whereby large fused HDL particles and preβ-HDL are generated. These two new particle populations can participate in cholesterol removal from macrophage-foam cells in a process called reverse cholesterol transport (Vikstedt et al., 2007, Figure 5); ii) it transfers post-lipolytic VLDL surface phospholipids to HDL (Figure 6).

Figure 6. PLTP-facilitated postlipolytic transfer of VLDL surface phospholipids to HDL (Courtesy of Riikka Vikstedt).
A role for PLTP in atherogenesis has been suggested (Cheung et al., 2006). Functions of PLTP which may influence the formation of atherosclerotic lesions include the generation of acceptors for lipid efflux from cells, regulation of plasma HDL levels, protection of lipoproteins from oxidation, and regulation of production of atherogenic lipoproteins (Jiang et al., 2001). In human plasma, two distinct forms of PLTP are present, one with high activity (HA-PLTP) and the other with low activity (LA-PLTP) (Oka et al., 2000). These two forms are associated with different apolipoproteins during their purification (Kärkkäinen et al., 2002). Interestingly, PLTP was secreted only in an active form from HepG2 cells associated with apoE (Siggins et al., 2003). Although the role of PLTP in lipoprotein metabolism and atherogenesis has been intensively studied in gene-targeted mouse models and using in vitro experiments, the physiological role of PLTP in human metabolism is far from resolved. Another unsolved question is: By what mechanisms do PLTP and CETP mediate lipid transfer between the lipid donors and acceptors? On the basis of kinetic evidence, it has been suggested that CETP facilitates lipid transport by a shuttle mechanism rather than by forming a ternary complex between CETP, HDL, and acceptor lipoprotein particles like VLDL and LDL (Connolly et al., 1996). In favor of this model of lipid transport, it was later demonstrated that CETP can absorb lipids from phospholipid monolayers residing at the air-water interface (Weinberg et al., 1994).

### 2.3.5 Animal models of phospholipid transfer protein

The physiological role of PLTP in lipoprotein metabolism has been extensively studied using different mouse models. Both PLTP-deficient and overexpression models, as well as the macrophage-specific targeting of PLTP in mice, have been applied.

**PLTP deficient mice**

In order to evaluate the role of PLTP in lipoprotein metabolism Jiang et al. created a PLTP knockout mouse model (Jiang et al., 1999). In chow-fed mice, targeted mutation of the PLTP gene resulted in strong decreases in HDL, but no changes in non-HDL lipid or apoAI levels. Low HDL levels in PLTP KO mice arise from a marked increase in HDL catabolism (Qin et al., 2000). The increased HDL catabolism is possibly related to a decrease in the phospholipids of the HDL particles. On a high-fat diet, both HDL and non-HDL lipoprotein levels were altered (Jiang et al., 1999), suggesting accumulation of surface components of triglyceride rich lipoproteins. These findings show the importance of PLTP activity for the transfer of surface components of triglyceride-rich lipoproteins to HDL. The decreased plasma levels and production rate of apoB-containing lipoproteins could explain why PLTP KO mice have decreased susceptibility to develop atherosclerosis (Jiang et al., 2001). An other explanation for the protective effect seems to be that circulating apoB lipoproteins are protected from oxidation due to the increased availability of free plasma vitamin E (Jiang et al., 2002). PLTP-deficient mice also exhibit an anti-inflammatory effect due to significantly lower levels of IL-6 (Schlitt et al., 2005).
**PLTP overexpressing mice**
Overexpression of PLTP drives to the formation of larger HDL species, demonstrating the involvement of PLTP in HDL remodeling \textit{in vivo} (Ehnholm et al., 1998). The overexpression of PLTP also lowers the total HDL levels of targeted mice, while it induces increased generation of preβ–HDL (Föger et al., 1997). Although preβ–HDL is only a minor subfraction of total HDL, it is very efficient acceptor of cellular cholesterol in the reverse cholesterol transport and thus it was suggested that overexpression of PLTP might have atheroprotective potential. However, the elevation of PTLP activity levels increased the susceptibility to development of atherosclerosis in dose-responsive manner (van Haperen et al., 2002) and also atherosclerotic lesions in proximal aorta were increased (Yang et al., 2003). The plausible explanation for this seems to be the decreased plasma vitamin E levels, which leads to increased oxidation of lipoproteins.

**Macrophage-specific targeting of PTLP in mice**
Macrophages, that are a key component of atherosclerotic lesions through foam cell formation, have been demonstrated to highly express PLTP (Desrumaux et al., 2003). In order to elucidate the role of macrophage-derived PLTP in atherosclerosis several bone marrow transplantation studies has been performed. The results have been controversial as both atheroprotective and pro-atherogenic results have been published (Valenta et al., 2008; Vikstedt et al., 2007), indicating that the small differences between the experimental conditions employed grossly affect the results. Thus, the role of macrophage-PLTP in lipoprotein metabolism and in the development of atherosclerosis is far form resolved.
3 AIMS OF THE STUDY

The aim of the present study was to investigate the role of PLTP lipid transfer mechanisms in TF and evaluate the effect of PLTP in dry eye. The specific aims were:

1. To investigate the presence and activity of the lipid transfer proteins PLTP and CETP in tears. (I)

2. To characterize the molecular mechanisms of PLTP-mediated lipid transport in air-water surfaces and in TF. (II, III)

3. To clarify PLTP-protein interactions in TF. (III)

4. To evaluate the effects of PLTP knock-out on the mouse ocular surface and compare these results to experimentally induced dry eye (EIDE). (IV)

5. To determine the PLTP activity among human dry eye patients and compare it to other dry eye tests. (IV)
4 MATERIALS AND METHODS

4.1. Materials

Antibodies:
- Mono- (Mab59 IgG) and polyclonal (R290 IgG) antibodies against PLTP: Produced and purified as described by Huuskonen et al. (2000).
- A monoclonal antibody against human CETP, TP-2: A kind gift from Dr. Yves Marcel, Ottawa, Canada.
- Polyclonal antibodies against human apoA-I and apoE: Raised in New Zealand white rabbits using antigens purified from human plasma.
- Enzyme conjugated secondary antibodies: Goat anti-mouse IgG Horseradish peroxidase (HRP) and goat anti-rabbit IgG HRP: Purchased from Bio-Rad (Bio-Rad Laboratories, CA, USA).
- Monoclonal antibodies against lipocalin and serum amyloid A: Purchased from Abcam (Cambridge, UK).
- Proline rich-protein 1 monoclonal antibodies: Purchased from Sigma (St. Louis, MO, USA).
- Polyclonal rabbit anti-occludin: Purchased from Zymed (San Francisco, CA).
- Alexa 488-conjugated goat anti-rabbit immunoglobulin: Purchased from Molecular Probes (Eugene, OR).

Proteins and lipids:
- PLTP: Purified from human plasma as described by Kärkkäinen et al. (2003).
- Purified tear lipocalin: A kind gift from Dr. B. Redl (University of Innsbruck, Austria).
- HDL₃: Isolated from human plasma by sequential ultracentrifugation.
- Bovine submaxillary gland mucin (type I-S) and lactoperoxidase: Purchased from Sigma (St. Louis, MO, USA).
- Lysozyme: Purchased from Abcam (Cambridge, UK).
- POPC (palmitoyloleoylphosphatidylcholine): Purchased from Avanti (Birmingham, AL).
4.2 Methods

The methods used in this thesis are listed in the table below. Details can be found in the original publications and references.

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4.2.1 Tear fluid analysis

Collection of tear fluid
Individual TF samples (approximately 20 µL) were collected from the lower conjunctival sac using Blaubrand intramark 10 µL micropipets (Brand GMBH, Wertheim, Germany). The samples were immediately cooled to +4 °C and stored at -70 °C until analyzed. To obtain large amounts of TF tear production was stimulated by onion vapor. Pooled samples were collected from 11 subjects, and stored like individual samples. Clinical investigation of the subjects before collection of tear fluid showed no signs of ocular inflammation or allergy.

Measurement of tear PLTP and CETP concentration and activity
The concentration of PLTP in TF and plasma was determined using an enzyme-linked immunosorbent assay (ELISA). PLTP activity was recorded by measuring the transfer of \[^{14}C\]-PC from radiolabeled PC vesicles to unlabeled HDL\textsubscript{3} acceptors. CETP activity was determined from the rate of transfer of radiolabeled cholesterol ester from LDL to HDL.

Immunoinhibition of tear PLTP activity
Increasing amounts of rabbit polyclonal anti-PLTP IgG (80-1280 µg) were added to human tear fluid (5 µL) and incubated for 2 h at room temperature. After volume adjustments, 20 µL aliquots were used to analyze PLTP activity. Control incubations contained PBS instead of anti-PLTP IgG.

Size-Exclusion Chromatography of Tear Fluid Proteins
Catalytically active PLTP can be separated from the catalytically low activity form by size-exclusion chromatography or by using the heparin-binding properties of the active protein. Pooled TF samples (500 µL) was subjected to a fast-performance liquid chromatography on a Superose 6 HR (10x30 cm) size-exclusion column (Amersham Pharmacia Biotech). The column was equilibrated with PBS (150 mM NaCl and 10 mM Na-phosphate at pH 7.4) containing 0.05% Tween 20. Chromatography was performed at a flow rate of 0.5 mL/min, and 0.5 mL fractions were collected for PLTP activity and mass measurements.

Heparin-Sepharose Chromatography of Tear Fluid Proteins
Pooled TF samples (70 µL) was mixed with 930 µL of buffer A (25 mM Tris-HCl at pH 7.4 containing 1 mM EDTA) and applied on 1 mL Hi-Trap Heparin-Sepharose column (Amersham Pharmacia Biotech) pre-equilibrated with buffer A. After elution of nonbound material, buffer B (buffer A supplemented with 0.5 M NaCl) was applied and 0.5 mL fractions were collected at a flow rate of 0.5 mL/min. After buffer B, the elution was continued with buffer C (buffer A supplemented with 1 M NaCl). Collected fractions were analyzed for PLTP concentration and activity.

Measurement of tear fluid osmolarity
Human TF osmolarity was measured with Tearlab osmometer (Ocusense Inc., San Diego, CA).
Measurement of mouse tear production
Mouse tear production was measured before and 7 days after induction of EIDE. A half of Schirmer paper was applied in the lateral canthus for 60 seconds. Wetting of the paper was measured in millimeters.

4.2.2 Lipid monolayers
Lipid monolayer, residing on an air-buffer interface, provides access to measure the penetration of protein(s) into membrane by monitoring the increase in surface pressure. Protein derived desorption of radioactively labelled lipids from monolayer can be measured from aqueous subphase.

Interaction of PLTP with Lipid Monolayers
Penetration of PLTP into lipid film, POPC/DPPC or [14C]-DPPC (80:20 ratio) was measured using circular Teflon-coated wells (1200 µL). Surface pressure (π) was monitored with a Wilhelmy wire attached to a microbalance (μTroughS; Kibron Inc., Helsinki, Finland) and connected to a Pentium personal computer. The monolayer with different initial surface pressures (π₀) was spread on an air-buffer (25 mM sodium phosphate buffer and 150mM NaCl, pH 6.8) interface by dropwise addition of phospholipid-chloroform solution from a Hamilton syringe. The resulting monolayers were allowed to equilibrate for 15 min before addition of PLTP to the subphase. The increment in π after the addition of PLTP was complete in <60 min, and the difference between π₀ and the value observed after binding of PLTP into the film (πₜ) was taken as Δπ.

Absorption of DPPC from the monolayer
A monolayer of [14C]-DPPC was spread on an air-buffer interface to an initial surface pressure of ~7 mN/m. The surface pressure was controlled as described above. Monolayer was allowed to equilibrate before the addition of 10 µL of either HA-PLTP, LA-PLTP, HDL₃ or a mixture into the subphase. Lipid/buffer system was incubated for 3 h, and one-fourth (300 µL) of the subphase volume was collected for liquid scintillation counting (Wallac Winspectral 1414 liquid scintillation counter, Turku, Finland).

4.2.3 Asymmetrical Flow Field-Flow Fractionation
Miniaturized and conventional asymmetrical flow field-flow fractionation (mAsFlFFF and AsFlFFF, respectively) were utilized. Detection was performed with a UV/vis detector (HP1050 model 79853C, Tokyo, Japan). For both systems, a regenerated cellulose acetate ultrafiltration membrane with a molar mass cutoff of 10 kDa (DSSRC70PP, Nakskov, Denmark) was laid on top of the porous frit. An HPLC pump (model PU-980, Jasco International Co., Ltd., Tokyo, Japan) was used to deliver carrier solution during the injection-relaxation-focusing and running periods.
outlet flow from the channel was monitored at 254 or 280 nm. The carrier liquid used in AsFIFFF was 10 mM phosphate buffer, 150 mM NaCl, 1 mM EDTA, and 0.02% NaN3 at pH 7.4.

AsFIFFF and Lipid Transfer Assays
A mixture of the donor (DPPC small unilamellar vesicles, 15 µL) and of acceptor (HDL₃, 250 µg) were placed in polypropylene microsample tubes (Eppendorf), and diluted to 400 µL with the phosphate buffer solution (10 mM phosphate, 150 mM NaCl, and 1 mM EDTA, pH 7.4). After incubation (45 min) the sample was diluted to 10 mL with the same buffer and then injected to AsFIFFF at 1.0 mL/min for 10 min. To elucidate the ability of PLTP to transfer lipids from donor SUVs to acceptor particles, either LA (c =32 µg/mL) or HA (c=10-15 µg/mL) PLTP was added separately to the mixture before incubation. To demonstrate the actual transfer of the lipid and movement of the PLTP protein itself, [¹⁴C]DPPC-labeled SUV and [³⁵S]-labeled PLTP were used. After AsFIFFF the radioactivity of collected fractions was measured with liquid scintillation counting.

4.2.4 Detection of PLTP-protein interactions
To determine possible PLTP-protein interactions in tear fluid Heparin-Sepharose affinity chromatography, immunoprecipitation, dot-blot analysis and Size Exclusion Chromatography of mucin-PC and PLTP were used.

Heparin trapping of PLTP
Pooled tear fluid samples (400 µl) were applied to a 1 mL HiTrap Heparin column (GE Healthcare, Buckinghamshire, UK) that was equilibrated with buffer A (25 mM Tris-HCl buffer, pH 7.4 containing 1 mM EDTA). The column was washed with Buffer A (flow rate 0.5 mL/min) and the bound material was eluted with a 0.5 M NaCl in buffer A. The active PLTP fractions were combined and dialyzed against buffer A and then applied to same 1 mL HiTrap Heparin column. The bound PLTP was eluted with a linear 0-0.5 M NaCl gradient. The fractions were analyzed for PLTP activity. The active fractions were pooled and concentrated with nanostep 10K omega system (Palli, Life Sciences, U.K.). Lyophilized material was loaded to 12.5% SDS-PAGE gel and stained with Coomassie R-250 stain. The detected protein bands were cut apart and transferred to mass spectrometry.

Immunoprecipitation of PLTP-protein complex
Tear fluid samples and pure proteins identified by mass spectrometry were studied. Anti-PLTP MAb 59 or rabbit polyclonal anti-PLTP (R290) was added to Protein G (100 µl) and incubated for 60 min, after which the beads were washed two times with TBS-Tween. Either pooled tear fluid samples (100 µl) or purified active PLTP and candidate proteins were added to the beads and incubated overnight. Protein G-anti-PLTP IgG bound material and supernatants were analyzed with SDS-PAGE using buffer with or without 2 mM β-mercaptoethanol. Adhered proteins were detected with Western blot analyses. Immunoprecipitation of the tear fluid PLTP-mucin complex
was performed also using polyclonal anti-MUC5B antibody. Separation on non-reducing 5% SDS-PAGE was done for mucin. PLTP was analyzed using 12.5 % SDS-PAGE under reducing conditions. The mucins were detected with the monoclonal antibody and PLTP with MAb 59.

**Dot-blot analysis**
The dot-blot assay was used to determine the PLTP-mucin interactions. Increasing concentrations (0 – 45 µg/well) of either ovomucin or BSM were transferred onto nitrocellulose membranes using a Minifold (Amersham Pharmacia Biotech) Slot Blot Apparatus. The membranes were blocked with defatted milk (5% wt/vol in TBS, 0.1% Tween 20, pH 7.5) and washed twice with TBS-Tween 20. Membranes were incubated with HA-PLTP (25 µg) and controls without PLTP over night and membranes were washed with TBS-Tween 20. Bound PLTP protein was detected by using monoclonal anti-PLTP MAb 59 antibody. Bovine mucin and ovomucin were detected with the monoclonal anti-MUC5AC antibody.

**Size Exclusion Chromatography of mucin-PC and PLTP**
Radioactively labeled ([14C]-DPPC) mucin-PC were incubated together with purified active PLTP for 30 minutes. Samples were subjected to fast-performance liquid chromatography on a Superose 6HR size-exclusion column (Amersham Pharmacia Biotech). Chromatography was performed at a flow rate of 0.5 mL/min, and 0.5 mL fractions were collected for radioactivity, phospholipid and protein measurements. Radioactivity of the collected fractions was measured with liquid scintillation counting (Wallac LS Counter, Turku, Finland). Phospholipid concentration was assayed using a commercial kit (DiaSys, Holzheim, Germany). Dot-blot analysis of the proteins was performed as described above using anti-PLTP and anti-mucin antibodies.

**4.2.5 Examination of ocular tissues**

**Lacrimal gland samples**
Pieces of main lacrimal gland and the adjacent conjunctiva were obtained from nine subjects with whom the lacrimal gland was excised for diagnostic purposes. Sections of formalin-fixed paraffin-embedded tissues were incubated with monoclonal antibodies to PLTP, MAb 59 and MAb66. Immunohistochemical stainings were performed using a commercial Elite ABC Kit (Vectastain, Vector Laboratories, Burlingame, CA). Non-immunized mouse serum served as a primary antibody. The bound peroxidase activity was visualized with a specific substrate, 3-amino-9-ethylcarbazole (Sigma, St. Louis, MO) solution (0.2 mg/mL in 0.05 M acetate buffer containing 0.03% perhydrol; pH 5). The slides were stained with hematoxylin, washed and mounted in Aquamount (BDH, Poole, UK).

**Corneal and eyelid histopathology**
Eyes and eyelids from mice were fixed in 10% formalin and embedded in paraffin. Sections (5 µm) were prepared and stained with hematoxylin-eosin (HE) and Periodic
acid-Schiff (PAS). Sections from three different eyes in each group were examined and photographed with a Nikon Eclipse TE2000-E (Nikon Corp., Tokyo, Japan) microscope equipped with a Nikon Digital Sight DS-5MC camera. The corneal epithelial damage was assessed by counting the number of detaching apical cells from a full microscope field-of-vision from three separate tissue sections, using a x20 objective. Three mice were evaluated in each treatment group.

**Corneal permeability to carboxyfluorescein**

CF (1 μL of a 0.3 % solution) was applied to the mouse ocular surface. After 10 minutes the mice were sacrificed with high-dose carbon dioxide. Corneas without scleral rims were excised, rinsed twice with 200 μl BSS, weighted and placed in 100 μl BSS. The CF concentration of the solution was measured (excitation 490 nm, emission 535 nm) using Wallac Victor2 1420 Multilabel Counter (Beckman Coulter, Fullerton, CA).

**Ocludin Immunohistochemistry**

Whole mice eyes were fixed in formalin, embedded in paraffin and mounted on chromium-gelatin-treated slides in 5 μm sections. The sections were soaked in xylene, dehydrated in graded alcohol series (100-70 %) and treated (12 min at 37 °C) with pepsin (0.5 % w/vol) containing 0.1 M HCl. The sections were permeabilized and blocked with 0.1 M Na₂HPO₄ (PB) containing 0.2 % Triton X-100 and 10 % normal goat serum for 60 min and labeled overnight with polyclonal rabbit anti-occludin primary antibody (dilution 1:25) diluted in 0.1 M PB containing 0.1% Triton X-100 and 2.5% normal goat serum. Tissues without primary antibody served as negative controls. Sections were labeled for 1 hour with Alexa 488-conjugated goat anti-rabbit immunoglobulin. The stained sections were rinsed in PB and mounted with Aquamount (BDH Chemicals, Poole, UK). The sections were examined under fluorescent illumination (excitation 460-500 nm, emission 510-560 nm) by Nikon Eclipse TE2000-E (Nikon Corp., Tokyo, Japan) microscope equipped with a Nikon Digital Sight DS-5MC camera.

4.2.6 Experimentally induced dry eye

Induction of EIDE was achieved by cholinergic receptor blockade. Transdermal scopolamine patches were applied by cutting the patches into four pieces, wrapping them around the depilated midtail, and securing them with cellophane tape. Patches were reapplied on days 0, 2, 4, and 6.

4.3 Animals

C57Bl/6 WT (Charles River, USA) and C57Bl/6 PLTP-KO mice (Jiang et al., 1999) were bred at Taconic Europe Facilities, Denmark (http://www.taconic.com), and then transferred to the National Public Health Institute, Helsinki, Animal Care Centre for further breeding. The mice were housed in a room under controlled temperature (23 ± 1 °C) and light cycle, with free access to water and standard mouse chow (18% protein, 5% fat; Harlan Teklad Global Diets, Denmark, No. 2018).
4.4 Ethical and patient information

This study was performed according to the principles of the Declaration of Helsinki, and approved by the Ethics Review Committee of the Department of Ophthalmology (University of Helsinki). Informed consent was obtained from each human subject. The permission to use tissue samples was obtained from the Finnish National Authority for Medicolegal Affairs (TEO).

All of the animal experiments were conducted under the National Public Health Institute guidelines (license # KTL 2004-02) for the humane treatment of laboratory animals. All procedures in the study protocol adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
5 RESULTS

5.1 PLTP is an active part of human tear fluid

To gain insight into the mechanism of tear film formation, the occurrence of the two lipid transfer proteins, PLTP and CETP, in human TF was investigated. Western blotting of human TF and plasma using a monoclonal anti-PLTP antibody revealed that both tear fluid and plasma displayed a single 80 kDa band corresponding to the predicted molecular weight of the mature, highly glycosylated form of human PLTP (Figure 7).

![Western blot analysis of phospholipid transfer protein in tear fluid.](image)

**Figure 7.** Western blot analysis of phospholipid transfer protein in tear fluid. Plasma samples derived from seven normolipidemic subjects were run on a SDS-PAGE and after protein transfer to nitrocellulose PLTP was detected with a monospecific antibody against human PLTP.

5.1.1 Concentration and transfer activity of human tear fluid PLTP

The mean PLTP concentration in TF was 10.9 ± 2.4 µg/mL. In plasma, the PLTP concentration was 4.5 ± 0.9 µg/mL (p < 0.001) (I, Fig. 2A). Thus, the PLTP concentration seems to be higher in TF than in plasma.

The mean phospholipid transfer activity in TF was 15.1 ± 1.8 µmol/mL/h and in plasma was 8.9 ± 1.8 µmol/mL/h (p < 0.001) (I, Fig. 2B).

The specific activity of PLTP in tear fluid compared to plasma was found to be significantly lower, 1.4 ± 0.3 nmol/µg/h versus 2.1 ± 0.6 nmol/µg/h (p < 0.006) (I, Fig. 2C). No correlation between tear and plasma PLTP concentration and activity was observed.
The contribution of PLTP in tear phospholipid transfer was studied by heat inactivation (Groener et al., 1986) (58 °C for 60 min) and by immunoinhibition with a polyclonal antibody specific for PLTP (Huuskonen et al., 2000). Heat inactivation of PLTP resulted in the loss of transfer activity. In addition, immunoinhibition with a polyclonal antibody specific for PLTP resulted in the almost total inhibition of PLTP activity (I, Fig. 3). Tear lipocalin did not show phospholipid transfer activity in the assay developed to measure PLTP-facilitated phospholipid transfer (I).

5.1.2 Only the high-activity form of PLTP is present in human tears

Catalytically active plasma PLTP can be separated from the catalytic low-activity form by size-exclusion chromatography (SEC) or by using the heparin-binding properties of the active protein form (Oka et al., 2000; Kärkkäinen et al., 2002).

When analyzed by SEC, PLTP activity and PLTP protein coeluted in fractions, corresponding to an apparent molecular weight of 160–170 kDa (I, Fig. 4B and inset). Catalytically inactive PLTP was not observed in any of the fractions. During heparin-Sepharose-affinity chromatography, both PLTP activity and mass bound to the column and could be eluted with 0.5 M NaCl (I, Fig. 5). The recovery of PLTP activity was over 90% and that of the PLTP protein mass was about 80%. These results demonstrate that PLTP in TF has similar characteristics to the high-activity form of PLTP present in human plasma.

5.1.3 Human tear fluid contains neither apolipoprotein A-I, E nor CETP

In human plasma, LA-PLTP is found to be associated with apolipoprotein (apo) A-I, while HA-PLTP associates preferentially with apoE (Kärkkäinen et al., 2002, Siggins et al., 2003). This suggests that PLTP activity may be regulated by the apoE/apoA-I molar ratio in the PLTP-apolipoprotein complex. Because PLTP is highly hydrophobic, it was reasoned that it might also be associated with these apolipoproteins in TF. TF was analyzed for the presence of these apolipoproteins using Western blotting and ELISA. However, neither apoA-I nor apoE could be detected (I).

CETP neither could be detected in TF by Western blotting using a monoclonal antibody. The presence of other possible neutral lipid transferring activities in TF was studied by an isotopic assay, which measures the transfer of radioactive cholesteryl ester from LDL to HDL₃. Consistent with the lack of immunoreactive CETP, no cholesteryl ester transfer activity could be detected in TF. PLTP and CETP can be separated by heparin-Sepharose-affinity chromatography (Tollefson et al., 1988),
whereby PLTP is quantitatively bound to heparin while CETP is not. In the flow-through fractions of heparin-Sepharose chromatography, we could not detect either CETP activity or immunodetectable CETP. These observations strongly suggest that TF does not contain CETP (I).

5.2 Mechanism of phospholipid transfer protein –mediated lipid transfer

To elucidate the mechanism(s) by which PLTP transfers its principal substrate, phospholipids, series of monolayer and flow field-flow fractionation experiments were conducted. The principle of these methods is presented in figures 8 and 9.

5.2.1 Interaction of phospholipid transfer protein with phospholipid monolayers

Phospholipid monolayers containing POPC/DPPC (80:20 molar ratio) were prepared in Teflon-coated wells to assess the partitioning of PLTP between the phospholipid monolayer and the aqueous subphase as a function of monolayer surface pressure. The initial surface pressures ranged from 4 to 41 mN/m.

Either HA-PLTP (1.6 µg) or LA-PLTP (1.5 µg) was injected into the subphase of these monolayers, and the adsorption of PLTP to the interface was monitored by the change in surface pressure (Δπ) (Figure 8 and II, Fig. 1). The binding of HA-PLTP to the lipid-buffer interface decreased linearly with increasing π0 (II, Fig. 1); linear extrapolation of the π0-Δπ curve indicated that HA-PLTP could not penetrate the PL monolayer surface at π0 >25 mN/m. At higher π0 values, no changes in surface pressure were detected. Similar results were obtained when using LA-PLTP, except that the critical surface penetration pressure was estimated to be 33 mN/m (II, Fig. 1).
5.2.2 PLTP is capable of transferring phospholipids from a monolayer

To study whether PLTP can cause desorption of PL from the fluid monolayer and transfer it to the aqueous subphase, [14C]DPPC monolayers were prepared. The initial surface pressure for these monolayers was 7 ± 0.5 mN/m. After a 3 h incubation following the addition of either LA-PLTP or HA-PLTP, the radioactivity recovered in the subphase had increased almost 7-fold as compared to that observed for the control monolayers (II, Fig. 2).

PLTP mass in the subphase was analyzed using the ELISA method. From the added total HA-PLTP, 10.3 % (134 ng) was in the subphase; i.e., 90 % of the HA-PLTP was associated with the lipid monolayer. Analogously, 11.0 % (167 ng) of LA-PLTP was found in the subphase, and therefore 89.0% is bound on the monolayer. From the added HDL (119 µg), equal amounts were recovered in the lipid monolayer and in the subphase (II).

The amount of total desorbed DPPC-associated radioactivity in the subphase was 4.2% without any added proteins. After 3 h of incubation with LA-PLTP, 26.4% of the total radioactivity was encountered in the subphase, whereas in the presence of HA-PLTP and HDL₃, 27.6% and 38%, respectively, were found in the subphase. In the presence of both LA-PLTP or HA-PLTP and HDL₃, 35.2% and 39.5%, respectively,
were found in the subphase (II, Fig. 2). The measured radioactivities of collected subphases clearly demonstrated the lipoprotein enhanced transfer of DPPC from the PL monolayer.

Estimation of the stoichiometry and the specific activity of DPPC/PLTP binding from the subphase indicated that on average, 5.5 mol of DPPC/mol of PLTP was desorbed to the subphase. Accordingly, in addition to the two defined lipid-binding pockets, more lipid molecules are attached to the surface of PLTP in accordance with previous results (Nishida and Nishida, 1997).

5.2.3 Studies of PLTP–mediated phospholipid transfer using asymmetrical flow field-flow fractionation method

Flow field-flow fractionation was firstly used for the separation and fractionation of HA-PLTP, HDL3, small unilamellar vesicles (SUVs), and their mixtures. The components were efficiently separated, and the diameter of HA-PLTP appeared to be \( \sim 6 \) nm, that of HDL3 \( \sim 8 \) nm, and that of SUVs \( \sim 25 \) nm (II, Fig. 3A). Discoidal reconstituted apoA-I-phospholipid-cholesterol particles were resolved into three peaks due to the formation of three discoidal subgroups (II, Fig. 3B), which have also previously been detected by native gradient gel electrophoresis (Matz et al., 1982; Jänis et al., 2005).

To clarify the mechanism whereby PLTP facilitates lipid transfer, radioactively labeled ([\(^{14}\)C]DPPC) egg PC-SUVs and HDL3 were incubated in the presence of either HA-PLTP (activity 10 \( \mu \)mol/mL/h) or LA-PLTP (activity 10 nmol/mL/h). Mixtures were subjected to AsFIFFF analysis before and after 45 min incubation.
Before incubation, at time 0 min, radioactively phospholipids were almost exclusively associated with the SUVs. During the 45 min incubation, approximately 30% of the radioactivity was transferred to a position corresponding to HDL-3 particles (II, Fig. 4A). This transfer only occurs in the presence of HA-PLTP, while the inactive form, LA-PLTP, does not mediate the transfer of phospholipids (II, Fig. 4B). Incubation of SUVs and HDL-3 in the absence of LA- or HA-PLTP did not cause any transfer of SUV-associated radioactivity. The size of the different particles remained unaltered (II, Fig. 4).

In order to record the distribution of PLTP among the different particles (either SUVs, HDL-3 or unbound PLTP) during the phospholipid transfer process, [35S]-labeled HA- and LA-PLTP were used. At the start, time point 0 min, both forms of [35S]PLTP were associated with the same fractions as the SUVs (II Fig. 4C,D). However, after a 45 min incubation, a marked transfer (approximately 26%) of the radioactively labeled [35S]-HA-PLTP to the HDL-3 position was detected (II, Fig. 4C). No such transfer of radioactively labeled [35S]-LA-PLTP was observed (II, Fig. 4D). These results demonstrate that while HA-PLTP has the ability to attach to and detach from lipid environments and transfer lipids to HDL-3, while LA-PLTP is unable to carry out the transfer function.

To determine the ability of discoidal apoA-I-phospholipid and apoA-I-phospholipid-cholesterol particles to receive lipids from PLTP molecules, these particles were incubated with [14C]DPPC containing SUV donors. After incubation for 45 min and following AsFlFFF analysis, no transfer of radioactivity was detected in any of our experiments (II, Fig. 5). The data indicate that these disc-shaped apoA-I particles do not act as acceptors in phospholipid transfer processes as mediated by active PLTP.

5.3 PLTP is secreted from the lacrimal gland

Sections of isolated lacrimal glands were immunohistochemically stained with monoclonal antibodies to PLTP (Mab 59). The staining patterns were similar for all nine lacrimal glands studied. The basal cells of intra-glandular ducts gave a positive, granular staining pattern. Accumulation of secreted PLTP was also seen in the lumen (III, Fig. 1). Yet, corneal epithelial cells also show strong PLTP immunoreactivity (unpublished data).

5.4 PLTP-protein interactions in tear fluid

To study possible protein-protein interactions of PLTP in TF, heparin affinity chromatography was used to separate PLTP from the TF. Following two consecutive heparin-Sepharose (H-S) affinity separations fractions containing active PLTP were collected and separated on SDS-PAGE gels mass spectrometry. Immunoblotting using Mab59 confirmed the presence of PLTP in the SDS-PAGE gels. Coomassie staining of the SDS-PAGE gel displayed several bands. The bands were excised and subjected
to MALDI-TOF analysis. Subsequent database searches showed significant hits for lactotransferrin, lactoperoxidase, proline-rich protein 1, and lysozyme C (Table I). In addition, lipocalin-1 and serum amyloid A were detected in some samples. All of these proteins have previously been detected in TF and may represent protein-protein interaction partners to PLTP. However, it is also possible that these proteins appear in the PLTP fraction due to a direct interaction with the immobilized heparin.

5.4.1 PLTP does not interact with lysozyme-C, lactoperoxidase, lipocalin, or serum amyloid A

Coimmunoprecipitation and Western blotting were used to investigate the interaction of PLTP with the proteins coeluted in H-S chromatography and identified by MALDI-TOF mass spectrometry. Yet, after several immunoprecipitation experiments both with and without detergent (0.1 % Triton X-100), no unambiguous binding was observed between lipocalin or serum amyloid A. A very weak band for lysozyme and lactoperoxidase was observed and these protein pairs (i.e., PLTP-lysozyme and PLTP-lactoperoxidase) were further analyzed by direct binding assays. PLTP and lysozyme or PLTP and lactoperoxidase (2 µg of each protein) were incubated and subjected to PLTP pull-down assay. The results clearly demonstrated that direct binding was not evident. (III) Although direct binding between PLTP and the two proteins could not be observed, an interesting observation was that incubation of PLTP with lysozyme reduced the binding of PLTP to the H-S column, suggesting some degree of masking of the heparin-binding domain of PLTP protein.

5.4.2 PLTP forms a complex with human tear fluid mucins

Interaction of PLTP with human TF mucins was studied using a PLTP immunoprecipitation assay. Mucin was not detectable in mass spectrometry analysis because ocular mucin did not enter the 12.5 % SDS-PAGE gel due to its high apparent molecular weight (about 220 kDa). Fifty microliters of human TF was incubated with polyclonal anti-PLTP antibody (R 290; 1 mg) in the presence of protein G. The immunoprecipitate was collected and analyzed by Western blotting using monoclonal antibodies against PLTP and mucin. The results suggested an interaction between PLTP and ocular mucins (Fig. 3).

Many studies have used commercially available mucins as substitutes for ocular mucins such as BSM. Prompted by the finding that active PLTP interacted with ocular mucins in human TF, interaction with purified BSM and non-mammalian ovomucin were studied using a dot-blot analysis. The results demonstrated that purified active PLTP was directly bound in a concentration-dependent manner to BSM, whereas ovomucin failed to interact with active PLTP (Fig. 4).
5.4.3 Characterization of the mucin–PLTP interaction

Possible functional implications for mucin–PLTP interaction in TF were elucidated through an in vitro experiment. First, a phospholipidation protocol was used to generate a BSM/ovomucin-phospholipid (phosphatidylcholine, PC) complex. The complex, which also contained a trace amount of $^{14}\text{C]}$-DPPC, was incubated together with purified PLTP. SEC was used to investigate the PLTP distribution. SEC elution profiles of the mucin-PC-PLTP samples in PBS or in PBS-Tween 20 can be seen in original paper (III, Fig. 5.)

When elution was performed with PBS, PLTP coeluted with BSM-PC in a broad range. BSM, $^{14}\text{C]}$-DPPC, cold PC, and PLTP coeluted in same fraction, suggesting a complex formation between these components. When an addition of 0.05 % Tween 20 was used in the elution PBS buffer, PLTP was released from the complex with mucins (both BSM and OVM) and it eluted around the apparent molecular weight of 160 kDa.

In SEC analysis, lipidated ovomucin elutes in two major peaks and PLTP elutes in three peaks, while two of these PLTP peaks mirror the ovomucin ones. The coelution of ovomucin, PLTP, and radiolabeled phospholipids in the same fractions suggests that the lipidated ovomucin can form a complex with PLTP. PLTP does not interact with ovomucin in the absence of phospholipids, and therefore sticks non-specifically to the column matrix (III, Fig 5.).

5.5 PLTP knock-out increases dry eye symptoms in mice

PLTP KO mice were used to study whether the lack of endogenous PLTP induces ocular changes. These findings were compared to the experimentally induced dry eye (EIDE) model presented by Dursun et al (2002).

5.5.1 PLTP is present in plasma and in tear fluid in wild-type mice

High immunoreactivity for PLTP was seen in the plasma of WT mice but was totally absent in the plasma of PLTP KO mice. Pooled TF samples of three eyes derived from both the normal and PLTP KO groups were analyzed by Western blot using the monoclonal anti-PLTP (MAb 59) antibody. Wild-type C57 Black mice showed a faint immunoreactivity (due to low yield of mouse tear fluid) at the apparent molecular weight of ~80 kDa, the predicted molecular weight of PLTP (IV).

5.5.2 Corneal histology of PLTP KO mice shows dry eye changes

Corneal histology was investigated in four groups of mice: WT or PLTP KO mice, and similar mice in which dry eye was induced experimentally (EIDE). The PLTP KO and EIDE WT and PLTP KO mice showed mild wing cell irregularity and some epithelial blebs compared to WT mice without EIDE. Compared to PLTP KO mice, both types of EIDE mice presented with more severe corneal alterations and
hyperkeratization (IV, Fig. 1 and 2). During the study period, a total of 253 PLTP KO mice were housed in our laboratory. In this group, six (2.4 %) spontaneous perforations were observed, while in the same period of time, WT C57black mice (n=631) had no corneal perforations.

5.5.3 Corneal epithelial permeability is increased in PLTP KO mice

Increased corneal epithelial permeability to fluorescein dye is a characteristic finding for dry eye (Göbbels and Spitznas, 1989). Corneal epithelial permeability to carboxyfluorescein (CF) (molecular weight 750 Da) was assessed in WT, PLTP KO, and EIDE WT and PLTP KO mice. Compared to WT mice, permeability to CF was significantly increased in PLTP KO mice, as well as in EIDE WT / PLTP KO mice groups (IV, Fig. 4), suggesting a role for PLTP in corneal epithelial homeostasis. Mice (on a C57BL/6 backround) that are homozygous null for the PLTP gene are viable, fertile, normal in size and do not display any gross physical or behavioral abnormalities. No PLTP mRNA is detected in the tissues that normally express PLTP (lung, liver). No plasma PLTP activity is detected (Jiang et al., 1999).

5.5.4 PLTP KO mice have decreased expression of the tight junction protein occludin

The normal cornea barrier function is maintained by tight junctions in the differentiated apical corneal epithelial cells. Increased occludin cleavage has been reported in dry eye (Pflugfelder et al., 2005). The corneal endothelium showed strong immunoreactivity with the polyclonal occludin antibody and this immunoreactivity was used to normalize occludin expression in the epithelium. That is, epithelial immunoreactivity was compared to endothelial immunoreactivity, which was considered to be constant in all the studied mouse groups.

In WT mice, occludin protein expression was seen in the endothelium, as well as in the epithelium, but not in the corneal stroma (IV, Fig. 5A). The expression pattern for occludin in PLTP KO mice was significantly different: The endothelium showed bright immunofluorescence but epithelial expression was significantly reduced (IV, Fig. 5B). Both types of EIDE mice showed decreased epithelial occludin protein expression (Fig. 5C&D). Quantitation of epithelial occludin protein expression confirmed these findings and showed a significantly decreased expression of occludin in PLTP KO mice, as well as in EIDE PLTP KO mice, compared to WT mice (p<0.05) (IV, Fig. 5). Although epithelial occludin expression was lower in EIDE WT mice compared to WT mice, this difference was not significant (p=0.11).

5.5.5 PLTP KO mice show normal histology of eyelids

In several dry eye knockout models, like ACAT-1 null (ACAT-1-/−) mice, the dry eye symptoms arise from the atrophy of the palpebral glands (Yagyuu et al., 2000). To
verify that the observed dry eye induction was not caused by atrophy of the Meibomian glands, we examined the histology of WT and PLTP KO mouse eyelids. The histological analysis of PLTP KO mouse eyelids was normal and the histology of Meibomian glands was similar to those in WT mice (IV, Fig. 6).

5.6 PLTP activity in human tears correlates to hyperosmolarity

Tear osmolarity is considered to be the most accurate single test to show dry eye (Tomlinson et al., 2006); a tear osmolarity level over 316 mOsm/kg is considered to show dry eye. A preliminary study was done to examine the relationship between human TF osmolarity and PLTP activity. Increased TF PLTP activity associates significantly with tear hyperosmolarity (> 316 mOsm/kg) (IV, Fig 7).
6 DISCUSSION

6.1 PLTP is a component of normal tear fluid

One of the main outcomes of this thesis project was to demonstrate that human TF displays phospholipid transfer activity. PLTP activity, as well as immunodetectable PLTP protein, was demonstrated in all human and WT mouse tear samples. Western blot using the monoclonal anti-PLTP antibody (Mab 59) of human and mouse TF samples displayed a single 80 kDa band corresponding to the predicted molecular weight of the mature, highly glycosylated form of human PLTP. Inhibiting PLTP by heat treatment or by a polyclonal anti-PLTP antibody resulted in almost total inhibition of PLTP activity in tears. These results suggest firmly that PLTP is a normal component of TF.

A recent analysis (Li et al., 2005) of the protein composition of human TF revealed only 54 proteins that were identified with high confidence. In this study, Li et al. also confirmed the presence of PLTP in TF. Yet, de Souza and collaborators (2006) were able to identify nearly 500 proteins in human TF using multiple mass spectrometric approaches. It seems that in the latter study, however, the authors may have had significant contaminants from the ocular tissues. This is evidenced by the relatively large number of intracellular proteins, as well as the fact that not all of the major proteins, such as phospholipases, were found. The relatively small amount of identified proteins in the study of Li et al. (2005) suggests a high stature for these key proteins in human TF. As the composition and functional regulation of TF remains largely unknown, it is important to characterize the factors that play a role in the homeostatic processes of TF such as the organization of lipids and viscoelasticity of the tear film.

6.2 PLTP is secreted from the lacrimal gland

In this thesis study, no correlation between tear and plasma PLTP concentration, activity, or specific activity was observed, and thus it is likely that TF PLTP does not originate from the circulation. The secretion route of PLTP to the TF was studied through the immunostaining of tissue samples of ocular origin. The PLTP was found to be secreted from lacrimal glands. Yet, also the corneal epithelium is likely to contribute to the TF PLTP concentration (unpublished data). The immunostaining pattern with a specific monoclonal antibody against PLTP in the lacrimal gland sections was very similar to that already demonstrated for another members of lipid transfer/lipopolysaccharide binding protein family (Peuravuori et al., 2006), and also to that of TF lipocalin (Glasgow, 1995). PLTP expression in the other ocular surface tissues is not presented in this study, but it seems that at least some degree of expression of PLTP is found in these tissues. The lacrimal gland serves as a predominant source of TF proteins, and thus it is likely that this is the main source of TF PLTP.
6.3 CETP and human tear fluid

CETP is a member of the lipid transfer/lipopolysaccharide binding protein family that also includes PLTP, LBP, and BPI. Of these, the BPI is a lacrimal gland–derived normal component of TF, while PLTP and CETP play an important role in plasma lipoprotein metabolism. The major function of CETP in plasma is to facilitate the transfer of cholesteryl esters from HDL to apolipoprotein B–containing lipoproteins and the reciprocal transfer of triglycerides (Tall et al., 1983).

In the present work, cholesteryl ester transfer activity was not detected in TF. A possible explanation for the absence of CETP in tear fluid may be that CETP cannot reach cholesteryl esters/triglycerides in the superficial lipid layer, or that there is no need for such lipid transfer activity. The superficial tear film is composed of 2–3 lipid layers. Phospholipids and other amphiphilic molecules are located next to the aqueous interface because of the hydrophobic effect (Tanford, 1980; Kulovesi et al., 2010). Externally to this, cholesteryl esters/triglycerides are located on a more disordered manner in 1–2 layers, depending on the surface pressure (Rantamäki et al., in press). Accordingly, phospholipids cause a steric obstruction for CETP or other neutral lipid transferring proteins to reach their ligands. Thus, there is no reason that these neutral lipids should be transported to the superficial lipid layer. The evidence for the presence of cholesterol esters and triglycerides in TF derives from chemical analysis of the composition of the Meibomian gland excretions (McCulley and Shine, 1997), which certainly differs from the actual lipid layer resting on the top of the tear fluid. In this thesis the lipid composition of the TF was analyzed with enzymatic assays. The majority of the lipids were phospholipids, whereas the concentration of cholesterol and neutral lipids was below the detection limit of the enzymatic assays (unpublished data). This scenario would provide a very convenient explanation for the lack of cholesteryl ester transfer activity in TF and would necessitate a revision of the arrangement of the superficial tear film lipid layer.

6.4 PLTP activity in tear fluid

In human plasma, PLTP exists in two different forms, one with high activity (HA) and the other with low activity (LA) (Oka et al., 2000). In the plasma of mice overexpressing human PLTP, these two different populations of PLTP have also been observed (Jaari et al., 2001). Catalytically active plasma PLTP can be separated from the catalytically low-activity form by SEC or by using the heparin-binding properties of the active PLTP (Kärkkäinen et al., 2002). In SEC, PLTP activity has been demonstrated to elute with an average mass of 160 kDa, whereas the inactive form of PLTP has been shown to elute in a position corresponding to a molecular size of 520 kDa (Oka et al., 2000). In this study, human TF-derived PLTP displayed characteristics very similar to the HA form in human plasma. PLTP was quantitatively bound on an H-S column and PLTP activity and PLTP protein comigrated in a position corresponding to the apparent molecular mass of 160–170 kDa during SEC. Catalytically inactive PLTP was not observed in any of the studied fractions. These
findings suggest that the TF compartment lacks the molecular machinery that facilitates the generation of the LA form of PLTP observed in plasma.

Only one gene has been reported to code for both forms of the human PLTP protein, indicating that PLTP activity regulation occurs at the posttranslational level (Tu et al., 1995). It remains to be established whether the two forms of PLTP possess different functions in plasma or if LA-PLTP is just a remnant of HA-PLTP. The binding of LA- and HA-PLTP to lipids needs to be studied and may give a clue as to whether lipid composition is related to the activity of PLTP in tear fluid. The finding that the specific activity of tear PLTP was lower compared to plasma implicates that enzyme activity could be differently regulated. It may be that the proteins have different sets of oligosaccharide structures, and their different specific activities might possibly reflect these variations. Another possibility for the difference in specific activity is that the activity of plasma PLTP is partly regulated by protein-protein interactions, that are different in TF.

6.5 PLTP activity and apolipoproteins in tear fluid

In plasma, an active PLTP has been demonstrated to coelute with apoE but not with apoA-I or apoB-100, while the low-activity form of PLTP associates with apoA-I (Kärkkäinen et al., 2002). The activity could be inhibited only with antibodies against PLTP or apoE (McCulley and Shine, 1997). These findings suggest an in vivo selectivity of the PLTP interaction between apolipoproteins. As a first step to identifying the proteins with which tear PLTP might associate, the presence of apoA-I and apoE in tear fluid were analyzed. Neither apoA-I nor apoE were found in TF, suggesting that the observed lowered specific activity of TF PLTP might reflect the absence of these proteins.

During the normal PLTP purification process from plasma, the final purified PLTP protein does not contain any other copurified proteins, and is nevertheless highly active (Jauhiainen et al., 1993). This is further supported by the observations that human PLTP produced in the baculovirus protein expression system (Siggins et al., 2004), as well as PLTP in human seminal plasma (Masson et al., 2003), are fully active but that neither contain apoA-I nor apoE. An explanation for this may be that as PLTP binds phospholipids, it is able to maintain the active conformation. This concept is supported by the finding that purified plasma PLTP indeed interacts with several phospholipid molecules (Nishida and Nishida, 1997). These observations might suggest a possible different physiological function of PLTP in TF compared to plasma. It remains possible that in TF, no protein will be found associated with PLTP and that PLTP is still catalytically active.
6.6 PLTP-protein interactions in tear fluid

To elucidate possible PLTP-protein interactions in TF, specific immunoprecipitation and binding assays were carried out. The heparin-bound active PLTP fractions were analyzed by MALDI-TOF mass spectrometry, which revealed several candidate proteins (lactoperoxidase, lactotransferrin, proline-rich protein 1, lysozyme C, apolipoprotein J [clusterin], serum amyloid A, and lachryoglobin). Of these proteins, apolipoprotein J and serum amyloid A, as well as PLTP and lipocalin, are known to interact avidly with phospholipids (Jenne et al., 1991; Bausserman et al., 1983). Thus, complex formation between these proteins seems to be physiologically reasonable. Yet, further analysis using Western blotting could not confirm binding of any of these proteins to PLTP.

Lysozyme and lactotransferrin are major TF proteins and thus most likely represent contaminants in the sensitive MALDI-TOF analysis. The results of binding assays with purified proteins clearly demonstrated that direct binding was not evident. Although direct binding between PLTP and the two proteins could not be observed, an interesting observation was that incubation of PLTP with lysozyme reduced the binding of PLTP to the H-S column (data not shown), suggesting some degree of masking of the heparin-binding domain of PLTP protein or that lysozyme might compete with PLTP for heparin binding sites in the column.

As PLTP does not show interaction with either of the proteins studied here, it seems likely that in TF, no other proteins are needed to maintain the PLTP capability of transferring phospholipids. As TF does not contain apoA-I or apoE, which seem to regulate the activity in plasma, it seems a plausible explanation that PLTP avidly binds phospholipids from TF and this phospholipidated form alone could maintain the active molecular conformation.

6.7 PLTP in an air-water environment

Lipid monolayers residing on an air-buffer interface provide a convenient means to mimic lipid transfer processes in TF, where the outermost layer consists of lipids. In this study, a series of monolayer experiments were performed to explore the surface properties of PLTP. To act efficiently in TF, a protein must be surface active; this was experimentally verified for both HA- and LA-PLTP (II, Fig. 1). HA-PLTP binds to monolayers with a critical surface penetration pressure of around 25 mN/m. This is somewhat lower than the 29–34 mN/m range of other plasma apolipoproteins and CETP (Weinberg et al., 1994), while the critical surface penetration pressure of LA-PLTP was estimated to be 33 mN/m LA-PLTP (II, Fig 1).

This may be important for the more efficient release of HA-PLTP from the hydrophobic environment compared to other apolipoproteins and LA-PLTP, which may be more strongly docked to the phospholipid monolayer surface of lipoprotein particles. The very stable tertiary structure and relatively low surface exclusion pressure of HA-PLTP suggest that, when bound to the monolayer interface, HA-PLTP does not unfold like many other apolipoproteins. Furthermore, it may be that HA-PLTP does not penetrate deeply into the fatty acyl chain region of the lipid layer but mainly resides at the headgroup level. This is in accordance with the proposed shuttle
mechanism of PLTP, in which PLTP should be able to efficiently attach to and detach from the donor and acceptor particles.

In monolayer studies, both HA- and LA-PLTP were able to absorb lipid molecules from the monolayer (II, Fig. 2). The calculated stoichiometry of one PLTP molecule to desorb 5–6 molecules of DPPC is within the same range when compared to earlier estimations of the unspecific phospholipid binding of the PLTP molecule outside the N-terminal pocket (Nishida and Nishida, 1997). This unspecific binding explains why LA-PLTP is also able to absorb phospholipids from the phospholipid monolayer. An increased absorption was seen when HA-PLTP was combined with HDL₃ molecules (II, Fig. 2). This may indicate that, after absorbing lipids from the lipid monolayer, PLTP attaches to the HDL₃ particle where it can release bound lipids. Another mechanism is that HDL₃ can directly absorb lipids from the lipid monolayer, which seems more likely since LA-PLTP and HDL₃ mixtures also show increased subphase radioactivity.

6.8 PLTP and mucins in tear fluid

Ocular mucins are thought to be located at the surface of the tear film, where they cause an increase in surface pressure by generating lateral reorganization of the lipids and alteration of surface viscoelastic properties (Millar et al., 2006). The contamination of ocular surface mucins with lipids from the superficial lipid layer can lead to drying of the corneal epithelium (Holly and Lemp, 1971). This can happen when the aqueous-mucin layer becomes too thin or the lipid contaminates the mucin layer. A lipid saturation of the corneal epithelium forms a hydrophobic area that prevents wetting of the surface above it, leading to the formation of DES. Thus, a mechanism to prevent contamination and remove lipids from the ocular surface is necessary.

Immunoprecipitation assay-suggested that PLTP directly interacts with ocular mucins in human TF (III, Fig. 3). These findings were confirmed by series of further studies to characterize the PLTP-mucin interactions. The collection of human ocular mucins in volumes for in vitro interaction experiments is challenging, and thus in many previous studies, commercially available mucins have been used as substitutes for ocular mucins such as BSM and avian-derived ovomucin. While each human mucin gene has a unique tandem repeat amino acid sequence, length, and variation in allele number of repetitions among individuals (Mantelli et al., 2008), it is reasonable to consider that BSM and ovomucin are an adequate model for tear mucins during in vitro experiments. In vitro a direct binding assay showed that PLTP avidly interacts with BSM, but not with avian-derived ovomucin. A possible explanation for this is that there are some structural differences between these proteins (Lang et al., 2006). The β-subunit of ovomucin, which is an avian ortholog to a secreted human mucin (MUC6), has an unusual organization, as the tandem repeats of the STP domain are encoded by multiple exons where each exon encodes one repeat (Lang et al., 2006).
Mucin-lipid-PLTP interactions were studied in more detail with SEC, where a PLTP-mediated transfer of radioactively labeled phospholipids from phospholipidated mucin was registered. The SEC analysis showed that phospholipids can act as interaction partners between mucin and PLTP or to promote the interplay between the two proteins. When PLTP was incubated with the phospholipidated mucin, PLTP eluted together with mucin in fractions corresponding to a wide molecular size range. Detergent in the buffer significantly changed the PLTP elution profile, as well as that of phospholipids, indicating a disruption of the mucin-PLTP interaction. It has been shown that mucin proteins have hydrophobic regions that can bind lipids (Smith et al., 1984). PLTP can function as a phospholipid transfer protein both in the presence of apolipoproteins such as apoAI and apoAII or alone when directly bound to phospholipids. The displacement of phospholipids and PLTP from phospholipidated mucin by detergent encourages the consideration that phospholipids play a key role in the mucin-PLTP interaction. The broad elution profile of mucin in PBS could be explained by the size heterogeneity of the gel-forming mucin. The wide heterogeneity of mucin molecules was also noted by preliminary AsFIFFF experiments, where mucin complexes were observed with a hydrodynamic diameter ranging from 25 up to 225 nm (data not shown).

6.9 PLTP and phospholipid transfer mechanism

Lipids can be exchanged and transferred between different lipoprotein classes by spontaneous, temperature-dependent transfer or by protein-mediated transfer (Tall, 1995). In plasma, protein-mediated transfer of lipids is facilitated by CETP and PLTP. Two different models of PLTP-mediated lipid transfer have been proposed: (i) a shuttle model in which PLTP physically transports lipids between lipoprotein particles and (ii) a ternary complex model in which PLTP forms a bridge between two lipoprotein particles, enabling them to exchange lipids by diffusion (Tall, 1995; Barter et al., 1994). On the basis of kinetic evidence, it has been suggested that CETP facilitates lipid transport by a shuttle mechanism between HDL—acceptor lipoprotein particles like VLDL and LDL (Barter and Jones, 1980; Epps et al., 1995). In favor of the shuttle model, it has also been demonstrated that CETP can absorb lipids from phospholipid monolayers residing at the air-water interface (Weinberg et al., 1994).

To supplement the monolayer studies where PLTP was able to absorb lipids from air-water interface, and to elucidate the actual lipid transfer mechanism of PLTP in more detail, a series of flow field-flow fractionation studies were done. With this method, the lipid components were more efficiently separated and their sizes were more homogeneous than previously described (Li and Giddings, 1996). In addition, reconstituted apoA-I-phospholipid-cholesterol particles were resolved in three peaks due to the formation of three discoidal subgroups, which have also previously been detected by native gradient gel electrophoresis.
With AsFF studies, the transfer of radiolabeled lipids from SUVs to HDL, following the addition of HA-PLTP, but not LA-PLTP, was noted. The particle size of HDL was not altered in either experiment, suggesting that no ternary complexes were formed. In addition, the radiolabeled HA-PLTP and LA-PLTP molecules showed that after incubation, the active PLTP itself was transferred from SUVs to HDL. These results strongly suggest that the shuttle mechanism like in the use of CETP (Epps et al., 1995) is the preferred mechanism of PLTP-induced lipid transfer.

In the monolayer series, both LA- and HA-PLTP showed lipid-binding capability in water-air interface, but LA-PLTP was unable to deliver lipids to the acceptor particle in AsFF studies. These results suggest that even if the surface of LA-PLTP can unspecifically bind lipids, the complex cannot dock on the acceptor. Similar results were seen when the structure of the acceptor particles was changed. Both apoA-I-phospholipid discoidal and apoA-I-phospholipid cholesterol particles were unable to receive lipids from PLTP. In AsFF assay where SUVs and HDL are used as substrates for PLTP, the curvature of the SUV and HDL may be an important determinant for the activity of PLTP. On a monolayer where lipids are resting on the surface of the buffer, they display a very small degree of curvature, while SUVs and especially HDL have a high degree of curvature. Within these molecules, a surface layer distortion is needed for the formation of sufficient gaps that may accommodate the interfacial binding site of HA-PLTP and proper interaction of the active site with the phospholipid to be transferred. It seems that because LA-PLTP is unable to adopt an active conformation, proper interactions between SUV and HDL are hindered.

6.10 PLTP-deficient mice and dry eye

To study the effect of PLTP deficiency on the homeostasis of eye, a KO mouse model was used. This model was compared to the dry eye mouse model (EIDE) described by Dursun et al. (2002), where TF production is attenuated by the cholinergic blockade with scopolamine. Compared to the cholinergic blockade, the PLTP KO mice showed similar morphological changes in the cornea. In addition, increased corneal permeability and cleavage of epithelial occludin were noted in these mice. During the follow-up time, 2.4% of PLTP KO mice suffered spontaneous perforation of an eye, while the WT animals grown under the same conditions had no eye problems.

PLTP KO mice were also treated with scopolamine to see whether the effects are additive. PLTP KO with EIDE did not further induce corneal permeability or occludin cleavage. Presumably, these two models can separately cause a maximal permeability to CF with the concentrations used. The activation of matrix metalloproteases, especially MMP-9, that are primarily responsible for occludin cleavage in corneal epithelium (Pflugfelder et al., 2005) seems to be involved in both PLTP KO and EIDE treatment.
Several animal models that mimic human dry eye have been introduced. Most of these models resemble autoimmune disease seen in human Sjögren syndrome, since the key factor in the formation of DES is diminished aqueous tear production. As the tear lipid film imbalance and the evaporative DES are common diseases of the eye, an experimental model that mimics this condition is needed. In addition, at the moment the techniques used to diminish the production of TF in different animal models mostly alter the presence of ocular surface system. Mechanical cauterization of Meibomian gland orifices (Gilbard et al., 1989) and other lid surgery techniques, as well as mutant and transgenic techniques to induce aplasia or atrophy, heavily change the function of the normal ocular surface structure even before formation of DES. In addition, the animal model of blepharitis (Chan et al., 1995) itself induces ocular surface irritation and alters the immunological profile of the ocular surface, which is an important factor in the development of DES. Among PLTP KO mice, TF production was similar to that recorded in WT littermates. In addition, the eyelid and Meibomian gland morphologies were normal in PLTP KO mice. It seems that the imbalance of TF is a key element in the development of DES in these mice, and thus the PLTP knockout model serves as a relevant model of human evaporative DES.

6.11 PLTP as a scavenger of the ocular surface

Tear lipocalin, a common tear lipoprotein, has been proposed to serve as a scavenger of lipophilic substances from the corneal surface (Glasgow et al., 1999). Even though lipids can be extracted from Tlc and it seems to be capable of binding lipids (Gasymov et al., 2005), it has been discussed whether Tlc can actually bind to lipid membranes and accept membrane lipids (Saaren-Seppälä et al., 2005). Tear PLTP has lipid transfer ability without any acceptor molecule and is active without any detectable protein-protein associations (I). In contrast to Tlc, which binds lipids at a 1:1 protein/lipid ratio (Glasgow et al., 1999), PLTP can accommodate up to 43 mol of phosphatidylcholine and 13 mol of cholesterol with one mol of PLTP (Nishida and Nishida, 1997). A high number of PLTP-bound lipids are seen even though only two specific lipid binding pockets have been identified in PLTP (Huuskonen et al., 1999). These pockets might only be needed for specific lipid transfer of this protein and the rest of the associated phospholipids may be needed for proper folding of the protein and stabilizing the enzymatic activity.

Human lung tissue, where PLTP is found at air-water atmosphere, displays high levels of PLTP compared to other tissues. In the lung, PLTP may serve an important role in maintaining the normal function of this organ, such as in the transport of surfactant components, primarily phospholipids. The PLTP gene is highly expressed in alveolar type II cells, and is induced during hypoxia and in emphysema (Jiang et al., 1998), indicating important surface protective properties. A somewhat analogous situation to emphysematous lungs might be what takes place in DES. More specifically, the superficial lipid layer provides a barrier to prevent evaporation of TF that would eventually lead to pathological damage of the corneal epithelium. In TF, PLTP could
facilitate the uptake of Meibomian lipids and ultimately transport them to the superficial lipid layer. Likewise, if the corneal epithelium were contaminated by hydrophobic material, PLTP could scavenge these and transport them to the superficial layer of the TF or, alternatively, scavenge lipids by binding them and transporting them through the naso-lacrimal duct. PLTP might play an integral role in tear lipid trafficking and in the protection of the corneal epithelium.

In line with these findings, human DES patients show significantly elevated levels of PLTP activity in tears, suggesting an induction of PLTP activity under hyperosmolar conditions (IV).

The results of this thesis do not contradict the previous findings, which propose that Tlc is the key lipid transfer molecule and a scavenger of TF lipids (Glasgow et al., 2010). However, it seems unreasonable to assume that only one protein would be solely responsible for removing ocular contaminants in the TF. The homolog of human Tlc is von Ebner’s gland protein (VEGP) in rats (Kock et al., 1994), Can f1 in dogs (Konieczny et al., 1997), and in pigs (Garibotti et al., 1995), a homologous protein, pVEG, is produced by the lacrimal glands. Like in humans, there is more than one gene encoding for rat VEGP (Kock et al., 1994). However, in contrast to humans, where only one form of Tlc was found to be expressed, in rats three different closely related proteins have been found to be expressed (Syed et al., 1999). It is interesting to note that in mice, cows, and guinea pigs, no homologous protein to Tlc has been detected so far, either by immunohistochemical methods or by hybridization. Thus, it seems that during evolution, several different scavenger proteins of TF have arisen and in humans it seems reasonable that PLTP acts together with Tlc to maintain a normal ocular surface.

Together, these findings suggest a notable role of PLTP in lipid transfer in human tears. PLTP could play a significant role in preventing instability of the lipid film and thus reduce the development of DES. The precise mechanisms of tear PLTP-facilitated phospholipid transfer and its association with different ocular structures remain to be clarified in the future.
7 SUMMARY AND CONCLUSIONS

The aim of this thesis was to verify the presence of PLTP in human tear fluid, investigate lipid transfer mechanisms in tear fluid, and evaluate the possible association of PLTP in dry eye.

First, the presence of the high-activity form of PLTP was demonstrated by Western blot in all human tear samples studied. Also tear fluid derived from the wild-type mice displayed immunoreactive PLTP. Inhibition of PLTP with heat treatment or by polyclonal anti-PLTP antibody resulted in the almost total inhibition of phospholipid transfer activity in tears. The secretion route of tear PLTP was demonstrated via immunostaining of tissue samples of ocular origin. PLTP was found to be secreted from lacrimal glands. These results suggest firmly that PLTP is a normal component of TF.

To characterize the role of PLTP in the lipid transport in an air-water interface, several monolayer and AsFIFFF lipid transfer assays were performed. PLTP seems to be surface active and is capable of accepting lipid molecules without the presence of lipid-protein complexes. The active movement of radioactively labeled lipid and HA-PLTP to acceptor particles suggested a shuttle model of PLTP-mediated lipid transfer. In this model, PLTP physically transports lipids between the donor and acceptor. In light of these results, a shuttle model is more evident than a ternary complex model, in which PLTP forms a bridge between two lipoprotein particles.

To further characterize the molecular mechanisms of PLTP-mediated lipid transfer in TF, the association of PLTP with TF proteins was elucidated. Apolipoproteins responsible for PLTP activity in serum were found to be absent in TF. PLTP was also able to transfer lipids without any associated protein partners. The only proteins interacting with PLTP in human TF were mucins. These results suggest a scavenger property of TF PLTP: If the corneal epithelium is contaminated by hydrophobic material, PLTP could remove it and transport it to the superficial layer of the TF or, alternatively, abolish lipids by binding them and transporting them through the naso-lacrimal duct. Thus, PLTP might play an integral role in tear lipid trafficking and in the protection of the corneal epithelium.

To reveal whether the results obtained in vitro bear any relevance in vivo, a mouse model with a selective deficiency in PLTP was used. In these mice, enhanced corneal epithelial damage, increased corneal permeability, decreased corneal epithelial occludin expression, and even perforations of the eye were detected. These findings were similar to those of previous dry eye models and correlate to observations in human dry eye. As the imbalance of TF is a remarkable cause of human DES, it seems that PLTP lipid transfer is needed to maintain the normal function of TF and a healthy ocular surface. Results represented in this thesis indicate that the mouse PLTP KO model serves as a relevant model of evaporative DES.
To study whether PLTP plays a role in human DES, a series of DES patients were examined. Tear osmolarity is found to be the single most efficient indicator of DES. In this study, a significant correlation between elevated osmolarity and increased PLTP activity was shown.

At the time being, lipocalin is thought to be responsible for the lipid transfer in human TF. However, it seems unreasonable to assume that only one protein would be solely responsible for removing ocular contaminants in the TF. The results of this thesis suggest that although PLTP is a minor constituent of normal TF, it possesses efficient phospholipid transfer activity, and thus it is likely that PLTP aids Tlc, and perhaps other proteins, to decontaminate the ocular surface. These results suggest that in the TF, PLTP may act in concert with human tear lipocalin to ensure efficient lipid transfer. The specific mechanism of PLTP in lipid transfer processes in tears and the detailed lipid composition of TF will be important targets to investigate in order to define the exact role of PLTP in DES.
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