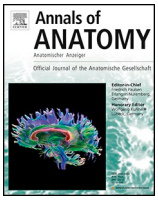
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# Molecular detection of lacrimal apparatus and ocular surface - related ABC transporter genes

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ABSTRACT

The ocular system is in constant interaction with the environment and with numerous pathogens. The ATP-binding cassette (ABC) transporters represent one of the largest groups among the transmembrane proteins. Their relevance has been demonstrated for their defense function against biotic and abiotic stress factors, for metabolic processes in tumors and for their importance in the development of resistance to drugs. The aim of this study was to analyze which ABC transporters are expressed at the ocular surface and in the human lacrimal apparatus. Using RT-PCR, all ABC transporters known to date in humans were examined in tissue samples from human cornea, conjunctiva, meibomian glands and lacrimal glands. The RT-PCR analyses revealed the presence of all ABC transporters in the samples examined, although the results for some of the 48 transporters known in human and analyzed were different in the various tissues. The present results provide information on the expression of ABC transporters at the mRNA level on the ocular surface and in the lacrimal system. Their detection forms the basis for follow-up studies at the protein level, which will provide more information about their physiological significance at the ocular surface and in the lacrimal system and which may explain pathological effects such as drug resistance.

## 1. Introduction

ATP binding cassette (ABC) transporters represent one of the most prominent families of membrane proteins in humans (Dean et al., 2022). These proteins share a typical structure, their ATP binding motif. Most of them also possess a transmembrane domain, with the exception of ABCE and ABCF. ABC transporters are mainly active transporters that transport substrates across membranes against the concentration gradient by consuming adenosine triphosphate (ATP). They play a crucial role in moving large molecules across membranes, metabolism, and drug resistance. To date, 48 different ABC family transporters are known in humans. They are divided into seven subfamilies based on amino-acid sequence similarities and phylogeny, ranging in nomenclature from ABCA to ABCG (Dean et al., 2022, Locher, 2016, Kos and Ford, 2009). They are expressed in a variety of tissues, e.g. in the liver, kidney or brain, where they are responsible for the uptake, distribution and excretion of substances. Some of these transporters are also involved in intracellular processes, e.g. cell division, osmotic homeostasis or the transport of lipids and cholesterol (Liu, 2019). Furthermore, these proteins transport a wide variety of molecules, ions, peptides, amino acids, vitamins, organic and inorganic degradation products, antibiotics, and proteins (Vasiliou et al., 2009). Some of these transport systems are not directly involved in the transport of substrates but mediate the transfer of ions across the membrane (Vasiliou et al., 2009). Several of the ABC transporters are known to be involved in the multidrug resistance mechanism in cancer cells. "Multidrug resistance" describes the means by which cells have or can develop resistance to drugs. Mutations in the ABC transporters are known to cause various diseases, such as cystic fibrosis, Stargardt disease, sideroblastic anemia with ataxia and ankylosing spondylitis (Molday and Zhang, 2010, Hunt et al., 2013, Vasiliou et al., 2009).

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### 1.1. ABCA family

The Group includes twelve different proteins, most of which are responsible for lipid transport and are expressed in numerous body regions (Arnould et al., 2001, Piehler et al., 2007). Some of them are associated with severe inherited diseases due to defective transport of lipids, such as a mutation in ABCA1 leading to Tangier disease (Molday and Zhang, 2010). Mutation of this gene results in decreased removal of cholesterol from the cell, leading to increased storage of cholesterol, particularly in macrophages, the nervous system, or the spleen. Patients have significantly reduced plasma HDL levels, develop yellowish plaques on the tonsils, and are at increased risk for atherosclerosis (Peelman et al., 2003). Neonates harboring mutations in the ABCA3 gene suffer of severe respiratory distress syndrome due to surfactant deficiency. However, it has yet to be fully elucidated in how the ABCA3 protein is responsible for providing surfactant (Piehler et al., 2007).

### 1.2. ABCB family

This family, which consists of eleven different genes, are known for the multidrug resistance mechanism in cancer cells, which is why this subfamily is also referred to as the "MDR family". ABCB1 (MDR1/PGP) is the best-known and best-studied transporter of the ABC transporter family. Physiologically, the protein is expressed in the various tissues, namely the liver, jejunum, and kidney. Their expression of MDR1 results in decreased enteric drug absorption and increased renal and biliary excretion. Pathological, it confers resistance through the efflux pumping to certain cytostatic drugs for cancer cells and plays a critical role in the human blood-brain barrier (Dean et al., 2001).

### 1.3. ABCC family

The 13 members include one of the best-known representatives of the ABC transporters: the cystic fibrosis transmembrane conductance regulator protein, also known as CFTR, encoded by ABCC7, their mutation being causative for cystic fibrosis (Hunt et al., 2013). A large section of the other members of the ABCC family confers efflux capacity to various tissues. For example, ABCC1 is involved in the physiological defence of the lung, testis, and placenta against xenobiotics and toxic molecules (Gu and Manautou, 2010). However, the appearance of ABCC transporters like ABCC1, ABCC2, ABCC4, ABCC5 can lead to multidrug resistance in neoplastic diseases like hepatic cell cancer or neural glioma (Kunicka and Soucek, 2014, Bronger et al., 2005).

### 1.4. ABCD family

ABCDs are located in the membrane of peroxisomes and lysosomes. ABCD1, ABCD2, and ABCD3 regulate the transport of long-chain fatty acids into the peroxisomes for the β-oxidation (Baker et al., 2015, Dean et al., 2001). ABCD4 governs the transportation of cobalamin out of the lysosomes into the cytosol (Kawaguchi and Imanaka, 2022). Mutations in the ABCD1, ABCD2, and ABCD3 are known causes of diseases like adrenoleukodystrophy (ALD for short) or Zellweger syndrome (Vasiliou et al., 2009).

### 1.5. ABCE family

The family consists exclusively of the gene ABCE1, which regulates exogenous RNA decay (Karblane et al., 2015, Annibaldis et al., 2020). Interestingly, ABCE1 lacks the transmembrane domain and is soluble in the cytosol (Navarro-Quiles et al., 2018). It is also highly conserved, and the analogues can be found in archaeons, mosquitoes, or catfish. The loss of function of ABCE is associated with reduced or slow growth of organs. The exact mechanism of action is still elusive though.

### 1.6. ABCF family

ABCF-transporter have been identified as a translational regulator of an inflammatory cytokine pathway, as ABCF1 is involved in tumor necrosis factor (TNF)-α (Richard et al., 1998).

### 1.7. ABCG family

This group plays a role in atherosclerosis and sitosterolemia. ABCG1 is involved in the export of cholesterol from macrophages. ABCG2 functions as a multidrug resistance transport protein (Kim et al., 2002). It transports cholesterol, progesterone and testosterone and is involved in macrophage lipid metabolism (Engel et al., 2001, Woodward et al., 2011, Hazard and Patel, 2007).

### 1.8. Significance of the ABC transporter

Today, the therapy of diseases of the anterior and partially posterior segment of the eye is predominantly carried out by topical application of eye drops via the ocular surface containing, for example, anti-infectives, anti-inflammatories or glaucoma drugs. Clinically, a wide spectrum of therapeutic effects with the same medication and dosage between patients is known. There are diverse reasons each drug acts differently in patients, which needs to be clarified. The ocular surface with cornea and conjunctiva is an epithelial association of cells that provides an effective permeability barrier by tight junctions. In addition, some active transport structures, such as efflux transporters (MRP1–7, MDR1, LRP), have already been described in the cornea, conjunctiva and retina (Mannermaa et al., 2009, Vellonen et al., 2010, Chen et al., 2013). A large proportion of ABC transporters, however, has yet to be investigated on the ocular surface. Not only might those transporters influence the deposition of pharmaceutical substances, but they could also be influential for the pathologic genesis of certain eye diseases. Knowing the qualitative mapping of the ABC transports to the different tissues of the ocular system may allow pharmacists to develop new strategies and formulations to circumvent the efflux barrier and affect therapeutic concentrations of substrates in target ocular tissues. The primary aim of this study was therefore to systematically analyze all ABC transporters known in humans in the tissues of the ocular surface and the lacrimal system, initially at the mRNA level, in order to create a basis for further investigations at the protein level.

## 2. Material and methods

### 2.1. Tissues

Tissue samples were obtained from six body donors (three men, three women; age range: 63–95 years) donated to the Department of Functional and Clinical Anatomy, FAU Erlangen-Nuremberg, Erlangen, Germany. Donors were free of recent trauma, eye and nasal infections, and diseases involving or affecting lacrimal apparatus or ocular surface function. The study was approved according to institutional review board regulations and is consistent with the goals of the Declaration of Helsinki. Samples were collected from the body donors within 24 hours post-mortem. Half of each sample was immediately frozen at −80°C for biological testing. Human brain and liver tissue from the same body donors were used as positive controls. Specifically, in PCRs detecting ABCB5, ABCB7, ABCB11, ABCC1, ABCC3, ABCC7, ABCD1, ABCF2, ABCG5, and ABCG8 we used liver tissue, for all other genes we used brain tissue as positive control.

### 2.2. Cell culture

Immortalized HCE and HCjE cells were cultured as previously described by Schicht et al., 2018 (Schicht et al., 2018). SV40-transformed human corneal epithelial cells (HCE cells, obtained

from Kaoru Ara-ki-Sasaki, Tane Memorial Eye Hospital, Osaka, Japan, passage number 18–27) (Araki-Sasaki et al., 1995) as well as a human immortalized epithelial cell line from normal human conjunctiva, HCjE-Gi obtained from Ilene Gipsons lab at Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston, USA were cultured as monolayer and used for further experiments (Gipson et al., 2003). Human meibomian gland epithelial cell line (hMGEC), obtained from David A. Sullivan (also Schepens in Boston), was cultured under standard conditions with and without 10% fetal calf serum to initiate differentiation as described before (Hampel and Garreis, 2017).

### 2.3. mRNA extraction and cDNA synthesis

Total mRNA was extracted from cell cultures as well as from human tissues: cornea (n=3), lacrimal gland (n=4), meibomian gland (n=4) and HCE, HCjE, hMGEC (n=2) using peqGOLD TriFast reagent (VWR International GmbH, Darmstadt, Germany). SpeedMill plus (Analytik Jena AG, Jena, Germany) was used for mechanical crushing. After centrifugation (10 minutes, 15,520 g), the supernatant was applied to mRNA isolation. DNA contaminations were removed using a standard protocol. Reverse transcription of mRNA samples into first-strand cDNA was performed using the RevertAid Reverse Transcriptase Kit (Thermo Fischer, Wesel, Germany) according to the manufacturer's protocol. Two micrograms of total RNA were used for each reaction to cDNA. Gene-specific intron-spanning primers previously synthesized at MWG Biotech AG (Ebersberg, Germany) were used for Reverse Transcription Polymerase Chain Reaction (RT-PCR).

### 2.4. Reverse transcription polymerase chain reaction

RT-PCR was performed according to the following standard protocol, with all primer pairs used for the 48 ABC genes listed in Table 1. To estimate the amount of amplified PCR product, a β-actin PCR was performed with specific primers: sense (5'-GAT CCT CAC CGA GCG CGG CTA CA-3') and anti-sense (5'-GCG GAT GTC CAC GTC ACA CTT CA-3'). The reference gene β-actin was used as an internal control for assessing the integrity and stability of the transcribed cDNA.

## 3. Results

The results of the gene expression of all 48 ABC transporters by RT-PCR in human corneas, lacrimal glands, meibomian glands and a human corneal epithelial cell line (HCE), a human conjunctival epithelial cell line (HCjE) and a human meibomian gland epithelial cell line (hMGEC) – the latter cultured as differentiated cells and as undifferentiated cells - are shown below. For the analyses of human tissue from body donors, different cDNA samples from different body donors were used. Human brain and human liver tissue from a body donor was used as a positive control.

### 3.1. Detection of the ABCA family

The amplicons of the ABCA family should have a size of 200 bp at the gene level. All transporters examined were visible as bands at 200 bp. The negative sample containing DEPC water instead of cDNA showed no detection in any investigations (Fig. 1). Expression of ß-actin demonstrated a band with the expected size of 298 bp in all samples used. In the cornea, only ABCA10 could not be detected. In the lacrimal gland, ABCA4 and ABCA12 were not detected in the samples examined. ABCA1, ABCA3 and ABCA4 could only be detected in three out of four samples in meibomian glands; ABCA13 could only be detected in two out of four samples. ABCA6 and ABCA8 could not be detected in HCE cells. In HCjE cells, ABCA4, ABCA6, ABCA9 and ABCA10 were expressed in only one. In undifferentiated hMGEC cells, ABCA9 and ABCA10 were not detectable. In contrast, all ABCAs were detectable in differentiated

**Table 1**
Primers used.

| Gene | Primersequenze 5'-3' | Annealing-temperatur | PRoduct size |
|---|---|---|---|
| ß-Aktin | caa gag atg gcc acg gct gct tcc ttc tgc atc ctg tcg gca | 60°C | 298 bp |
| ABCA1 | tac tgg ttt ggc gag gaa ag gag gtg atc tgg ccc tca ta | 60°C | 200 bp |
| ABCA2 | tat cac agc atg ccc acc ta atg gcc acg atg atg aag at | 60°C | 200 bp |
| ABCA3 | gta cga caa ctg ctc gtc ca aaa gcg gga aaa ggg aag ta | 60°C | 200 bp |
| ABCA4 | gaa aag acc gag ccc cta ac aat gcg gtg atc tgg ttc tc | 62°C | 200 bp |
| ABCA5 | tca aag gga tac cag cca ac aga aca ggg gtc cat tcc ag | 60°C | 200 bp |
| ABCA6 | tga gca gct gaa tgt tcc tg tgg tca gga gga cac ctc tc | 62°C | 200 bp |
| ABCA7 | gca cct cag cat ggg ata ct gtt gtt ccc tcc gct gta g | 60°C | 200 bp |
| ABCA8 | cat gca atg cca aaa gat tg aat gta agg tgg gca act cg | 60°C | 200 bp |
| ABCA9 | gac tgc tgg att gga tcc tc agc cta tgc ccc att tct tc | 60°C | 200 bp |
| ABCA10 | ctt agc cct ttt gcc ttc ac cca tgg cca tct tta tca gg | 58°C | 200 bp |
| ABCA12 | agc acg gaa gca ctt gtc tc tgc ggt ggg gaa tag tta aa | 60°C | 200 bp |
| ABCA13 | gtg tgt tgc cgt tat tgt cg gca tgg tac aaa ggc caa ag | 60°C | 200 bp |
| ABCB1 | tgg aga gat cct cac caa gc ttc ctg tcc caa gat ttg ct | 60°C | 200 bp |
| ABCB2 | ttg cag gga gag gtg ttt g gac act gat ccc cag agc at | 60°C | 200 bp |
| ABCB3 | cgg ata cca ccc tga tga gt gat ctc ccg aag cac ttc ct | 60°C | 200 bp |
| ABCB4 | aca ggc agc tct gga taa gg gga tct ggc ttc ctg atg tc | 60°C | 200 bp |
| ABCB5 | gcg gca tta tcg aga cca ta gag ctc ctt ttt ccc cta cc | 60°C | 200 bp |
| ABCB6 | tac aac gcc gag agt tac ga agc aca tag tcc cca acc tg | 60°C | 200 bp |
| ABCB7 | ctc tgt tag cgg ctc agg tc cat gcc aac atg tcc tct ttt | 60°C | 200 bp |
| ABCB8 | tgg aag ctc ttc tgg cag tt ata agc agg tgg gtg ctg ag | 60°C | 200 bp |
| ABCB9 | gca tcg tca tcc aga aaa gc gcg gtt ctc atc aaa gaa gc | 60°C | 200 bp |
| ABCB10 | gca ttt tgc cta tcc agc tc ctg ggt tta gct gac gga tg | 60°C | 200 bp |
| ABCB11 | gtc gatt agt cca ggg cag a agg caa aca aca ctg gtt cc | 60°C | 200 bp |
| ABCC1 | gga ttt ttg ctg tgg atc gt acc agc cag aaa gtg agc at | 60°C | 200 bp |
| ABCC2 | gag ctg gcc ctt gta ctc ac tga aat tga aag tgc cac a | 60°C | 200 bp |
| ABCC3 | ggc agc tac tca gag gag ga gct gtgg cct cgt cta aaa c | 58°C | 200 bp |
| ABCC4 | aat acc ctt ggt tcc cct tg cct ggt gtg cat caa aca gt | 60°C | 200 bp |
| ABCC5 | ctc atc ctg tcc atc gtg tg cgg taa ttc aat gcc caa gt | 60°C | 200 bp |
| ABCC6 | gtg gtg ttt gct gtc cac ac acg aca cca ggg tca act tc | 62°C | 200 bp |
| ABCC7 | gcc gac act ttg ctt gct at aga ggc aga agg tca tcc aa | 60°C | 200 bp |
| ABCC8 | gca agt cct cgc tcc ttc ta ctc cac agt ggc att tag ca | 60°C | 200 bp |
| ABCC9 | ttg gta ggc ttg ggt ctt ct ttg atc tcc cct tct tgt gg | 60°C | 200 bp |
| ABCC9 SUR2A | tgg atg agg caa cag ctt c att ctt gtg ggc gaa caa at | 60°C | 200 bp |
| ABCC9 SUR2B | tgg atg agg caa cag ctt c cat ttt cct gag cca aga gg | 60°C | 200 bp |
| ABCC10 | gag aac ctg cga ctc ctt ga gtc agg gac agg gca taa ga | 60°C | 200 bp |

(continued on next page)

**Table 1** (*continued*)

| Gene | Primersequenze 5'-3' | Annealing-temperatur | PRoduct size |
|---|---|---|---|
| ABCC11 | cct gag ctc cat cca tgt ct agt agg ggg tgg agg aaa tg | 60°C | 200 bp |
| ABCC12 | aac aga gac gca agc caa at tgt tca agt tca ggc tgt cg | 60°C | 200 bp |
| ABCD1 | atg cat ctg ctc atc aca gg tgc atg tcc tcc act gag tc | 60°C | 200 bp |
| ABCD2 | ggc ttc cag gct aaa ctt ca gat ccc gaa gac ttc caa ga | 60°C | 200 bp |
| ABCD3 | gac cct tgg aac act tcg ag tcg cca ttc ttt gct ttt ct | 60°C | 200 bp |
| ABCD4 | tgg agc aca tga gga cag ac gac cag ggt gct aag ctc tg | 60°C | 200 bp |
| ABCE1 | ggt tac acc cca gag caa aa cca aaa ctt cac ctg gac ga | 60°C | 200 bp |
| ABCF1 | aaa agg agc ctc cca aac aa atg agc gga gat gct gaa ct | 60°C | 300 bp |
| ABCF2 | aga gag ggt cgt gag cga ta gag ttg act tcc ctg ctc ca | 60°C | 200 bp |
| ABCF3 | aat ggg gct ggg aag tct ac tgg tga cgg tac tcc tcc tc | 60°C | 200 bp |
| ABCG1 | agg tgg tct cgc tga tga aa gtt gtg gta ggt tgg gca gt | 60°C | 200 bp |
| ABCG2 | ggc aga tgc ctt ctt cgt ta gaa gcc atg aca gcc aag at | 60°C | 200 bp |
| ABCG4 | gag acc tga acc cca tgt tg gga cag gaa ggt cct ctt ga | 60°C | 200 bp |
| ABCG5 | tgc tga acg ctg tga atc tg ata tcc aaa tcg ggc aac ct | 60°C | 200 bp |
| ABCG8 | cct gtc ctc gct aca gca at gct ttc cac aca ggt gtc ct | 60°C | 200 bp |

hMGEC.

### 3.2. Detection of the ABCB family

The amplicons of the ABCB family were expected to be 200 bp in size at the gene level. All transporters examined were visible as bands at 200 bp. The negative sample containing DEPC water instead of cDNA showed no detection in any of the investigations (Fig. 2). Expression of ß-actin showed a band with the expected size of 298 bp in all samples used.

The transporter ABCB5 could only be detected in one cornea sample. In the lacrimal gland, all ABCB transporters could be detected. In the meibomian gland, ABCB1, ABCB8, and ABCB9 were expressed in only three of four samples. In HCE cells, ABCB11 could only be detected in one sample; ABCB5 was not detectable. In HCjE cells, ABCB4, ABCB5, ABCB6, ABCB9, and ABCB11 were only expressed in one sample. In undifferentiated and differentiated hMGEC cells, ABCB5 was not expressed.

### 3.3. Detection of the ABCC family

The amplicon of the ABCC family was expected to be 200 bp in size at the gene level. All transporters examined were visible as bands of 200 bp. The negative sample containing DEPC water instead of cDNA revealed no detection in any of the analyses (Fig. 3). Expression of ß-actin showed a band with the expected size of 298 bp in all samples used. In the cornea, ABCC4, ABCC5, ABCC7, ABCC9, ABCC9/SUR2B, and ABCC11 were only expressed in two cornea samples; ABCC6 and ABCC10 were only identified in one sample; ABCC8, ABCC9/SUR2A, and ABCC12 were not detected in any of the samples of the cornea. In the lacrimal gland, except of ABCC6, which was found in only two of the analyzed samples and ABCC12 which was expressed only in one sample, all members of the ABCC family were expressed. In the meibomian gland ABCC9/SUR2A was not detectable in any sample, ACBCC6, ACBC8, ACBC12 were only identified in two of the four samples. All other members of the ACBC family were expressed. In the HCE cells ABCC7, ABCC9/SUR2A, ABCC9/SUR2B, and ABCC12 were identified only in one sample; ABCC8 and ABCC9 were not identified in samples of the HCE cells. In the HCjE cells, ABCC2, ABCC9, ABCC9/SUR2B, ABCC10, and ABCC12 were expressed in only one sample; ABCC7, ABCC8, ABCC9/SUR2A could not be identified in the analyzed samples. In the undifferentiated hMGEC cells ABCC7, ABCC8, ACBCC9, and their variants SUR2A and SUR2B were not expressed. In the differentiated hMGEC cells, ABCC8, ABCC9/SUR2A, ABCC9/SUR2B, and ABCC12 were not expressed.

### 3.4. Detection of the ABCD family

The amplicon of the ABCD family was expected to be 200 bp in size at the gene level. All transporters examined were visible as bands of 200 bp. The negative sample containing DEPC water instead of cDNA

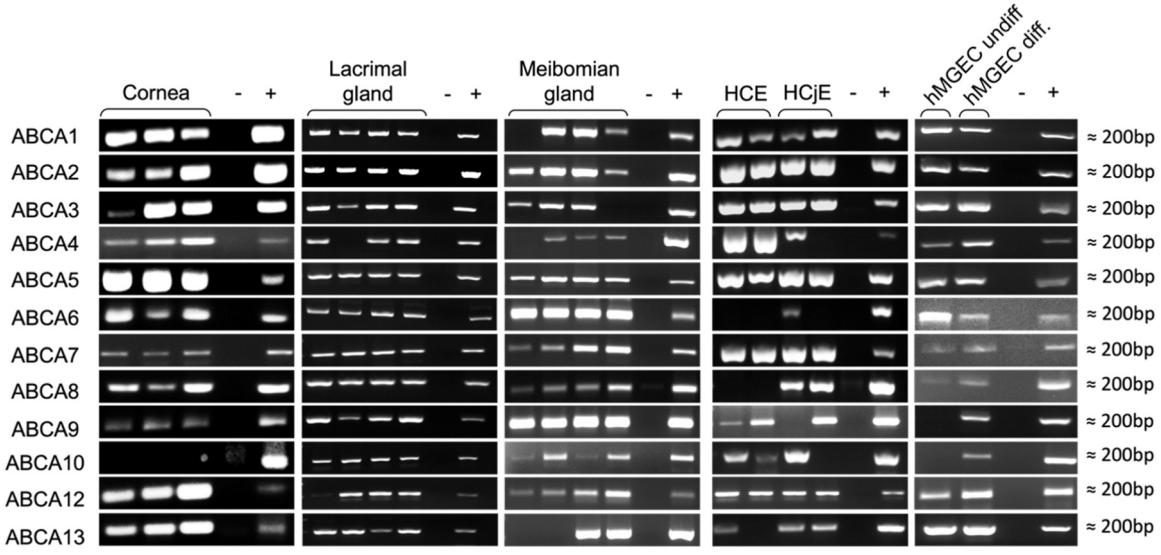



**Fig. 1.** Expression of the ABCA transporters in human tissues and cell lines by RT-PCR. The following samples were used: cornea (n=3), lacrimal gland (n=4), meibomian gland (n=4), human corneal epithelial cells (HCE, n=2), human conjunctival epithelial cells (HCjE, n=2) as well as human differentiated (n=1) and undifferentiated (n=1) meibomian gland cell line (hMGEC), negative control without cDNA, positive control with human liver cDNA or human brain cDNA. All samples are based on 200 bp.

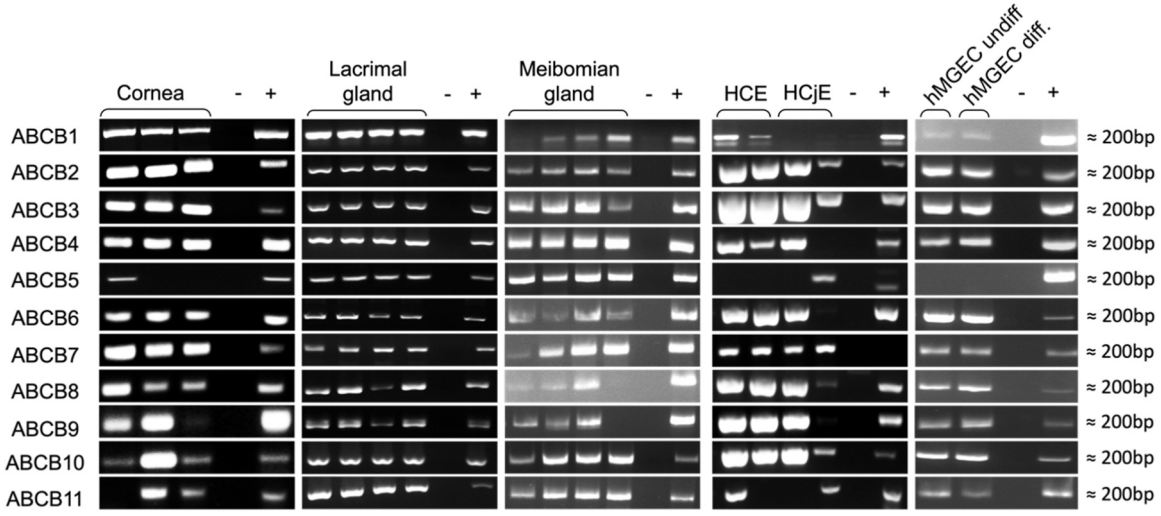



**Fig. 2.** Expression of the ABCB transporters in human tissues and cell lines by RT-PCR. The following samples were used: cornea (n=3), lacrimal gland (n=4), meibomian gland (n=4), human corneal epithelial cells (HCE, n=2), human conjunctival epithelial cells (HCjE, n=2) as well as human differentiated (n=1) and undifferentiated (n=1) meibomian gland cell line (hMGEC), negative control without cDNA, positive control with human liver cDNA for ABCB5, ABCB7, and ABCB11 and human brain cDNA for all other genes. All samples are based on 200 bp.

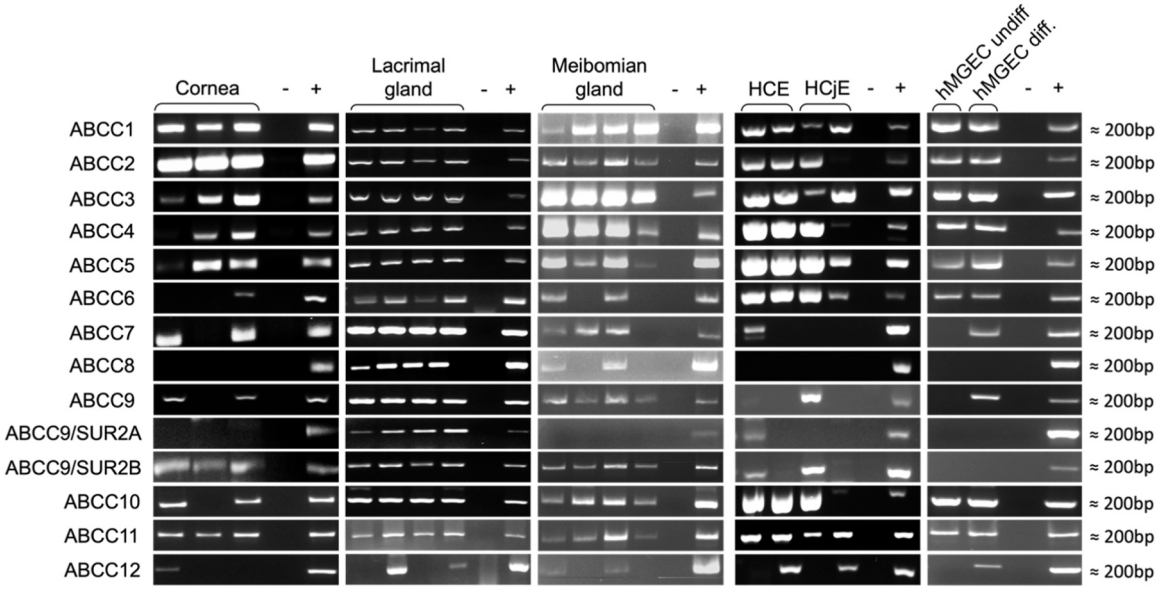



**Fig. 3.** Expression of the ABCC transporters in human tissues and cell lines by RT-PCR. The following samples were used: cornea (n=3), lacrimal gland (n=4), meibomian gland (n=4), human corneal epithelial cells (HCE, n=2), human conjunctival epithelial cells (HCjE, n=2) as well as human differentiated (n=1) and undifferentiated (n=1) meibomian gland cell line (hMGEC), negative control without cDNA, positive control with human liver cDNA for ABCC1, ABCC3, and ABCC7 or human brain cDNA for all other samples. All samples are based on 200 bp.

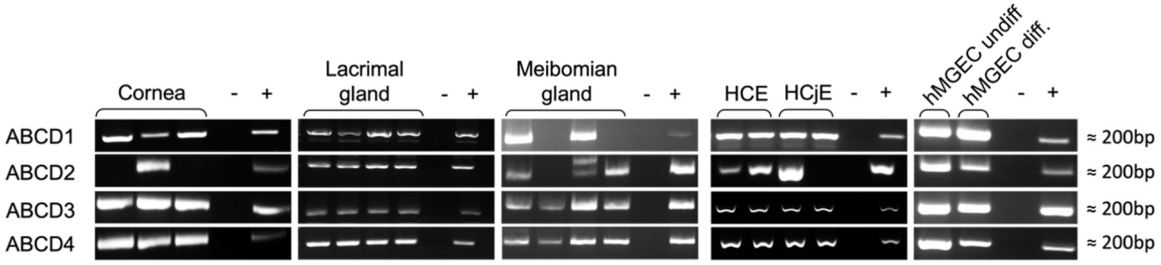



**Fig. 4.** Expression of the ABCD transporters in human tissues and cell lines by RT-PCR. The following samples were used: cornea (n=3), lacrimal gland (n=4), meibomian gland (n=4), human corneal epithelial cells (HCE, n=2), human conjunctival epithelial cells (HCjE, n=2) as well as human differentiated (n=1) and undifferentiated (n=1) meibomian gland cell line (hMGEC), negative control without cDNA, positive control with human liver cDNA for ABCD1 or human brain cDNA for all other genes. All samples are based on 200 bp.

revealed no expression in any of the investigations (Fig. 4). Expression of ß-actin showed a band with the expected size of 298 bp in all samples used. ABCC4 was not expressed in one cornea sample. In the lacrimal gland, all members of the ABCD family were identified. In the meibomian gland, ABCD1 was only detected in two out of four samples, ABCD2 in three of four samples. ABCD3 and ABCD4 were detectable in all samples. In HCE cells, all genes of the ABCD family were expressed. In the HCjE cells, ABCD2 could only be detected in one sample. In undifferentiated and in differentiated hMGEC cells, all members of the ABCD family were expressed.

### 3.5. Detection of the ABCE and ABCF family

The ABCE and -F families were expected to be 200 bp in size at the gene level, except ABCF1 with an expected size of 300 bp. All genes examined were visible as bands of 200 bp or 300 bp, respectively. The negative sample containing DEPC water instead of cDNA showed no detection in any of the investigations (Fig. 5). Expression of ß-actin demonstrated a band with the expected size of 298 bp in all samples used. ABCE1 and all members of the ABCF family were detected in the investigated specimens.

### 3.6. Detection of the ABCG family

The amplicon of the ABCG family was expected to be 200 bp in size at the gene level. All genes examined were visible as bands of 200 bp or 300 bp. The negative sample containing DEPC water instead of cDNA revealed no detection in any of the investigations (Fig. 6). Expression of ß-actin reveled a band with the expected size of 298 bp in all samples used. In the cornea, ABCG1 was only expressed in two specimens; ABCG4, ABCG5, and ABCG8 were not detectable. In the lacrimal gland, ABCG4 was expressed in three out of four samples; ABCG5 and ABCG8 were not found in the analyzed samples. In the meibomian gland, ABCG8 could also not be detected in any of the sample, whereas ABCG4 only was found in one sample. In cultured HCE cells, ABCG5 and ABCG8 were not expressed. In HCjE cells, ABCG1 was only expressed in one sample; ABCG5 and ABCG8 were not visible in the investigated samples. In the undifferentiated and differentiated hMGECs ABCG5 and ABCG8 were not expressed.

## 4. Discussion

The ATP-binding cassette transporters represent one of the largest transmembrane transporter subfamilies. They show a variety of functional processes, mechanisms of action and influencing factors in the human body. The primary active transporters are involved in the transportation of numerous molecules, immunological processes, detoxification mechanisms, and support the functioning of membrane channels (Seyffer and Tampe, 2015, Dean et al., 2001). The association of these transporters with genetic diseases and failures in therapies highlight the importance of ABC transporters in the human body, making it of utmost importance to take a closer look at the unexplored areas of these molecules. The known modes of function and areas of expression also suggest an important role in the ocular system of humans, and therefore scientific investigations in this regard contribute significantly to the understanding of ABC transporters.

The original aim of this work was to systematically analyze the expression of all ABC transporters on the ocular surface and in the lacrimal apparatus and to establish immortalized cell lines of this body area for research groups interested in this field. We are aware - and this is of course a limitation of this study - that the detection of an ABC transporter at the mRNA level does not of course imply its existence at the protein level, let alone a significant function of the corresponding transporters (Krawczenko et al., 2017). However, the determination of ABC transporters transcribed mRNA in tissues of the ocular surface and lacrimal apparatus shall serve as a first step for further investigations. On the one hand, it narrows down the search for functional ABC transports and, on the other hand, helps to further differentiate the often-overlapping functions of these transports.

### 4.1. ABC transporter in the human cornea

The cornea is from uttermost importance for the main function of the eye. It supports the vision through the high optical refraction and protects the interior of the eye against xenobiotics and microorganisms through its specialized layered structure. Using RT-PCR, the expression of 40 of the 48 ABC transporters in the human cornea was clearly detected at the mRNA level. Only the expression of the transporters ABCA10, ABCB5, ABCC6, ABCC8, ABCC9, ABCC12, ABCD2, ABCG4, ABCG5 and ABCG8 was not detectable or not detectable at all in the corneal samples (Figs. 1–5). By demonstrating the expression of all other ABC transporters in the cornea, it can be assumed that ABC transporters have an integral regulatory function for the cellular homeostasis, and also influence the translation and transcription (Kerr, 2004) in the cornea. This, in turn, could explain their constitutive expression in all tissues and cells without apparent specificity to a particular organ. Our findings are generally in line with other works about the expression of ABC transporters in the cornea, mainly of the ABCC family, with some differences to the work of Vellonen et al. The latter may also be due to the methods used, as Vellonen et al. employed absolute quantification on the used samples. Also, the sources of the investigated samples differ. The non-expressed transporter ABCA10 is thought to play a role in lipid homeostasis and transport in the immune system (in macrophages) and monocyte differentiation, respectively (Wenzel et al., 2003). This result was expected because the cornea lacks blood vessels and is immunologically privileged. ABCB5 has been frequently described in association with progenitor cells (stem cells), malignant melanomas, colorectal carcinomas, and liver carcinomas (Schatton et al., 2008, Frank and Frank, 2009, Ksander et al., 2014, Muriithi et al., 2020, Wilson et al., 2011, Setia et al., 2012). ABCB5 is also described as a mediator of

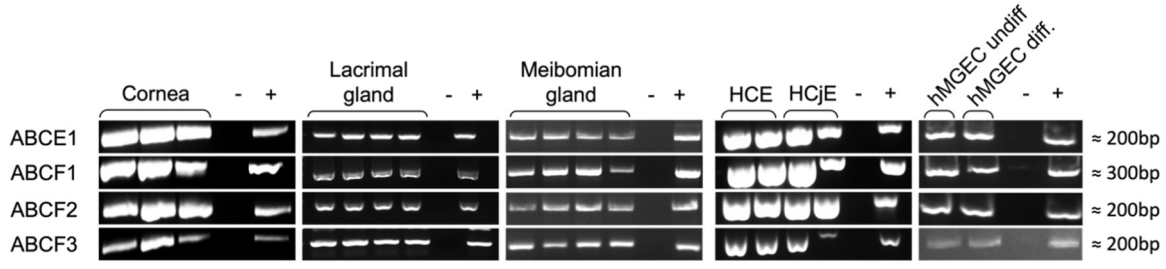



**Fig. 5.** Expression of the ABCE and F transporters in human tissues and cell lines by RT-PCR. The following samples were used: cornea (n=3), lacrimal gland (n=4), meibomian gland (n=4), human corneal epithelial cells (HCE, n=2), human conjunctival epithelial cells (HCjE, n=2) as well as human differentiated (n=1) and undifferentiated (n=1) meibomian gland cell line (hMGEC), negative control without cDNA, positive control with human liver cDNA for ABCF2 or human brain cDNA for all other genes. All samples are based on 200 bp or 300 bp.

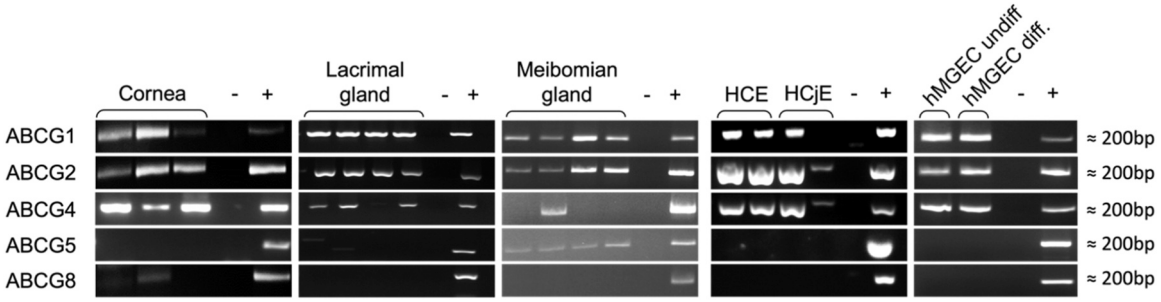



**Fig. 6.** Expression of the ABCG transporters in human tissues and cell lines by RT-PCR. The following samples were used: cornea (n=3), lacrimal gland (n=4), meibomian gland (n=4), human corneal epithelial cells (HCE, n=2), human conjunctival epithelial cells (HCjE, n=2) as well as human differentiated (n=1) and undifferentiated (n=1) meibomian gland cell line (hMGEC), negative control without cDNA, positive control with human liver cDNA for ABCG5 and ABCG8 or human brain cDNA for all other genes. All samples are localized at 200 bp.

chemoresistance by exporting drugs through the cell membrane (Frank et al., 2005). The detection in only one corneal sample suggests that corneal limbal stem cells were present in the sample examined, as corneal limbal stem cells express ABCB5. In this context ABCB5 is associated with corneal development and repair (Ksander et al., 2014, Gonzalez et al., 2018). ABCC6 is expressed mainly in the liver and the kidney (Jansen et al., 2014, Lau et al., 2014). A mutation in ABCC6 leads to pseudoxanthoma elasticum, a degenerative systemic disease of elastic tissues in the skin (Moitra et al., 2017). Also chronic kidney disease can lead to a deficiency of ABCC6 (Lau et al., 2014). In addition, a pathophysiological involvement of gene polymorphisms of ABCC6 in the development of atherosclerosis has been described (Dean et al., 2001). The detection in only one cornea sample suggests that due to the age of the body donors could be possible. Also, it is conceivable that elastic fibers, from the transition area to the sclera, mixed into the sample are the reason for the detection. ABCC8 is predominantly found in the pancreas, which promotes insulin release from beta cells (Busiah et al., 2014). Thus, it can be explained that no detection in the cornea was possible. ABCC9 is primarily expressed in skeletal and cardiac muscle, which is involved in the influx of potassium through specialized ion channels into the muscle (Bienengraeber et al., 2004, Aubert et al., 2019). A detection of ABCC9, more detailed, the detection of the splice variant ABCC9/SUR2A, but not the variant ABCC9/SUR2B in the corneal tissues is noteworthy. The formed potassium channels have distinct ligands, that differ from ABCC9/SUR2A and ABCC9/SUR2B (Davis-Taber et al., 2000). ABCC12 is one of the multidrug resistance proteins, but the functional properties of the translated protein MRP9 are currently unknown (Slot et al., 2011). However, it can be highly expressed in breast cancer (Bera et al., 2002), suggesting a role in tumor genesis. ABCD2 expresses transporters for long-chain fatty acids in peroxisomes. Since the cornea does not produce lipids, it can be assumed that ABCD2 cannot be detected in the cornea. ABCG4 is highly expressed in spleen, brain, eyes, and bone marrow (Dean et al., 2001). As in the brain, the detection in the eye refers to neuronal cells. ABCG4 is highly expressed in neurons in the human and mouse retina (Bojanic et al., 2010), which plays a role in cholesterol homeostasis (Koshiba et al., 2008). ABCG5 and ABCG8 play an essential role in transporting cholesterol and dietary sterols, especially in enterocytes and hepatocytes (Zein et al., 2019). Those transport functions have been investigated intensively; knockout mouse models show a protective effect against sterol accumulation in plasma and tissues. Those functions are crucial for the gastrointestinal tract. Therefore, the absence of ABCG5 and ABCG8 in the cornea can be explained (Kerr et al., 2021).

### 4.2. ABC transporter in the human lacrimal gland

The lacrimal gland supports the healthy ocular surface by providing major part of the water component as well as the mucous component of the tear film, which contains among others different antimicrobial molecules. Analysis of the lacrimal glands in our study showed the expression of 45 ABC transporters by RT-PCR. Only the transporters ABCC12, ABCG5 and ABCG8 could not be detected or could only be detected in some lacrimal gland samples (Figs. 1–5). By detecting all other ABC transporters in the lacrimal gland, it can be assumed that they are involved in any way in physiological secretory function (Uematsu et al., 2003, Lapczuk-Romanska et al., 2019) and could also influence the function of translation and transcription in addition to their transport function as in the cornea (Kerr, 2004). ABCC12's functional properties on the ocular surface are largely unknown, as described in the cornea section. Also, the ABCG5 and ABCG8 are essential for sterol transport in enterocytes and hepatocytes, so the absence in the lacrimal gland would be logical. The secretory function of the lacrimal gland can be described as diverse, secreting for example anti-microbial proteins like lactoferrin, lysozyme, and growth factors like transforming growth factor alpha or the epidermal growth factors, important for the wound healing ability at the ocular surface. The expression of mRNA of 45 ABC transporters in the lacrimal gland is also remarkable. Investigating the function of all these transporters in the lacrimal gland cells might be essential to understand further the lacrimal gland's function and their pathological relevance for the ocular surface and lacrimal apparatus.

### 4.3. ABC transporter in the human meibomian gland

The meibomian glands are situated in the upper and lower eyelids (Dietrich et al., 2021). Through lipid accumulation and consecutive apoptosis, they provide the lipid layer of the tear film. Through this layer, the evaporation of the tear film reduces, thus prolongs the guard function of the tear film for the ocular surface. Lately, also the secretion of proteins has been associated with the meibomian gland, making the investigation of trans-membranous transporters like the ABC transporters even more compelling (Green-Church et al., 2011). In the meibomian gland, 45 of the 48 analyzed ABC-transporters could be detected on the mRNA level. Only ABCC9/SUR2A, ABCG4, and ABCG8 were not expressed. Since all other tested transporters could be detected, it can be assumed that they play a physiological role in the human meibomian gland. Interestingly, ABCC9/SUR2A is a splice variant of the ABCC9 gene. The absence of the SUR2A but the presence of the SUR2B variant suggests a particular function of the ABCC9 gene in the meibomian gland. As mentioned, ABCC9 has been described as playing a role in the potassium influx into striated muscle tissue. Also, comparing the described corneal tissue, an expression of the SUR2B variant, and therefore the formed potassium channels, differ from their regulating ligands. ABCG4 and ABCG8 could also not be detected in the meibomian gland. As mentioned, ABCG4 is primarily involved in the transport of cholesterol. Interestingly, ABCG5 and ABCG8 are located closely on the second chromosome (Zein et al., 2019). It has been found that both genes have bidirectional promoters and similar regulatory elements. Also, in the protein stage, ABCG5 and ABCG8 are found together as

obligate heterodimers (Zein et al., 2019). The detection of ABCG8, but not ABCG5, indicates the possibility of an individual promoter or regulatory elements. As the meibomian gland accumulates a vast of lipids, for example cholesteryl esters, free cholesterols, and free fatty acids, it can be hypothesized, that might use the same transporters of such molecules like other tissues, for example the liver (Green-Church et al., 2011). Considering this, the absence of ABCG5 and ABCG4 are particularly remarkable.

### 4.4. ABC transporter in the human cell lines

Our PCR results show that the immortalized HCE, HCjE, and hMGEC cell lines also express ABC transporter and thus can serve as *in vitro* models for further ABC transporter studies. Immortalized epithelial cells differ in most cases from the vital primary cells, which represents a study limitation. This issue is known from numerous experiments and other cell lines (Verstraelen and Reichl, 2013, Endter et al., 2009, Dolberg and Reichl, 2016, Hilgendorf et al., 2007). Nevertheless, using these cells can provide first insights into the regulation and function of ABC transporters.

In contrast to the primary tissue, the cornea, the HCE cells do not express ABCA6, ABCA8, ABCC6, ABCC9, or ABCD2; however, ABCA10, ABCC6, and ABCC12 were detectable. Knowledge regarding ABCA6 is sparse. Distribution-wise, ABCA6 mRNA has been detected in the human liver, lung, and ovary (Kaminski et al., 2001). The function of ABCA6 is yet to be clarified; however, it has been linked to macrophage differentiation (Klucken et al., 2000). ABCA8 has a high degree of homology with ABCA6 and has been detected in human brain tissue, which also plays a role in cholesterol transport (Kaminski et al., 2001, Kim et al., 2008). The other ABC transporters, which have a different expression quality in HCE cells in contrast to their tissue of origin, have already been discussed.

In contrast to their original origin, the meibomian gland, the ABC transporter expression profile of the (un-) differentiated hMGEC cell lines differs. Specifically, ABCA8, ABCA9, ABCC7, ABCC8, ABCC9, ABCC9/SUR2B, ABCC12 or ABCG5 were not detectable in the undifferentiated hMGEC cells. There was no expression of ABCB5, ABCC8, ABCC9/SUR2B, ABCG5 in the differentiated hMGEC cells. In contrast, ABCG4 was expressed in the undifferentiated and differentiated hMGEC cells, but not in the tissue of the meibomian gland.

In summary, the differentiated hMGEC cells differ from the undifferentiated hMGEC cells, as ABCA9, ABCA10, ABCC7, ABCC9, and ABCC12 were only detectable in the differentiated cells. ACBA9 has been mainly detected in the human heart, brain, and liver tissues. Several correlations to neoplastic diseases have been described (Hwang et al., 2023, Chen et al., 2019), but the exact function is yet to be established. The difference in expression between the undifferentiated and the differentiated hMGEC cells is noteworthy. The function of ABCC7 and rather the translated protein, the cystic fibrosis transmembrane conductance regulator (CFTR), is crucial for the homeostasis of several body fluids. Best known are the disturbed alveolar or pancreatic fluids in patients with cystic fibrosis caused by mutations in ABCC7 (Riordan et al., 1989, Meng et al., 2018). The actual transport molecule of the CFTR protein, the chloride ions, change the viscosity of the fluids. Overall, the detection of ABCC7 after the differentiation of hMGEC cells seems logical, as the function of these cells is also individualized.

## 5. Limitation

In the present study, we systematically investigated the presence of the 48 ABC transporters known in humans in tissues of the ocular surface and lacrimal apparatus. However, our expression study has several limitations: The number and source (body donor tissue) of our analyzed samples could influence the results. The body donors are usually preselected by age, so physiologic changes due to age and undiagnosed pathologic processes may have influenced the outcome of the study. In addition, we only analyzed the samples at the mRNA level and did not examine the protein level. The mRNA-protein translation can be disturbed by several known effects, such as post-transcriptional gene silencing by siRNA or miRNA, which have been shown to influence the occurrence of functional ABC transporters. (Wang et al., 2021, Yang et al., 2015). Finally, we did not carry out any functional tests. Therefore, the discussion of the following results is merely hypothetical and can be seen as an outlook for further investigations without detracting from our results.

## 6. Conclusion

In summary, it can be said that numerous eye diseases are treated by the topical application of drugs. The large group of ABC transmembrane proteins is responsible for the successful transport of substrates (drugs, lipids and proteins) across the cell membrane. The expression and physiological significance of most ABC transporters in the ocular system are unknown in detail. We show here that ABC transporters are expressed to a relevant extent at the ocular surface and in the lacrimal apparatus, and thus may play a central role in pathophysiological conditions such as glaucoma, dry eye disease or retinal diseases. The characterization of all transporters under pathophysiological aspects is particularly important for pharmacological questions, as they can transport endogenous and exogenous substances (xenobiotics) out of the cell. Loss, altered expression and/or activities of individual transporters most likely contribute to an altered uptake and distribution of drugs, influence tear production and pain sensation and are therefore crucial for the efficacy of drug treatment.

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## CRediT authorship contribution statement

**Zeynep Kutlu:** Writing – review & editing, Investigation. **Barbara Kral:** Writing – review & editing, Investigation. **Ingrid Zahn:** Writing – review & editing, Validation, Methodology. **Martin Schicht:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Friedrich Paulsen:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Afsun Sahin:** Writing – review & editing. **Maximilian Musialik:** Writing – review & editing. **Fabian Garreis:** Writing – review & editing, Validation, Resources, Methodology. **Benedikt Kleinsasser:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Benedikt Kleinsasser reports financial support was provided by Sicca Forschungsförderung of the professional Association of German Ophthalmologists. Martin Schicht reports financial support was provided by Sicca Forschungsförderung of the professional Association of German Ophthalmologists. Martin Schicht reports financial support was provided by Ernst und Berta Grimmke Stiftung. Martin Schicht reports financial support was provided by Ernst Muck Stiftung. Friedrich Paulsen reports financial support was provided by German Research Foundation. Friedrich Paulsen is Editor-in-Chief of the Annals of Anatomy If

there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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### *Institutional Review Board Statement*

This study was conducted in compliance with institutional review board regulations, informed consent regulations, and the provisions of the Declaration of Helsinki and its later amendments.

### *Informed Consent Statement*

Written informed consent was obtained from each patient concerning diagnostic procedures, treatment, and the subsequent use of these specimens for research purposes, including data analysis, prior to inclusion in this study.

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