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(54) Title: A PROCESS FOR IDENTIFICATION OF BIOMARKERS FOR KERATOCONUS PROGRESSION

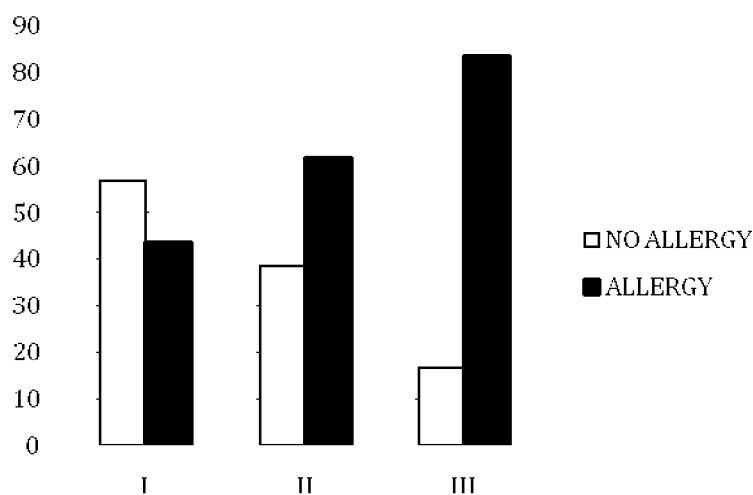


Figure 1

(57) Abstract: The present invention relates to a process of identification of biomarkers for disease progression of Keratoconus, a corneal thinning disorder. Proteomic profile of the KC patient tear was analyzed to identify up and down regulated proteins, compared to control and the proteins that indicated association with clinical grades were considered as biomarkers for KC progression. The biomarkers are used in form of diagnostic kit for detection of Keratoconus disease.



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TITLE OF THE INVENTION**A process for identification of biomarkers for Keratoconus progression****[001] Technical field of the invention**

[002] The present invention relates to a process of identification of biomarkers for disease progression of Keratoconus, a corneal thinning disorder. The invention further relates to use of biomarkers, obtained from mammalian tear samples, to identify proteins specifically associated to up or down regulation with respect to clinical grades of Keratoconus.

[003] Background of the invention

[004] Keratoconus (KC) is a corneal thinning disorder that reduces visual acuity through ectasia and irregular astigmatism. KC can cause substantial distortion of vision, with multiple images, streaking and sensitivity to light all often reported by the patient. It is typically diagnosed in the patient's adolescent years and attains its most severe state between the ages of 20 and 40. If afflicting both eyes, the deterioration in vision can affect the patient's ability to drive a car or read normal print. It seems to occur in populations throughout the world, although it is observed more frequently in certain ethnic groups, such as South Asians. Environmental and genetic factors are considered possible causes, but the exact cause is uncertain.

[005] Clinically, the symptoms of KC are highly variable and in part, depend on the stage of the progression of the disorder. Early in the disease, there may be no symptoms, and KC may be noted by the ophthalmologist simply because the patient's best corrected vision does not improve to 6/6 or 20/20. In the advanced stages there can be significant visual distortion which may be accompanied by profound visual loss. As the disease progresses, vision deteriorates, sometimes rapidly. Visual acuity becomes impaired at all distances, and night vision is often poor. The exact etiology is unknown, however, recent literature suggests that inflammatory molecules and abnormal levels of enzymes are present in subjects

with KC (Cristina Kenney and Brown 2003, Lema and Duran 2005, Lema et al. 2008).

[006] As far as diagnosis of KC is concerned, prior to any physical examination, the diagnosis of KC often begins with an ophthalmologist's or optometrist's assessment of the patient's medical history, particularly the chief complaint and other visual symptoms, the presence of any history of ocular disease or injury which might affect vision, and the presence of any family history of ocular disease. An eye chart, such as a standard Snellen chart of progressively smaller letters, is then used to determine the patient's visual acuity.

[007] There is currently no medication for containing KC, nor any adequate biomarkers to predict the disease. Furthermore, there is considerable confusion in the field regarding the pathophysiology of the disease and involvement of inflammation. Hence there is a need to have diagnostic methods and tools such as diagnostic kit for early and accurate diagnosis, severity and progression of KC to ensure timely treatment of the patient.

[008] Summary of the invention

[009] A process of identifying biomarkers for detecting Keratoconus progression consists of collecting tear samples from the patients suffering from KC, identifying proteomic profile of tear samples, correlating proteomic profile with different clinical grades of KC, comparing proteomic profile to the control with a cutoff rate at 1% and finally identifying proteins from proteomic profile that up regulated or down regulated with respect to control and showed association with different clinical grades of KC that is used as biomarkers.

[0010] The tear samples collected from KC patients were taken on Schirmer strips and subjected to mass spectrometric analysis to identify the proteomic profile. The proteins that showed up regulation of fold increase cutoff of >1.5 and proteins that showed down regulation of fold decrease cutoff of < 0.75 over control were considered. Among these up and down regulated proteins some showed tight association with clinical grades of KC representing good quality biomarkers.

[0011] Brief Description of Drawings

[0012] The features of embodiments will become more apparent from the following detailed description of embodiments when read in conjunction with the accompanying drawings. Furthermore, elements that are known to be common and well understood to those in the industry are not depicted in order to provide a clear view of the various embodiments of the invention. Thus, in the interest of clarity and conciseness, the drawings are generalized in form.

[0013] **Figure 1** shows a graph depicting correlation between allergy symptoms and KC in accordance with the embodiments of the present invention.

[0014] **Figure 2** shows a graph depicting average levels of proteins up regulated in patient cohort in accordance with the embodiments of the present invention.

[0015] **Figure 3** shows a graph depicting average levels of proteins down regulated in patient cohort in accordance with the embodiments of the present invention.

[0016] **Figure 4** shows a graph depicting proteins up regulated with increasing grades of KC in accordance with the embodiments of the present invention.

[0017] **Figure 5** shows a graph depicting proteins down regulated with increasing grades of KC in accordance with the embodiments of the present invention.

[0018] Detailed description of the invention

[0019] In order to more clearly and concisely describe and point out the subject matter of the claimed invention, the following definitions are provided for specific terms which are used in the following written description.

[0020] By the term “**Biomarker**”, we mean up and down regulated proteins that indicate occurrence and progression of Keratoconus (KC).

[0021] By the term “**Control**”, we mean samples collected from healthy individuals who are not suffering from the disease Keratoconus (KC).

[0022] By the term “*Down regulation*” we mean the process by which a cell decreases the quantity of a cellular component, such as RNA or protein, in response to an external variable

[0023] By the term “*Keratoconus*”, we mean a degenerative disorder of the eye in which structural changes within the cornea cause it to thin and change to a more conical shape than its normal gradual curve.

[0024] By the term “*Up regulation*” we mean the process by which a cell increases the quantity of a cellular component, such as RNA or protein, in response to an external variable

[0025] Unless otherwise defined, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of this invention. Indeed, this invention is in no way limited to the process and materials described.

[0026] The present invention relates to a process of identification of biomarkers for disease progression of corneal thinning disorder called KC. A biomarker, also termed as biological marker, is an indicator of a biological state or occurrence and progression of a disease. It is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. Biomarkers are of various kinds, but in the present studies, the prime focus has been KC biomarkers that enable identification of occurrence and progression of the disease.

[0027] Another preferred embodiment of the present invention relates to accurate diagnosis of KC by the use of biomarkers identified in this study, along with various techniques and methods, corneal topographic and tomographic techniques which generate color coded maps and topographic indices have been used for diagnosis of KC in this study. In addition to this, video keratography has been used herein to identify Forme fruste keratoconus (FFKC) in the absence of clinical

signs of KC. Video keratographic indices such as the Klyce or Maeda criteria, the Rabinowitz criteria, and others have been used to quantitatively analyze video keratography and screen for KC. These indices have been shown to identify KC with a high degree of sensitivity and specificity. The Orbscan II, a three dimensional slit scan topography system for analysis of the corneal surfaces and anterior chamber has been used on all patients in the study. It uses calibrated video and a scanning slit beam to measure x, y, and z locations of several thousand points. These points are used to construct topographic maps. The Pentacam, a corneal tomographer technology which generates data on topography and elevation of anterior and posterior using a rotating Scheimpflug camera has also been used on all subjects in this study. The clinical data, so obtained shall be correlated with the keratoconus biomarker data and shall be validated. The biomarkers identified may hence be used as representative of the clinical condition.

[0028] Allergic symptoms including itching and eye rubbing in the patients have been known to contribute to disease progression. In the present studies, it has been observed that within this cohort, nearly 56% of the patients presented allergic symptoms. These patients have been further categorized under the different clinical grades of KC and analyzed if the allergic symptoms correlate with disease progression. **Figure 1** demonstrates allergy symptoms correlation with increasing grades of KC, where more than 83% of the patients in grade III had allergy compared to only 43% of grade I.

[0029] Another preferred embodiment of the present invention relates to identification of biomarkers of KC progression. Tear samples from the patient were taken on Schirmer strips and subjected to mass spectrometric analysis to identify the proteomic profile of tears from each patient eye. The proteomic profiles of KC patients with different clinical grades and clinical features in a cohort of 54 patients are correlated with their proteomic signatures. Compared to the control, the tear proteome from KC patient tear content was found to be about 300 to 400 proteins more in 50% of the cases with a cutoff for false discovery rate at 1%. A number of proteins were elevated across the patient cohort wherein a small sub group showed tight association with increasing clinical grades. About

48 proteins were identified to be present in every sample and more than 1000 proteins were detected in at least 3 samples in the cohort. Protein levels were measured as fold difference compared to baseline control samples from unaffected individuals.

[0030] In another preferred embodiment of the present invention, the levels of the proteins were measured as fold difference compared to baseline control samples from unaffected individuals. The proteins found to be up regulated in proteomic profile as shown in **Figure 2** includes LCN1 (Lipocalin 1), PLA2G2A (phospholipase A2, group II, isoform A), SCGB2A1 (secretoglobin family 2A member 1), MSLN (mesothelin) and CRYAB (crystallin alphaB). Conversely, another group of proteins was observed to be significantly down regulated as shown in **Figure 3**. These proteins include ALB (albumin), IGHG2 & G3 (immunoglobulin heavy chain gamma isoforms), A2M (alpha-2 macroglobulin), complement factors C3, C4A, C6 & H, ORM1 (orosomucoid 1), KNG1 (kininogen1), PRDX1 (peroxiredoxin1), SERPIN-F1, GSN (Gelsolin), etc. Therefore, these specific proteins were used as biomarkers for predicting progression or severity of the disease. Furthermore, these proteins also shed light on the underlying deregulation of important inflammation and immunobiology related pathways that may be driving KC pathophysiology. The data in these graphs represent average fold increase or decrease over normal, healthy controls. For up regulation, the fold increase cutoff of >1.5 over control and for down regulation, fold decrease cutoff of <0.75 has been used.

[0031] Number of proteins that demonstrated increase in average levels across the patient cohort corresponding to clinical grades has been depicted in graphs. Specific proteins that are up and down regulated with respect to clinical grades of KC as shown in **Figure 4** and **Figure 5** are used as biomarkers for predicting progression or severity of the disease. Proteins such as, LCN1 (Lipocalin 1), PLA2G2A (phospholipase A2, group II, isoform A), SCGB2A1 (secretoglobin family 2A member 1), MSLN (mesothelin) and CRYAB (crystallin alphaB) demonstrate the potential to be considered as biomarkers for predicting the progression of KC. Many other proteins that were up regulated did not show specific association with disease progression and were therefore considered to be

indirectly associated with the disease pathology.

[0032] In another preferred embodiment of the present invention, a group of down regulated proteins that corresponded to clinical grades of KC as shown in **Figure 5** includes ALB (albumin), IGHG2 & G3 (immunoglobulin heavy chain gamma isoforms), A2M (alpha-2 macroglobulin), complement factors C3, C4A, C6 & H, ORM1 (orosomucoid 1), KNG1 (kininogen1), PRDX1 (peroxiredoxin1), SERPIN-F1, GSN (Gelsolin), etc. The proteins that were significantly down regulated, but did not show association with clinical grades were not considered as biomarkers for KC progression.

[0033] In order that this invention to be more fully understood the following preparative and testing examples are set forth. These examples are for the purpose of illustration only and are not to be construed as limiting the scope of the invention in any way.

[0034] Example 1: Diagnosis of KC

A total of 54 patients diagnosed with KC and 12 healthy subjects were engaged for the current study. The patient's cohort consisted of 33 males with an age range of 18-32 years, median age of 26 years and 21 females with an age range of 12-31 years, median age of 26 years. The screening of KC involves KC related clinical signs like retinoscopy scissors reflex, Munson sign, stromal thinning, Vogt's striae, and Fleischer's ring, but corneal topography is the most useful method in the diagnosis of KC, especially in the absence of clinical signs. This index has been used for the final gradation of KC stages of all subjects in this study.

[0035] Example 2: Tear sampling and proteomics

In order to identify biomarkers of KC progression, tear samples from each patient was taken on Schirmer strips. The tear samples were then subjected to mass spectrometric analysis at SERI laboratory to identify the proteomic profile of tears from each patient eye. Thus, the present invention utilizes a process of identification of biomarkers for disease progression of Keratoconus, wherein the power of up and down regulated proteins has been used as biomarkers.

Claims

We claim

1. A process of identifying biomarkers for detecting Keratoconus (KC) progression, the process comprising steps of:
 - a) collecting tear samples from patients suffering from KC;
 - b) identifying proteomic profile of tear samples;
 - c) correlating proteomic profile with different clinical grades of KC;
 - d) comparing proteomic profile to the control with a cutoff rate at 1%;
 - e) identifying proteins from proteomic profile that are up regulated or down regulated with respect to control and show association with different clinical grades of KC and use of these as biomarkers.
2. The process as claimed in claim 1, wherein said tear samples were taken on Schirmer strips and subjected to mass spectrometric analysis to identify the proteomic profile.
3. The process as claimed in claim 1, wherein said up regulated proteins showed fold increase cutoff of >1.5 over control.
4. The process as claimed in claim 1, wherein said down regulated proteins showed fold decrease cutoff of <0.75 over control.
5. The process as claimed in claim 1, wherein said proteomic profile of certain proteins showed tight association with clinical grades of KC, representing good quality biomarkers.
6. The process as claimed in claim 1, wherein said protein that is up regulated in proteomic profile is at least one of:
 - a) Lipocalin 1,
 - b) phospholipase A2 group II isoform A,
 - c) secretoglobin family 2A member 1,
 - d) mesothelin, or
 - e) crystallin alphaB.

7. The process as claimed in claim 1, wherein said protein that is down regulated in proteomic profile is at least one of:

- a) albumin,
- b) immunoglobulin heavy chain gamma isoforms,
- c) alpha-2 macroglobulin,
- d) complement factors including C3, C4A, C6, H and ORM,
- e) kininogen1,
- f) peroxiredoxin1, or
- g) Gelsolin.

8. A kit for predicting and detecting the severity and progression of Keratoconus disease using biomarkers.

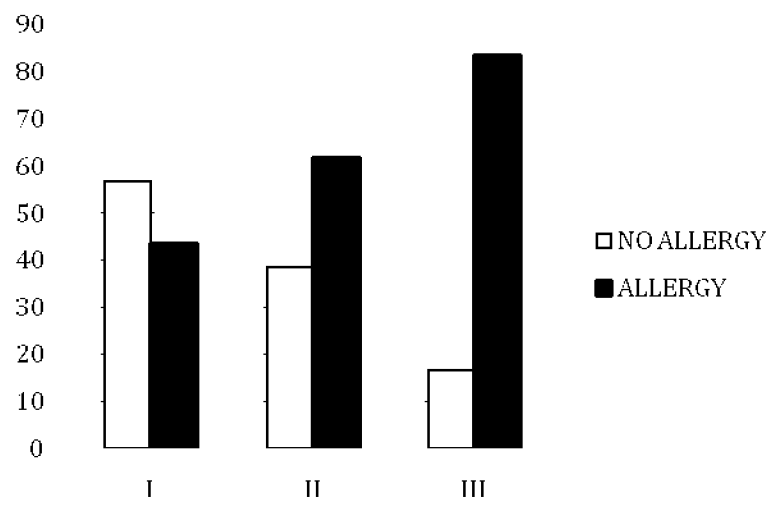


Figure 1

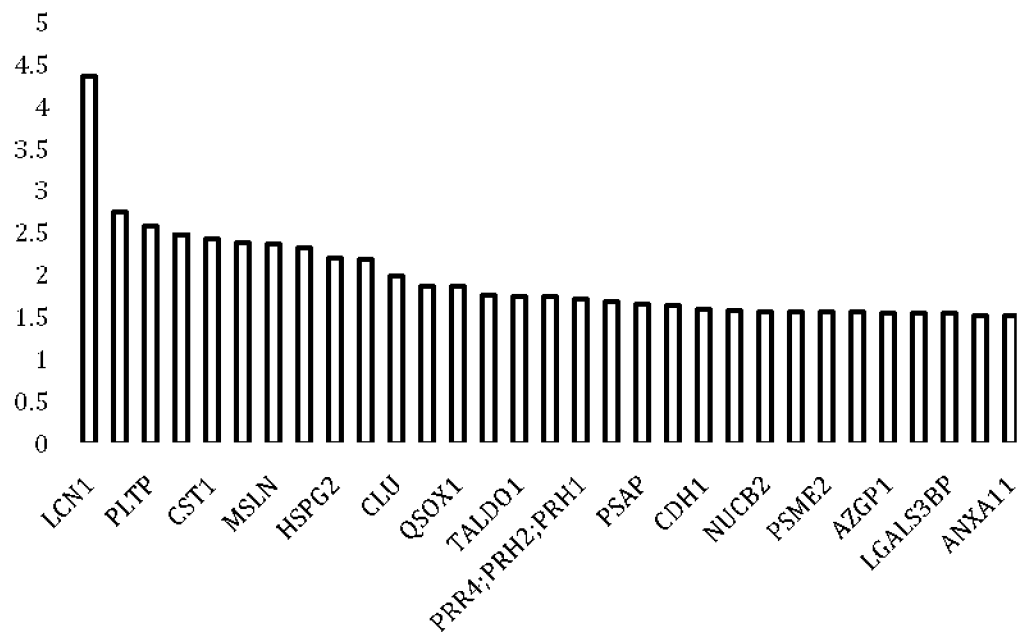


Figure 2

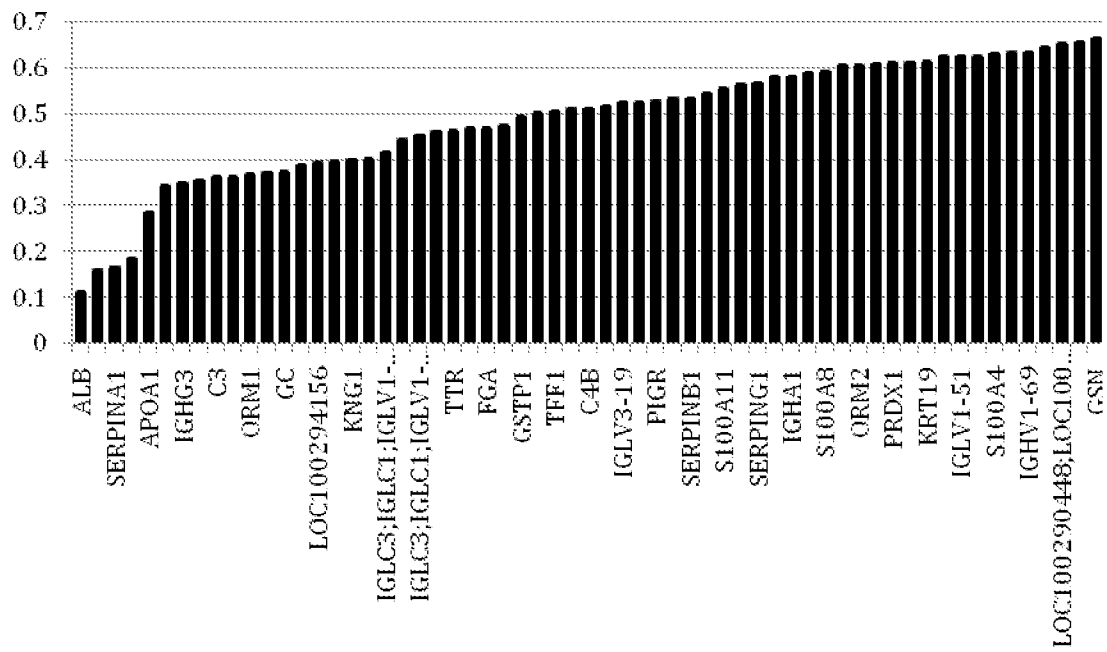


Figure 3

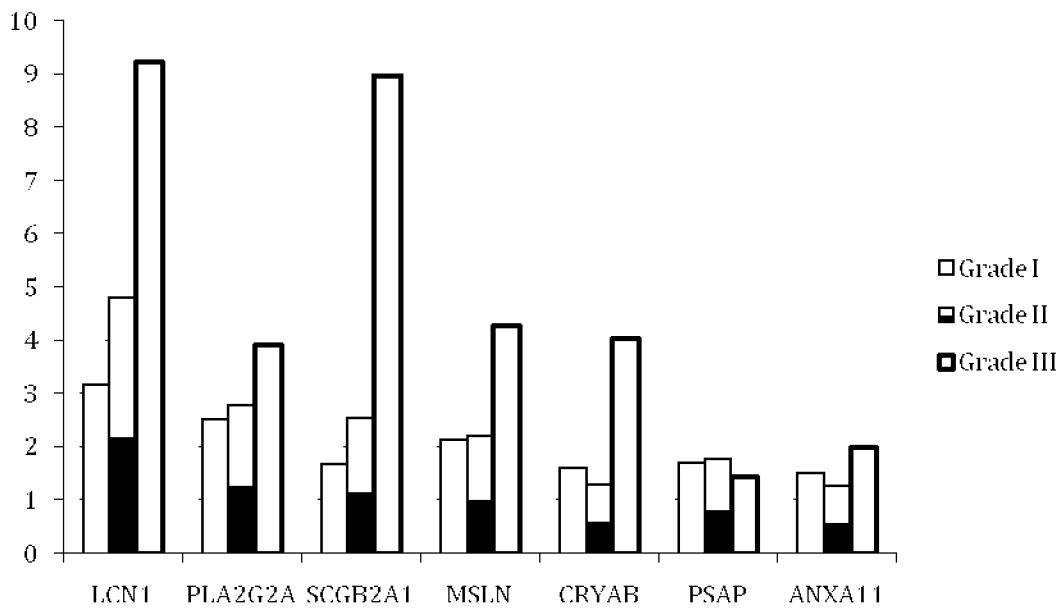


Figure 4

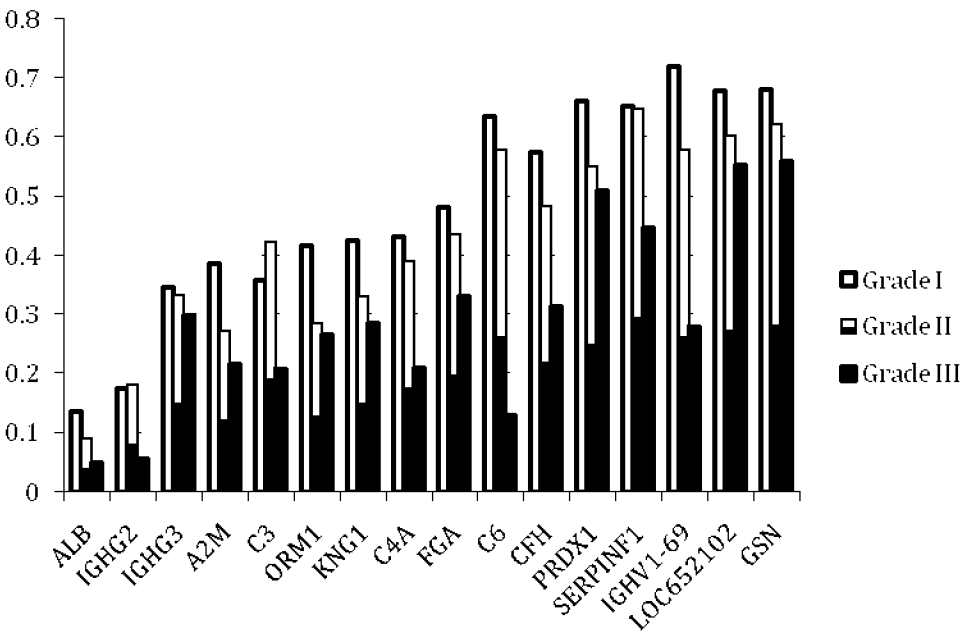


Figure 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 14/59328

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C40B 20/08, G01N 33/68, G01N 30/72 (2014.01)

USPC - 436/173, 506/6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC (8): C40B 20/08, G01N 33/68, G01N 30/72 (2014.01)

USPC: 436/173, 506/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

IPC(8): C40B 30/10, C40B 40/10 (2014.01)

USPC: 436/86, 506/7, 506/12, 506/18, 435/4, 435/6.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Google Patents, Google Scholar, Google Web, Patbase, Proquest Dialog

biomarker, predicting, Keratoconus (KC)/ectasia, tear, sample, proteomic profile/analysis, grade/progression/severity, proteins/polypeptides/peptides, Schirmer strip/test, Mass Spec, up regulate, Lipocalin, down regulate, alpha-2 macroglobulin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NIELSEN et al., Proteome profiling of corneal epithelium and identification of marker proteins for keratoconus, a pilot study. Experimental Eye Research, February 2006, Vol. 82, No. 2, pp 201-209. page 202, Col. 1, para 1; page 203, Col. 1, para 3; page 203, Col. 2, para 1-2; page 207, Col. 2, para 4; page 208, Col. 1, para 1; page 205; Table 1	1-8
Y	LEMA et al., Inflammatory molecules in the tears of patients with keratoconus. Ophthalmology. April 2005, Vol. 112, No. 4, pp 654-659. page 655, Col. 1, para 5-6; page 655, Col. 2, para 1; page 656, Col. 1, para 4; Figure 1; page 654, Col. 2, para 2; page 655, Col. 1, para 1	1-8
Y	LEMA et al. Proteomic analysis of the tear film in patients with keratoconus. Molecular Vision. 13 October 2010, Vol. 16, pp 2055-2061. page 2056, Col. 2, para 5; page 2057, Col. 1, para 1-3	2
Y	ACERA et al. Changes in tear protein profile in keratoconus disease. Eye (London, England), September 2011, Vol. 25, No. 9, pp 1225-1233. page 1226, Col. 1, para 3; page 1229, Col. 2, para 3; page 1230, Col. 1, para 1; page 1230, Col. 2, para 1	6
Y	SAWAGUCHI et al., Alpha 2-Macroglobulin levels in normal human and keratoconus corneas. Investigative Ophthalmology and Visual Science, November 1994, Vol. 35, No. 12, pp 4008-4014. page 4010, Col. 2, para 3; page 4011, Col. 1, para 1	7
Y	US 2007/0248970 A1 (RABINOWITZ et al.) 25 October 2007 (25.10.2007), para [0006]-[0008]; para [0005]	8

☒ Further documents are listed in the continuation of Box C.



* Special categories of cited documents:

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Date of the actual completion of the international search

09 May 2014 (09.05.2014)

Date of mailing of the international search report

03 JUN 2014

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 14/59328

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2012/0191685 A1 (Albar Ramirez et al.) 26 July 2012 (26.07.2012) para [0024], [0032]	1
A	VANDERMEID et al., A method to extract cytokines and matrix metalloproteinases from Schirmer strips and analyze using Luminex. Molecular Vision, April 2011, Vol. 27, No. 17, pp 1056-1063. page 1056, Col. 1, para 2	2
A	WO 2001/50133 A1 (KARIN et al.) 12 July 2001 (12.07.2001), Abstract; page 4, ln 22-32; page 5, ln 11-18; page 37, ln 12-13	1, 3
A	PANNEBAKER et al. Tear proteomics in keratoconus. Molecular Vision, 02 October 2010, Vol. 16, pp 1949-1957. page 1953, Col. 1, para 2; page 1953, Col. 2, para 1; page 1954, Col. 1, para 1	1
A	RABINOWITZ. Keratoconus. Survey of ophtalmology, January-February 1998, Vol. 42, No. 4, pp 297-319. page 304, Col. 1, para 2-4; page 304, Col. 2, para 1	7
A	US 6,444,791 B1 (QUAY) 03 September 2002 (03.09.2002), Abstract	7