Tear Fluid Inflammatory Proteome Analysis Highlights Similarities Between Keratoconus and Allergic Conjunctivitis

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Purpose. Keratoconus is characterized by the progressive thinning of the cornea, which leads to a cone-like appearance of the eye over time. Although conventionally defined as a noninflammatory condition, a number of recent studies have associated keratoconus (KC) with allergic conjunctivitis (AC) based on clinical parameters. This study aimed to consolidate this association by performing a proteomic analysis of tear fluid from patients with keratoconus and/or allergic conjunctivitis.

Methods. Of 51 patients, 17 were diagnosed with KC, 17 were diagnosed with AC, and 17 were diagnosed with both KC and AC (combined). Nine of 34 patients with KC had a progressive form of the disease. Tear fluid samples ($n = 51$, one eye per patient) were collected by the Schirmer's strips. Tear proteins were extracted from the Schirmer's strips. Proteomic profiling of 384 inflammatory proteins was assessed by a multiplex proximity extension assay (Olink Explore 384 Inflammation Panel I).

Results. A total of 384 inflammatory proteins were measured. Two hundred seventy-two of the 384 proteins passed stringent data cleaning and were compared among the patient groups. Compared to the 2 other groups, LGALS9 was upregulated uniquely in KC, whereas FGF19, PDGFB, HPCAL1, OSM, and FCAR were downregulated in KC. Similarly, TNFRSF4 and CCL13 were specifically upregulated in AC, whereas ectodysplasin A receptor (EDAR) was uniquely downregulated in AC.

Conclusions. High-throughput proteomic profiling of tear fluid confirms the association between KC and AC on a molecular level and raise the importance of redefining KC as an inflammatory condition.

Keywords: keratoconus (KC), allergic conjunctivitis (AC), tear biomarkers, proteomics

Keratoconus (KC) is a bilateral, progressive condition associated with corneal ectasia. It is characterized by asymmetric thinning of the central or paracentral cornea, leading to cone-shaped corneal protrusion.[1] As a consequence, patients often suffer from distorted vision and astigmatism.[2] The disease onsets during puberty and develops progressively throughout the second and third decades of life. Globally, it has been estimated that the incidence of KC is between 1.5 and 25 individuals per 100,000, whereas the prevalence is between 0.2 and 4790 individuals per 100,000 per annum.[3] The cause and underlying pathological mechanism are unknown, but both environmental and genetic factors are thought to contribute to the development of the disease. Frequent associations include history of allergies, atopy (asthma, hay fever, and eczema), eye rubbing, eye injuries, rigid or hard contact lens wear, and family history of KC.[4]

Eye rubbing and associated atopic risk factors for KC have received considerable attention in recent years, with 4 systematic reviews and meta-analyses published on this topic since 2020.[5] Seth et al. observed significant associations among KC and eye rubbing, family history, and allergy, but not with allergic eye disease, atopy, asthma, and allergic rhinitis.[6] Lo et al. found significant associations among KC and eye rubbing, allergic diseases, atopic diseases, asthma, atopic dermatitis/eczema, and allergic eye diseases, but not between KC and allergic rhinitis.[7] Hashemi et al. showed that eye rubbing, family history of KC, allergy, asthma, and eczema were the most important risk factors for KC.[8] Sahebjada et al. identified a strong association between eye rubbing and KC.[9] Eye rubbing seems to be the most consistent risk factor for KC, whereas more controversial results exist for other risk factors. The exact mechanism of how eye rubbing is associated with KC is still not clear. Thus,

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there remains a need for further elucidation of the molecular mechanisms of the relationship of the eye rubbing with KC.

Ocular itching is the pathognomonic symptom of allergic conjunctivitis (AC), one of the most common allergic conditions worldwide.[10] Although the prevalence of AC in patients with KC,[6–9,11] the effect of AC on corneal biomechanics,[12,13] as well as the effect of AC on KC severity[14,15] have been investigated extensively, a comparative analysis of tear fluid factors between AC and KC has never been performed before, to our knowledge. Studies comparing patients with KC, or AC, to healthy controls (HCs) have identified a number of notable protein biomarkers present in both conditions. Previous studies that examined the tear proteome in patients with KC as compared to controls found an imbalance and induction of inflammatory cytokines, proteases, free radicals, and oxidants, including IL-6 and MMP9.[16–20] Papers investigating tear fluid of patients with AC and HCs found elevated levels IgA, MMP9, and a number of cytokines (IL-6, IL-9, IL-10, IL-1beta, TNFalpha, etc.).[21–23] The aim of this paper was to search for similarities and differences in the underlying inflammatory markers between KC and AC.

Targeted tear fluid analysis is often limited to one or two multiplex immunoassays, due to its small sample volume. Recent advances in multiplex technologies make it possible to simultaneously measure a large number of proteins. The proximity extension assay (PEA) uses two oligonucleotide-coupled antibodies that allow hybridization of the oligonucleotides upon binding to the target protein in close proximity. Next, quantitative polymerase chain reaction (qPCR) enables amplification and quantification of the signal. The results are given as normalized expression units (NPX) and can provide the relative quantification of proteins. The advantages of the PEA technology, including simultaneously measurement of a large number of proteins in a low volume of samples, has been used for many body fluids. For tear fluid, PEA has been investigated only a few times so far in the context of wound healing after glaucoma surgery[24] and for method optimization[25] but not yet for KC nor AC.

To date, many studies assessed the association of AC with KC, however in-depth studies at the protein level are still lacking. Hence, this study endeavored to elucidate differences and similarities in tear fluid inflammatory protein profiles of patients with KC or AC, and to identify differentially expressed tear fluid proteins between patients with KC with and without AC using high-throughput PEA.

Methods

This study was approved by the Maastricht University Medical Ethical Review Board (ID number 2023-3624) in accordance with the tenets of the Declaration of Helsinki. Written informed consent was obtained by each person before performing the study visit and related procedures. All subjects were recruited at the University Eye Clinic Maastricht, Maastricht University Medical Center, The Netherlands.

Subjects

A total of 51 patients were grouped in one of three categories: the KC group = subjects with a diagnosis of keratoconus without signs of allergic conjunctivitis (n = 17); the KC + AC group = subjects with a diagnosis of keratoconus with signs of allergic conjunctivitis (n = 17); and the AC group = subjects with a diagnosis of allergic conjunctivitis without keratoconus (n = 17). Diagnosis of each condition was established according to the patient's history and the clinical signs and symptoms. Subjects were excluded if they were under 16 years of age, having vernal keratoconjunctivitis, a bacterial, or viral infection, or if there was a history of cross-linking, ocular surgery, or autoimmune disease. Keratoconus was defined as the presence of corneal irregularity by Scheimpflug Tomography (Belin Pentacam, Oculus, Lynnwood, WA, USA). Based on keratometric readings, patients were classified as mild (steep keratometry [K] < 45 diopters [D]), moderate (steep K between 45 D and 52 D), or severe (steep K > 52 D).[26,27] KC was considered progressive if there was an increase in the maximum corneal curvature (K_{max}) by ≥1.00 D for over 1 year. AC was defined as being in an active disease phase with presence of one or more of the following signs and symptoms (self-reported and/or by clinical examination): eye rubbing, ocular itch, tearing, mucus, swelling of eyelids, chemosis, hyperemia, or conjunctival presence of papillae.

Tear Fluid Sampling

Schirmer's strips from TrueBlue Optics (Contacare Ophthalmics and Diagnostics, Gujarat, India) were used throughout the study. Tear fluid was collected from the left and right eye without topical anesthesia (Fig. 1). Care was taken not to touch the strip with the fingertips. The migration length was read after 5 minutes from the preprinted scale bar on the strips. In case of completely wetted strips (35 mm), the strips were removed in <5 minutes. Immediately after sampling, samples were stored at −80°C. One tear sample (left or right eye) per patient was randomly selected for subsequent analysis.

Protein Extraction

Tear fluid was extracted from the Schirmer's strip by agitating small cut pieces of the entire strips in 120 µL phosphate-buffer saline (PBS, pH 7.4), and cOmplete Protease Inhibitor Cocktail (Roche, Basel, Switzerland) at 4°C for 1.5 hours[28] (Fig. 2). Tear fluid was then eluted by centrifugation ("piggy-back method") and stored at −80°C until further use. Total tear protein content was measured by the bicinchoninic acid (BCA, Pierce, ThermoFisher) according to the manufacturer's protocol. Proteins concentrations were normalized to the original migration length and elution buffer volume.

Protein Analysis by PEA

Tear fluid levels of 384 inflammatory proteins (Supplementary Table S1) were measured using the Olink Explore 384 Inflammation Panel I (Olink Proteomics, Uppsala, Sweden, www.olink.com) that uses a PEA technique. Briefly, 1 µL of extracted tear fluid was incubated with pairs of antibodies coupled to cDNA strands (Fig. 3). Upon binding of the target protein by the antibody pair, the close proximity of the antibodies allows dimerization of the complementary DNA strands and amplification by polymerase chain reaction (PCR). The final protein concentration output from these assays is reported in NPX values. The NPX is an arbitrary unit on a log base two scale wherein higher NPX values indicate higher protein concentrations. For example, a one-

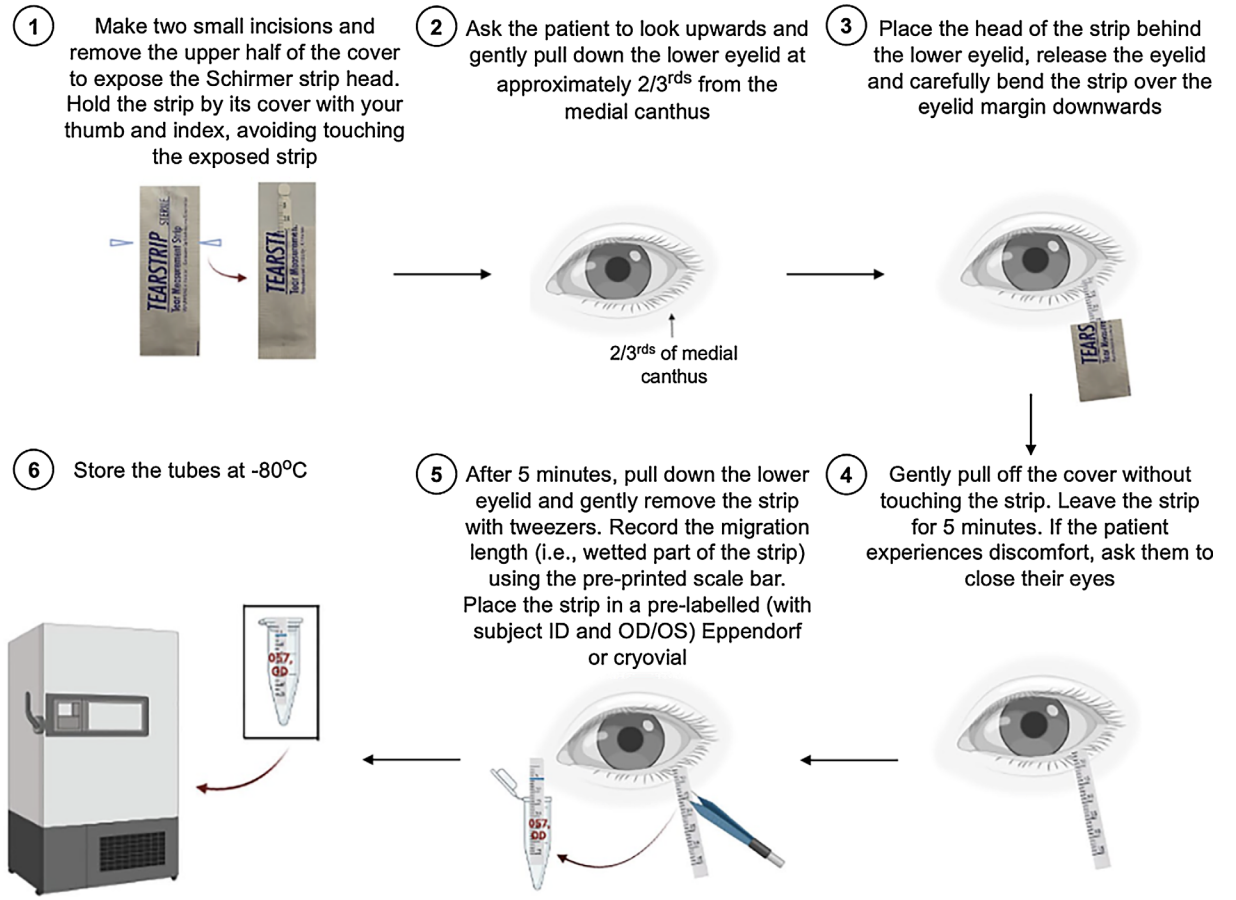

Figure 1. Tear fluid collection. Created in BioRender.com.

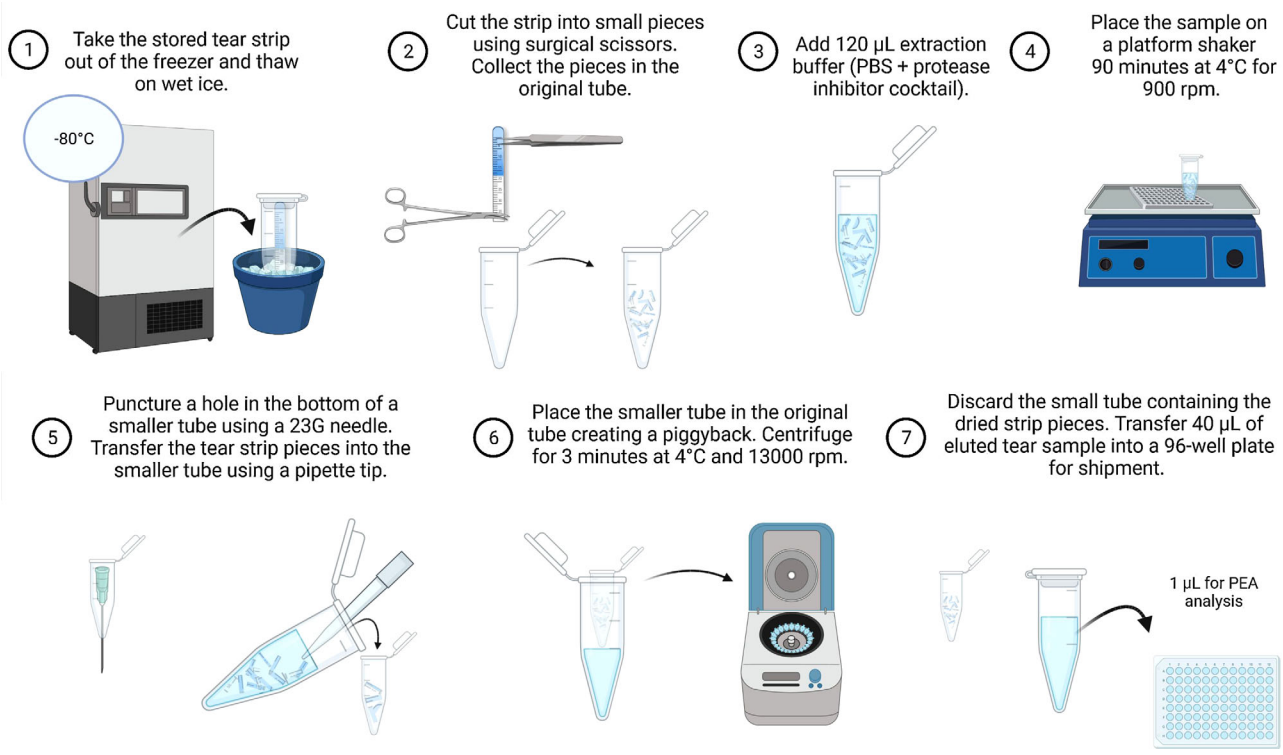

Figure 2. Tear fluid extraction. Created in BioRender.com.

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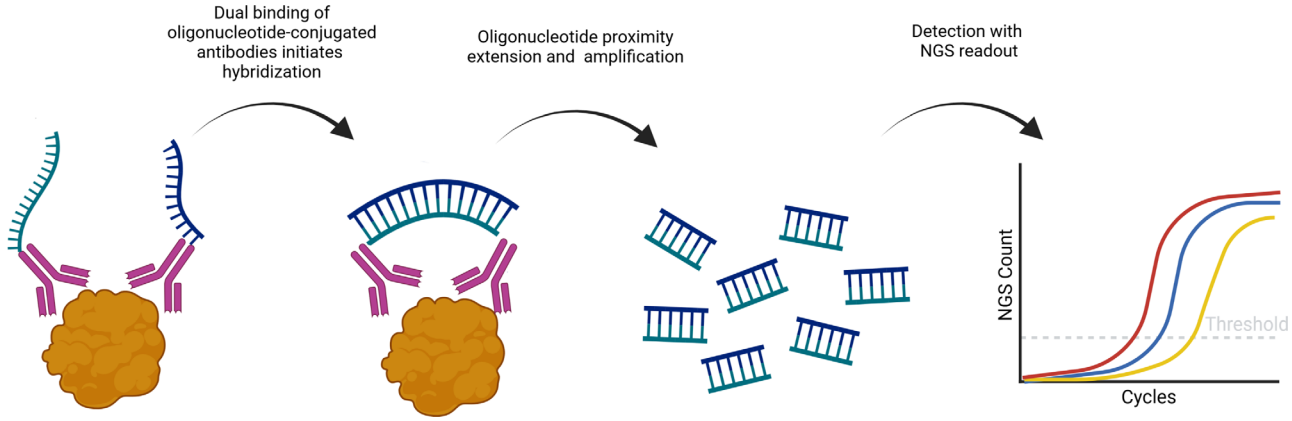

Figure 3. Proximity extension assay (PEA). Created in BioRender.com (adapted from Bodén et al.[29]).

point difference in an NPX value is equivalent to a twofold change in protein concentration.

Data Cleaning

In accordance with the manufacturer's recommendations, proteins with NPX values below the limit of detection (LOD) and those that did not pass the manufacturer's quality control test were omitted from further data analysis. In addition, we set a detection limit of 50%, meaning that proteins detected in <50% of the tear fluid samples were excluded and referred to as undetectable. In other words, any proteins which were not detected in at least 50% ($n = 26$) of all 51 samples and at least 50% ($n = 9$) of one or more diagnostic group's 17 samples were excluded.

Statistical Analysis

Statistical analysis was performed in SPSS Statistics 25 for Windows (SPSS, Chicago, IL, USA). GraphPad Prism version 9.5.1 (GraphPad Software, San Diego, CA, USA) was used for data visualization. Fisher's exact test was used to assess differences in categorical variables. Differences in protein expression levels between diagnostic groups were tested by multivariable linear regression adjusted for age and sex. The level of statistical significance was set at $P < 0.05$. Volcano plots were generated where the x-axis represented the Log2 (fold change) and the y-axis the −log10 (P value). The Log2 (fold change) values were determined per protein by subtracting the mean NPX value of a specific protein in one group from that in another group (i.e. mean NPX of protein X in KC − mean NPX of protein X in AC, where X represents any one of the 384 proteins, for AC versus KC). A Log2 (fold change) value of +1 indicates a doubling of the relative protein concentration, whereas a value of −1 indicates a halving of the relative protein concentration. The threshold of significance was set at a −log10 (P value) of 1.3, which is equivalent to a P value of 0.05.

Bioinformatics Analysis

Information about the subcellular location of proteins was derived from the UniProt database (www.uniprot.org). The STRING functional protein associations network program was used to create a protein-protein interaction map of identified differentially expressed proteins. Gene ontology (GO) term enrichment analysis was performed using the function enrichment interaction analysis (FunRich) tool to obtain information about the biological processes in which any significant proteins were involved in.

Results

Patient Characteristics

Table 1 summarizes the patient characteristics per diagnostic group. The average age of patients with KC was 36.9 ± 13.5 years, whereas it was 29.1 ± 8.8 years for the KC + AC group, and 35.5 ± 14.9 years for the AC group ($P = 0.174$). The KC and KC + AC groups were 29% female patients, whereas the AC group was 76% female patients ($P = 0.007$). No significant differences were observed for K_{mean}, K_{max}, or K_{steep} values between patients with KC and patients with KC + AC. Nine of the 34 patients with KC had a progressive form of the disease, whereas the remainder ($n = 25$) were stable. AC was seen in most (7/9, 78%) patients with progressive KC but only in 40% of stable patients ($P = 0.118$). There were no differences in tear migration length or total tear protein content across the three diagnostics groups.

Differential Protein Detectability

Specific proteins that are more detectable in one diagnostic group compared to another group may bear fundamental information about the disease. To search for group differences in protein detectability (prior to further data cleaning based on 50% detectability), we analyzed the proteins that had a higher detectability (minimally 2-fold) in the tear samples in one group compared to another (Table 2). Nine proteins were predominantly present in patients with KC, 7 proteins in patients with AC, 5 proteins in patients with KC + AC, and 2 proteins in patients with AC and KC + AC. The largest differences between the two groups were 7-fold for MLN and PTH1R, 5-fold for IFNG and TPSAB1, and 4-fold for IL17F, ITGA6, and ISM1.

Proteins that were undetectable in all tear samples of one group are listed in Table 3. Only two proteins were undetectable in all samples from patients with KC, whereas detectable in samples from patients with AC and KC + AC. Five proteins were undetectable in patients with AC samples and 6 proteins in patients with KC + AC samples. No proteins

Table 1. Patient Characteristics Per Diagnostic Group

	KC (n = 17)	KC + AC (n = 17)	AC (n = 17)
Demographics			
Age (mean ± SD) y	36.9 ± 13.5	29.1 ± 8.8	35.5 ± 14.9
Sex (F/M)	5/12	5/12	13/4
KC parameters			
K_{mean} (mean ± SD)	45.4 ± 2.8	46.1 ± 4.4	
K_{max} (mean ± SD)	50.4 ± 4.8	53.2 ± 7.7	
K_{steep} (mean ± SD)	46.7 ± 3.1	47.8 ± 5.3	
KC severity (mild/moderate/severe)	6/10/1	4/11/2	
KC progression (stable/progressive)	15/2	10/7	
Tear fluid parameters			
Eye used for protein analysis (OD/OS)	11/6	10/7	8/9
Tear migration length (mean ± SD) (mm)	20.9 ± 13	24.9 ± 12.8	25.1 ± 10.5
Tear total protein content (µg/mL, mean ± SD)	1218.9 ± 570.5	1438.9 ± 1051.8	1369.9 ± 919.7

Data are expressed as mean ± SD or number (n/n). Based on keratometric readings, patients were classified as mild (steep keratometry [K] < 45 diopters [D]), moderate (steep K between 45 D and 52 D), or severe (steep K > 52 D).

Table 2. List of Proteins That Were Detectable in More (Minimal 2-Fold) Tear Samples in One Patient Group Over Another

	Acronym	Protein	AC	KC	KC + AC
Predominantly present in AC	CCL25	Chemokine (C-C motif) ligand 25	11	4	8
	CCL7	C-C motif chemokine 7	7	2	5
	CNTNAP2	Contactin-associated protein-like 2	9	3	4
	IFNG	Interferon gamma	5	1	3
	IL17F	Interleukin-17F	4	2	1
	IL5RA	Interleukin-5 receptor subunit alpha	13	6	9
	TNFRSF13C	Tumor necrosis factor receptor superfamily member 13C	6	4	3
Predominantly present in KC	FCRL6	Fc receptor-like protein	5	6	2
	L15RA	Interleukin-15 receptor subunit alpha	2	6	4
	ITGA6	Integrin alpha-6	2	4	1
	MLN	Promotilin	1	7	4
	NCR1	Natural cytotoxicity triggering receptor 1	4	6	2
	PNLIPRP2	Pancreatic lipase-related protein 2	9	16	13
	PRELP	Prolargin	6	11	9
	SCG3	Secretogranin-3	9	16	13
	SULT2A1	Bile salt sulfotransferase	4	10	8
Predominantly present in KC + AC	IL33	Interleukin-33	5	9	10
	ISM1	Isthmin-1	3	1	4
	PTH1R	Parathyroid hormone/parathyroid hormone-related peptide receptor	1	3	7
	PTPRM	Receptor-type tyrosine-protein phosphatase mu	6	11	12
	TNR	Tenascin-R	7	4	9
	TPSAB1	Tryptase alpha/beta-1	3	1	5
Predominantly present in AC and KC + AC	IL11	Interleukin-11	8	3	8
	SCGN	Secretagogin	4	2	4

The numbers indicate number of patient samples with protein levels >LOD. Proteins are listed in alphabetical order.

Table 3. List of Proteins That Were Undetectable in all Tear Samples of a Diagnostic Group

	Acronym	Protein	AC	KC	KC + AC
Undetectable in AC	CCL11	Eotaxin	0	2	4
	FCRL3	Fc receptor-like protein 3	0	2	3
	IL2	Interleukin-2	0	1	5
	IL24	Interleukin-24	0	2	1
	IL2RB	Interleukin-2 receptor subunit beta	0	1	1
Undetectable in KC	IL22RA1	Interleukin-22 receptor subunit alpha-1	2	0	2
	WNT9A	Protein Wnt-9a	1	0	1
Undetectable in KC + AC	CD200	OX-2 membrane glycoprotein	3	1	0
	DGKZ	Diacylglycerol kinase zeta	5	2	0
	DPP10	Inactive dipeptidyl peptidase 10	3	2	0
	EPO	Erythropoietin	4	3	0
	ITGA11	Integrin alpha-11	2	3	0
	MEPE	Matrix extracellular phosphoglycoprotein	1	2	0

Numbers indicate number of patient samples with protein levels >LOD. Proteins are listed in alphabetical order.

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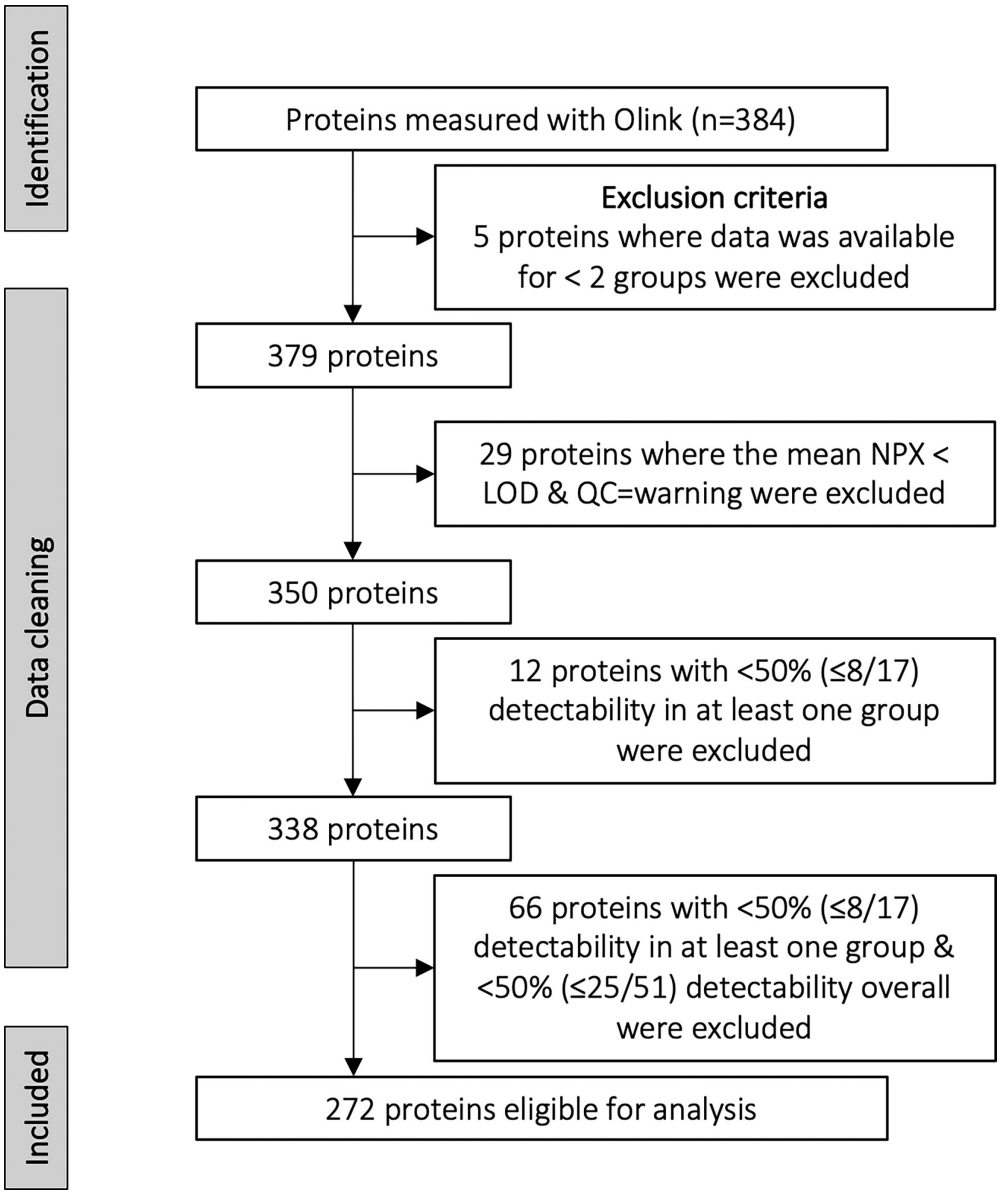

Figure 4. Stringent data cleaning steps were applied and removed a total of 112 proteins. The resulting 272 proteins were found eligible for subsequent statistical data analysis.

were undetectable in all patient samples from two groups at the same time.

Overall Protein Detectability

A total of 384 proteins were measured. Five proteins were undetectable in >2 groups and therefore excluded (Fig. 4). Twenty-nine proteins had NPX values below the LOD or did not pass quality control (QC). Twelve proteins were not detectable in 50% of the samples per diagnostic group (≤8/17 samples) in at least one group. Sixty-six proteins were not detectable in the majority of all samples (≤25/51 samples). After these 4 stringent data cleaning steps, 272 were found "detectable" and used for further analysis.

Differentially Expressed Proteins

Multiple linear regression adjusted for age and sex was performed on the 272 detectable proteins, where the majority (243/272, 81%) were similar and 11% (29/272) were found to be significantly differentially expressed between diagnostic groups (Fig. 5). This included 15 proteins when comparing patients with AC and patients with KC, most of which (11/15, 73%) were downregulated in KC (Fig. 6A). Similarly, 14 proteins were observed when comparing patients with AC and patients with KC + AC, with the majority (12/14, 85%) downregulated in patients with KC + AC (Fig. 6B). Nine significantly differentially expressed proteins were observed when comparing patients with KC and patients with KC + AC with comparable upregulation patterns (Fig. 6C).

LGALS9 was upregulated in patients with KC and patients with KC + AC, but not patients with AC, indicating its uniqueness to KC. Similarly, CCL13 and TNFRSF4 were expressed in both patients with AC and patients with KC + AC, but not patients with KC, likely indicating its specificity to AC. ectodysplasin A receptor (EDAR) was downregulated in the patients with AC and patients with KC + AC, but not in

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	ADA	AGER	ALDH3A1	CCL13	CD79B	CDON	CSF3	CXCL6	EDAR	FABP1	FCAR	FGF19	GMPR	HCLS1	HPCAL1	IL32	JUN	KLRB1	KLRD1	LGALS9	LY9	OSCAR	OSM	PCDH1	PDGFB	PRSS8	SLAMF7	TNFRSF4	VEGFA
AC versus KC	*			*					*	*	*	*	*		*					*		*	*	*	*			**	*
AC versus KC+AC					**	*	*	**			*	*			*	**	*		**	*			*		**	*			
KC versus KC+AC		*	*	*					*					*				*			*						*	*	

Figure 5. Differently expressed proteins amongst diagnostic groups. Proteins that were significantly differentially expressed in more than one comparison are highlighted in *green* (AC versus KC and KC versus KC + AC) or *orange* (AC versus KC and AC versus KC + AC). $^{*}P < 0.05$, $^{**}P < 0.01$.

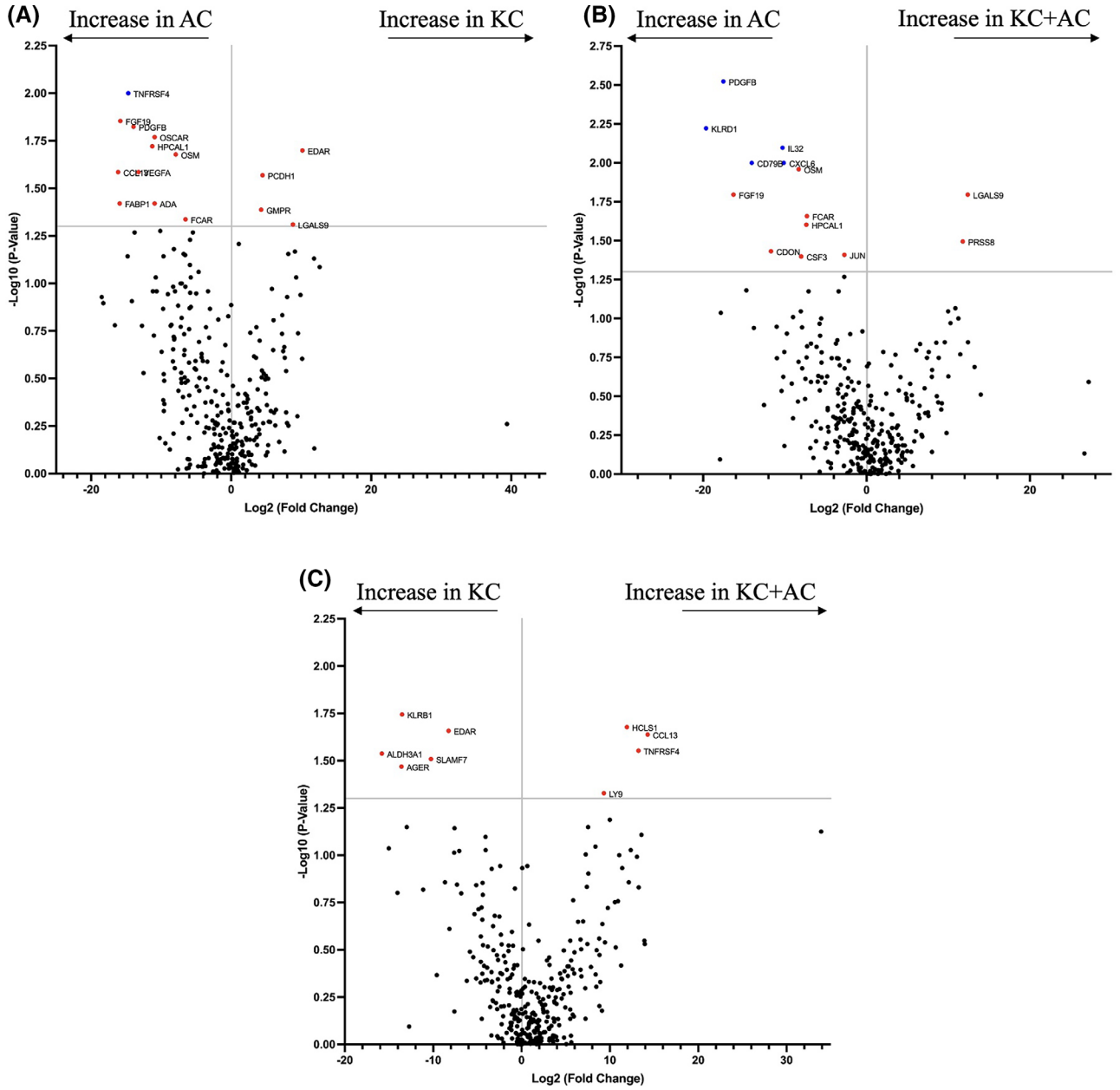

Figure 6. Volcano plots of all "detectable" proteins ($n = 272$) significantly differentially expressed among (**A**) AC versus KC, (**B**) AC versus KC + AC, and (**C**) KC versus KC + AC. The analysis was performed using multiple linear regression adjusted for age and sex. Fold change (displayed on the x-axis) was calculated as the mean Log2 NPX values differences between groups. A Log2 (fold change) value of +1 indicates a doubling of the relative protein concentration (upregulation), whereas a value of −1 indicates a halving of the relative protein concentration (downregulation). Proteins where $P < 0.05$ (−Log10 (P value) > 1.3) and $P < 0.01$ (−Log10 (P value) > 2.0) are indicated with *red* and bIlue dots, respectively.

Table 4. The Subcellular Location and Type of the Proteins Which are Uniquely Differentially Expressed in KC or AC

	Acronym	Protein	Subcellular Location	Type of Protein
Unique to KC	**LGALS9**	**Galectin-9**	Cytoplasm and nucleus	Intracellular and secretory
	FGF19	Fibroblast growth factor 19	N/A	Secretory
	PDGFB	Platelet-derived growth factor B	N/A	Secretory
	HPCAL1	Hippocalcin like 1	Membrane*	Transmembrane
	OSM	Oncostatin M	N/A	Secretory
	FCAR	Fc fragment of IgA receptor	Plasma membrane	Transmembrane
Unique to AC	**CCL13**	**Chemokine (C-C motif) ligand 13**	N/A	Secretory
	TNFRSF4	**Tumor necrosis factor receptor superfamily member 4**	Plasma membrane	Transmembrane
	EDAR	Ectodysplasin A receptor	Plasma membrane	Transmembrane

The upper, bold proteins are the upregulated proteins. N/A = not applicable due to the protein being secreted.
* Which exact membrane(s) is to be determined.

the patients with KC. This suggests EDAR is uniquely downregulated in AC. FGF19, PDGFB, HPCAL1, OSM, and FCAR were downregulated in patients with KC and patients with KC + AC, but not patients with AC. This suggests that these five proteins are downregulated specifically in KC. In summary, 3 of the 29 differentially expressed proteins were likely specific to AC and 6 to KC. The NPX values (mean with standard deviation) and the protein detectability per diagnostic group for these proteins can be found in Supplementary Table S2.

Of the six proteins unique to KC, four of them are secreted by the cell and thus do not have a defined subcellular location (Table 4). LGALS9, one of these secretory proteins, can also be localized intracellular, where it is then found either within the nucleus or the cytoplasm. The remaining two proteins, HPCAL1 and FCAR, are membrane-spanning proteins. Two of the proteins unique to AC (TNFRF4 and EDAR) are also membrane-spanning, including one of the upregulated proteins. The remaining upregulated protein (CCL13) is secreted by the cell, and thus not bound to a subcellular location.

String Analysis

The STRING protein interaction analysis of the differentially expressed proteins among diagnostic groups is shown

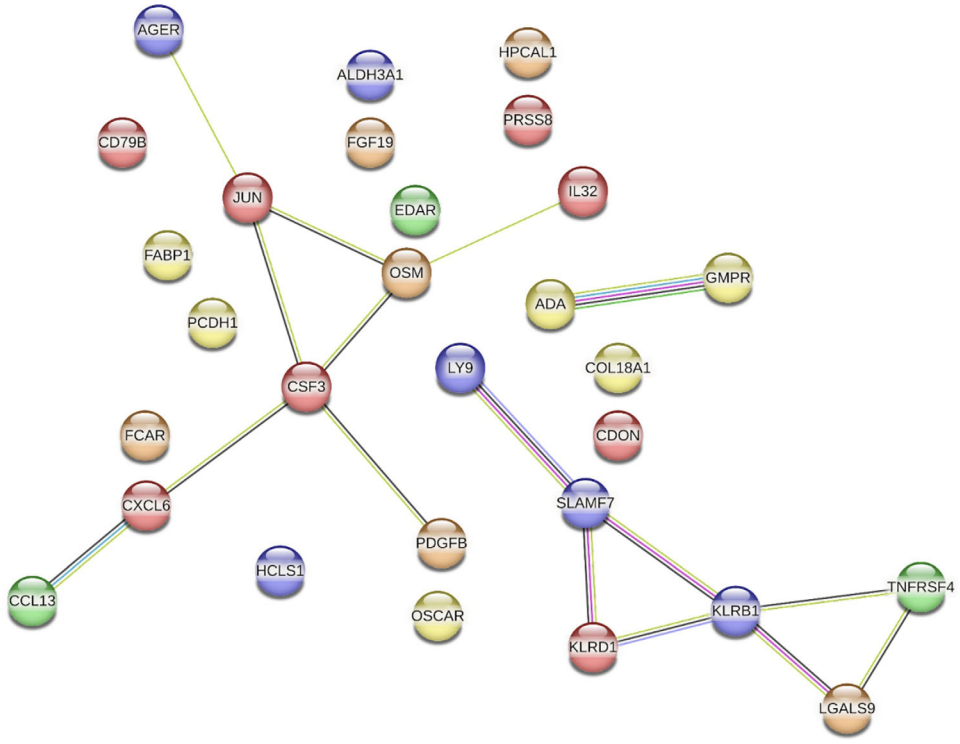

Figure 7. The combined STRING Interaction Network of the detectable proteins that were differentially expressed in AC versus KC (*yellow* nodes), AC versus KC + AC (*red* nodes), and KC versus KC + AC (*blue* nodes). The *orange* nodes represent proteins which are differentially expressed in AC versus KC and AC versus KC + AC, whereas the *green* nodes represent proteins which are differentially expressed in AC versus KC and KC versus KC + AC. Each colored line connecting the nodes indicates the type of interaction. (Teal and magenta lines indicate known interactions determined from databases or experiments, respectively. *Green*, *red*, and *dark blue* lines indicate predicted interactions based on gene neighborhoods, fusions, or co-occurrences, respectively. Other types of interactions are based on text mining, co-expression, or protein homology, displayed by *yellow-green*, *black*, and *indigo* lines, respectively.)

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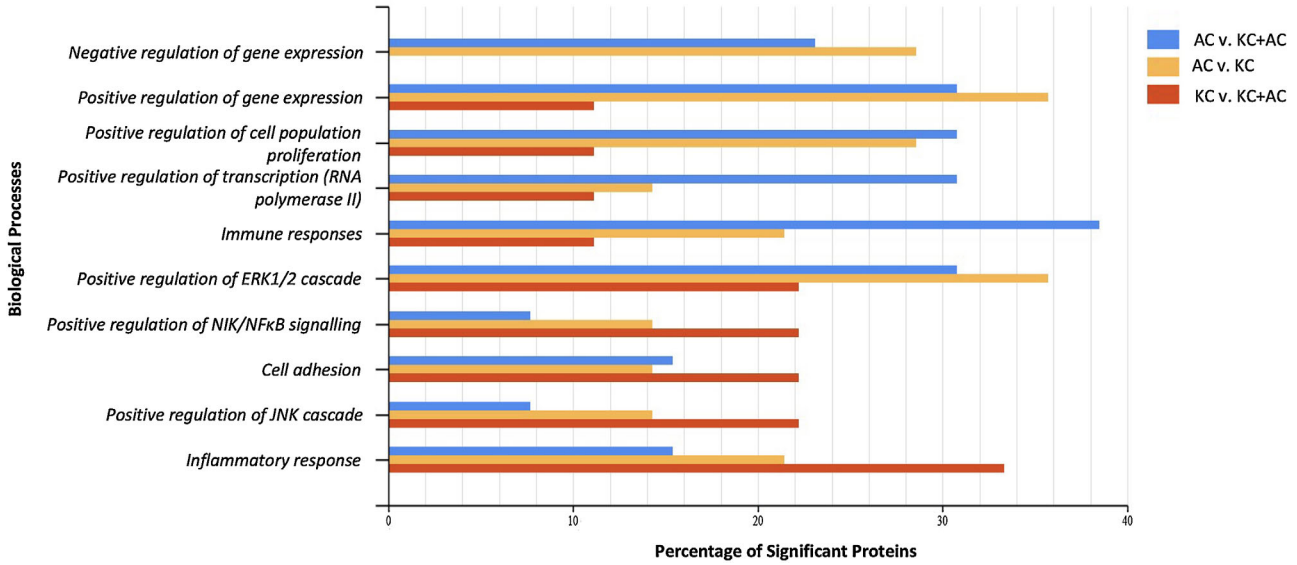

Figure 8. GO enrichment analysis reveals the main biological processes related to the significantly differentially expressed proteins. The bars represent the main biological processes that the proteins were involved in. The y-axis displays the biological processes, and the x-axis shows the percentage of the proteins involved in these processes compared to the total proteins inputted.

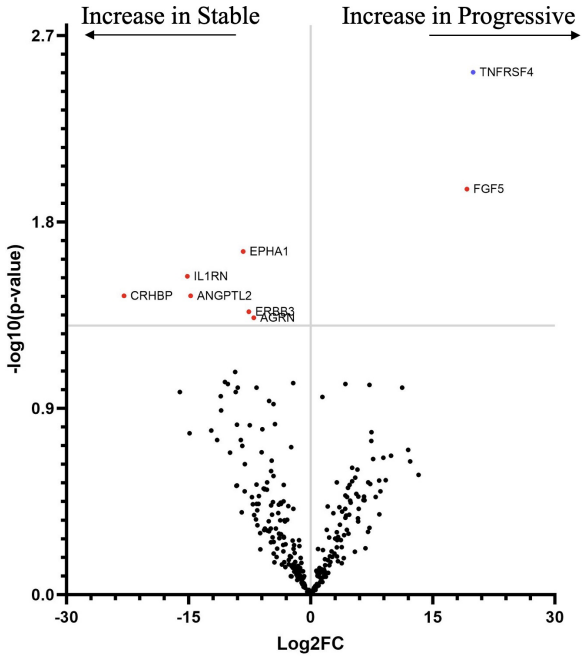

Figure 9. Volcano plots of all "detectable" proteins significantly differentially expressed between KC progressive and KC stable. The analysis was performed using multiple linear regression adjusted for age and sex. Fold change (displayed on the x-axis) was calculated as the mean Log2 NPX values differences between groups. A Log2 (fold change) value of +1 indicates a doubling of the relative protein concentration (upregulation), whereas a value of −1 indicates a halving of the relative protein concentration (downregulation). Proteins where $P < 0.05$ (−Log10 (P value) > 1.3) and $P < 0.01$ (−Log10 (P value) > 2.0) are indicated with *red* and *blue* dots, respectively.

in Figure 7. There were two protein-protein interactions in the AC versus KC comparative groups (ADA-GMPR and LGALS9-TNFRSF4), six in the AC versus KC + AC group (CXCL6-CSF3, JUN-CSF3, OSM-CSF3, PDGFB-CSF3, JUN-OSM, and IL32-OSM), and three in the KC versus KC + AC group (LY9-SLAMF7, SLAMF7-KLRB1, and KLRB1-TNFRSF4). Thirteen out of 29 proteins were not found to interact with the other identified proteins.

GO Enrichment

GO enrichment analysis revealed that most (21/29, 72%) of the significantly differentially proteins were involved in the same 10 biological processes (Fig. 8). More proteins belonging to the AC versus KC and AC versus KC + AC groups were involved in the upper biological processes (regulation of gene expression, cell population proliferation, transcription by RNA polymerase II, ERK1/2 cascade, and immune responses), with fewer involved in the lower ones (regulation of NIK/NFκB signaling, cell adhesion, JNK cascade, and inflammatory responses). The opposite was the case for the KC versus KC + AC groups, where more proteins were involved in the lower processes than the upper ones.

KC Progression and Severity

This study also allowed to investigate differences in expression levels of inflammatory proteins between patients with KC that were stable (n = 25) or progressive (n = 9). We identified 6 proteins that were significantly downregulated in progressive KC (or upregulated in stable KC), namely ANGPTL12, AGRN, CRHBP, EPHA1, ERBB3, and IL1RN (Fig. 9). TNFRSF4 and FGF5 were the only 2 proteins identified as upregulated in progressive KC.

Discussion

Keratoconus is a chronic, noninflammatory disease of the cornea with an incidence of approximately 1 in 2000 in the general population. Given the close interplay between allergic conjunctivitis and KC,[6–9] we hypothesized that the analysis of inflammatory tear proteins could be relevant in this respect. In the current study, we compared three

groups of patients (patients with KC without AC, patients with KC with AC, and patients with AC) with each other to identify differences and similarities between both ocular conditions.[30] A total of 272 detectable inflammatory proteins were shared among patients with KC, patients with AC, and patients with KC + AC. Of the significantly differentially expressed proteins, only LGALS9 was upregulated explicitly in KC, whereas FGF19, PDGFB, HPCAL1, OSM, and FCAR were downregulated in KC. Both TNFRSF4 and CCL13 were specifically upregulated in AC whereas EDAR was uniquely downregulated in AC.

A novel finding of our study is the upregulation of LGALS9 (galectin-9) in the tear samples of patients with KC and KC + AC. The galectin-9 receptor, TIM-3, is expressed on CD4+ and CD8+ T effector cells. When galectin-9 engages TIM-3, the selective deletion of these cells or the formation of regulatory T cells is triggered. The upregulation of galectin-9 and TIM-3 have been previously reported in corneal allografts in mice by Shimmura-Tomita et al., where these 2 proteins protected the graft from immune rejection.[31] The upregulation of two other galectins (galectin-1 and −3) has been reported previously in the corneal tissue (but not in tear fluid) of patients with KC compared to HCs.[32]

Dysregulated corneal extracellular matrix (ECM) remodeling underlies KC pathogenesis.[33] We identified three proteins unique to KC that have been shown to play a role in ECM remodeling in other diseases. A recent study on LGALS9 (galectin-9) in breast cancer demonstrated a positive correlation between Galectin-9 levels and invasiveness of cells through the ECM.[34] Platelet-derived growth factor beta (PDGFβ) is a member of the PDGF family of proteins. PDGFs play a significant role in the growth, proliferation, survival, and chemotaxis of the mesenchymal stem cells (MSCs). As such, coupled with its receptor (PDGFRβ), the PDGFβ also plays a vital role in ECM remodeling, through the activation of the MAPK/ERK, PI3K/AKT, and PKC pathways.[35] Oncostatin M (OSM) is a known pro-inflammatory factor which has a suggested role in the plaque vulnerability, and therefore development and progression of atherosclerosis. It is then believed that OSM binds to its receptor, OSM receptor (OSMR), present on the vascular smooth muscle cells (VSMCs), resulting in upregulated levels of the matrix metalloproteinases (MMPs). The MMPs are well known to cause degradation of collagen and elastin, and thus result in ECM remodeling.[36]

We are the first to report TNFRSF4 (OX40) upregulation in the tear fluid of patients with AC. Its binding partner, the OX40 ligand (OX40L), is expressed on T cells and other immune cells (including antigen-presenting cells). When OX40 engages with OX40L, it triggers CD4+ and CD8+ T cell clonal expansion and survival, and inhibits the activity and differentiation of T regulatory cells. The role of OX40-OX40L in the pathogenesis of allergic conditions, such as asthma, experimental allergic encephalomyelitis, and experimental AC, has been assessed using mice models.[37–40] The upregulation of OX40 in other ocular conditions, including corneal allograft rejection, experimental uveitis, and retinal pigment cell immunosuppression, has also been reported.[41–44] Akin to the allergic conditions, OX40-OX40L appears to exacerbate these conditions.

Elevated levels of CCL13 (MCP-4) have been reported in several ocular conditions, including uveitis, retinal vein occlusion, and retinal detachment, but also in allergic rhinitis, atopic dermatitis, and asthma.[45] In these conditions, CCL13 has been reported to induce a state of chronic inflammation and, in the case of allergic asthma, has been associated with the condition's progression.[46,47] Our study appears to corroborate these findings, given the unique upregulation of CCL13 in patients with AC.

Another distinctive finding of our study is the downregulation of EDAR in AC. Under normal physiological conditions, EDAR is highly expressed in the cornea, conjunctiva, and Meibomian and lacrimal glands. Its ligand, the ectodysplasin A (EDA) protein, is highly expressed in the Meibomian glands and secreted into tear fluid. EDA gene mutations are associated with X-linked hypohidrotic extodermal dysplasia (XLHED) and Meibomian gland dysfunction (MGD).[48] Intriguingly, patients with XLHED often present with asthma and other allergic conditions (such as rhinitis), but also chronic dry eye disease.[49]

In our study, 78% (7/9) of the patients with progressive KC had AC, whereas 40% (10/25) of patients with stable KC had AC. Previous studies have associated AC and KC severity based on clinical symptoms and parameters. Naderan et al. compared patients with KC, KC, and vernal keratoconjunctivitis (VKC; a more severe and rare form of AC), and KC and AC, and found that the latter two patient groups had thinner, more curved corneas compared to the first group.[14] Similarly, Cingu et al. compared patients with KC to patients with KC and VKC and found that patients in the latter group had thinner, more curved corneas and lower visual acuity.[15] Other studies have shown similar findings, with both Wang et al. and Mazzotta et al. implicating AC in the progression of KC.[12,50]

In the current study, tear fluid was used as source of molecular markers. Tear fluid contains local factors of the ocular surface and is therefore the best approach to get molecular information of the pathological situation. Tear fluid is noninvasively collected via Schirmer's strips, a paper strip which is gently placed in the lower eyelid where it absorbs tear fluid. This method, which is used as a standard test in clinical practice, is a simple and quick procedure to collect basal tears and is becoming increasingly relevant. Tear fluid (when extracted from paper strips) can be used directly in (immuno-)assays.

Advancement of technology that makes it possible to simultaneously measure a large number of proteins in small sample volumes provides new opportunities for unbiased discovery of novel biomarker, as well as for the identification of targeted hypothesis driven biomarkers. A particular interesting development in this context is the antibody-based PEA platform offered by Olink. One advantage of the Olink technology, that is of particular interest in ophthalmology, is that it only requires a small volume (1 µL) of tear fluid to perform multiplex immunoassays quickly without compromising the data quality. Further, PEA utilizes detection antibodies tagged with unique oligonucleotides preventing the cross-reactivity often seen in standard ELISAs. As such, PEA has a higher specificity and sensitivity. A drawback of using Olink is that the fixed panels may not contain all your proteins of interest. For example, the Explore Inflammation Panel I that was used in this study, does not contain previously identified KC markers, such as MMP9 or LOX.[20,51] Another limitation is that the Olink technology has known little coverage in ophthalmology so far. A standardized methodology to analyze tear fluid, aqueous humor of vitreous, has yet to be established. The first steps in this regard have been taken by Vergouwen et al.[25] They compared the use of tear extracts with a paper punch of the Schirmer's

strip and showed that the punch method identified more proteins than tear elutes.

Despite these strengths, a few limitations must be considered. First, although the Olink proximity extension technology is a good screening tool, it only generates semiquantitative data. Our findings thus not only require validation in larger, independent cohorts but also require the use of other methodologies to allow quantitative cutoffs in tear fluid before translation into daily clinical practice. Second, protein expression levels were provided as NPX values, so normalization to the tear fluid volume (migration lengths) was not possible. Third, we performed differential expression analysis only on proteins that passed our stringent data cleaning criteria. Although the majority of proteins (272/384) passed these criteria, the remaining proteins may contain relevant information about the disease as well. Finally, HCs were not included in this study as we aimed to search for differences and similarities between the two disease conditions. However, baseline levels of the measured proteins might have provided valuable additional information.

Conclusions

Based on the findings of this study, although KC and AC are two different conditions, the degree of similarity in the proteomic profiles indicates a likeness in the underlying mechanisms. This further validates the idea of keratoconus as an inflammatory condition, contrary to previous beliefs, and consolidates the association between AC and KC, as reported by previous studies. However, future work is needed to confirm these findings.

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