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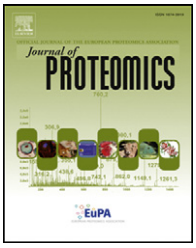

Tear proteome and protein network analyses reveal a novel pentamarker panel for tear film characterization in dry eye and meibomian gland dysfunction☆,☆☆

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ABSTRACT

Dry eye and meibomian gland dysfunction are common ocular surface disorders. Discrimination of both conditions often may be difficult given the overlapping of signs and symptoms, and the lack of correlation with clinical parameters. A total of 144 individuals were included in this study. To search for proteome differences, tear proteins were collected by Merocel sponge and analyzed using 2D-PAGE. Comparative tear protein profile analysis indicated changes in the expression levels of fifteen proteins. Subsequent to MALDI-TOF/TOF protein identification, network analysis revealed expression/interaction connections with other proteins, thereby identifying additional putative markers. A screening validation assay demonstrated the discriminative power of six candidate biomarkers. A further validation study using multiplexed-like ELISA assays in tear samples collected with both sponge and capillary confirmed the high discriminatory power of five biomarkers: S100A6, annexin A1 (ANXA1), annexin A11 (ANXA11), cystatin-S (CST4), and phospholipase A2-activating protein (PLAA) with an area under ROC curve (AUC)≥97.9% (sensitivity≥94.3%; specificity≥97.6%) when comparing dry eye and control individuals. This panel also discriminated between dry eye, meibomian gland dysfunction and control individuals, with a global correct assignment (CA) of 73.2% between all groups. Correct assignment was not found to be significantly dependent on the tear collection method.

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1. Introduction

Dry eye (DE) and meibomian gland dysfunction (MGD) are common inflammatory ocular surface diseases affecting tear film stability and ocular surface integrity. These pathologies may coexist and interact in the same patient, in this case, the severity of the coexisting diseases could be higher than that of the isolated diseases [1]. Dry eye (DE) is currently recognized as a disturbance of the lacrimal functional unit (LFU) resulting in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface [1]. The etiopathogenic classification updated by the DEWS Subcommittee, classified DE as aqueous-deficient (ADDE) when a failure of lachrymal secretion occurs, or evaporative (EDE) which is due to an excessive water loss from the exposed ocular surface [1]. Dry eye is usually a symptomatic disorder that varies in severity and must be differentiated from other forms of symptomatic ocular surface disease (SOSD). MGD is a chronic, diffuse abnormality of the meibomian glands, commonly characterized by terminal duct obstruction and/or qualitative/quantitative changes in the glandular secretion. This may result in alteration of the tear film, symptoms of eye irritation, clinically apparent inflammation, and ocular surface disease [2].

According to previous clinical studies, posterior blepharitis including MGD has been frequently reported in dry eye patients, with significant overlapping of the symptoms and signs. Patients commonly complain of sandy-gritty irritation, a burning sensation, red eye, irritation, photophobia and blurred vision [3,4]. MGD can lead to alterations in the normal lipid composition in meibomian gland secretions, which in turn induces abnormalities on the tear film composition and function resulting in evaporative dry eye [4].

The pathophysiology of both conditions is complex and thought to represent the interaction of multiple mechanisms including tear film hyperosmolarity, instability, and subsequent activation of an inflammatory cascade, with release of inflammatory mediators into the tears, which in turn can damage the ocular surface epithelium [1,5]. Diagnosis of DE and MGD sometimes is difficult and frustrating given the overlapping symptoms and signs. Currently, dry eye diagnosis is performed based on questionnaires and clinical tests (the Schirmer test, tear breakup time, staining of the cornea and conjunctiva, among others). However, most of these tests do not provide reproducible results which correlate well with objective signs [6]. Although a general consensus for clinical diagnosis of dry eye employing a wide number of tests has been reported [1], the use of specific biomarkers would be a relevant clinical improvement in differentiation from other symptomatic ocular surface diseases (SOSD), determination of etiology and severity grades of the disease, and the selection of proper treatment strategy.

Molecular markers to diagnose ocular surface diseases have been intensively investigated by means of the analysis of tear film proteins, employing multiple proteomic techniques, such as one and two dimensional electrophoresis [7–10], nano-liquid chromatography tandem mass spectrometry [11–15] or surface-enhanced laser desorption ionization time of flight mass spectrometry [16], among others. Some of these studies have focused on the compositional analysis of tear film [11,17], while others have centered on the identification of protein markers for ocular diseases, such as dry eye [15,16] and blepharitis [8] separately, and the relative quantification of their abundance. Differences in the identification of protein biomarkers were thought to be due to the technique used for analyzing the samples, and to the tear sample collection method employed [12,16]. The limitations in these studies include the lack of extensive validation of candidate biomarkers using new samples, the lack of determination of the specificity of the candidate markers as well as the absence of predictive models and the testing of their accuracy as a tool for diagnosis.

The purpose of the present study was to perform a differential tear film protein expression study of dry eye and MGD patients in comparison to healthy control individuals by using 2D-PAGE to identify novel candidate protein biomarkers. A wide validation study was also carried out in order to determine the validity of the candidate biomarkers, as well as the possible effect on marker concentration of the tear collection method, and to evaluate which of these biomarkers provides optimal accuracy when employed as a clinical tool. The biomarkers described here contribute to a better knowledge of physiopathology of the diseases, and might aid to a correct diagnosis in those cases of confusing and/or insufficient signs, and also to monitoring response to different treatments.

2. Materials and methods

2.1. *Patients*

A prospective case-controlled study was carried out, in which 144 patients were enrolled, 63 DE, 38 MGD and 43 CT subjects. Patients with dry eye or MGD, and normal subjects were recruited from four Spanish centers: Cruces Hospital (Baracaldo), Instituto Clínico Quirúrgico de Oftalmología (Bilbao), Instituto Oftalmológico Fernandez Vega (Oviedo), and Hospital de Valladolid (Valladolid). Diagnosis was based on clinical examinations including the Schirmer I test with anesthesia, slit lamp examination of the lid margin and meibomian glands, fluorescein staining, and subjective symptoms. Each patient answered a modified "National Eye Institute Visual Functioning Questionnaire 25" (VFQ-25) which includes some statements about problems involving their vision or feeling.

Patients were classified as having aqueous-deficient dry eye if they had dry eye symptoms with at least one abnormal measure of clinical tests, Schirmer I test dynamics abnormalities ($\leq$5 mm/5 min) and fluorescein staining according to Oxford scale. Patients were diagnosed as having MGD if they presented symptoms including eyelid inflammation, a Schirmer I test result of >5 mm/5 min and alteration in meibomian glands. In the control group (CT), healthy subjects were recruited who were not suffering from any ocular disease (no allergic or atopic history), Schirmer I test values of >5 mm/5 min, no corneal fluorescein staining or sensations of discomfort, and no evident eyelid inflammation. The exclusion criteria included the presence or history of any systemic or ocular disorder or condition (including ocular surgery, trauma and disease) and contact lens users. Patients previously diagnosed with Sjögren syndrome

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