



- (51) **International Patent Classification:**
G01N 33/68 (2006.01) *G01N 33/58* (2006.01)
- (21) **International Application Number:**
PCT/SG2014/000219
- (22) **International Filing Date:**
21 May 2014 (21.05.2014)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
201303949-0 21 May 2013 (21.05.2013) SG
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(81) **Designated States** (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) **Title:** METHOD OF DIAGNOSING THYROID DISEASE

(57) **Abstract:** This invention relates to methods of diagnosing thyroid disease or diagnosing the risk of developing thyroid disease, with or without thyroid orbitopathy, in a patient. Further, the present invention relates to a kit for detecting at least one of the proteins identified as a thyroid disease marker protein according to the present invention or at least one gene encoding such protein.



METHOD OF DIAGNOSING THYROID DISEASE

FIELD OF THE INVENTION

[0001] The present invention lies in the field of biochemistry and relates to methods of diagnosing thyroid disease or diagnosing the risk of developing thyroid disease in a patient. Further, the present invention relates to a kit for detecting at least one of the proteins identified as a thyroid disease marker protein according to the present invention or at least one gene encoding such protein.

BACKGROUND OF THE INVENTION

[0002] Tears are a complex biological mixture, containing electrolytes, proteins, lipids, mucins, some small organic molecules and metabolites. Proteomics provides a comprehensive approach for cataloguing all the proteins of the tear proteome, which will help to elucidate disease pathogenesis and make clinical diagnoses [Zhou L and Beuerman RW, Progress in Retinal and Eye Research, 2012, 31(6), 527-550; Zhou L et al., Ann Acad Med Singapore, 2006, 35(6), 400-7; Zhou L et al., Journal of Proteome Research, 2009, 8(4), 1992-2003; Chen L et al., Journal of Proteome Research, 2011, 10(10), 4876-82; Zhou L et al., Journal of Proteomics, 2012, 75(13), 3877-3885; Zhou L et al., Proteomics, 2013, 13(16), 2469-2481]. Changes in the tear proteomics are correlated with conditions such as ocular surface wounding and inflammatory disease [Zhou L et al., Journal of Proteome Research, 2004, 3(3), 410-416; Zhou L et al., Proteomics, 2007, 7(17), 3194-3206; Zhou L et al., Invest. Ophthalmol. Vis. Sci., 2009, 50(5), 2077-2086; Zhou L et al., Journal of Proteome Research, 2009, 8(11), 4889-4905; Wong TT et al., Invest. Ophthalmol. Vis. Sci. 2011, 52(10), 7385-91; Tong L et al., British Journal of Ophthalmology, 2011, 95(6), 848-52]. S100 A8, A9 and A12 are key pro-inflammatory and stress-related proteins of the S100 calcium binding family of proteins. Abundantly expressed in neutrophils, elevated levels of S100 A8 and A9 proteins have been reported in patients with

systemic inflammatory disorders such as rheumatoid arthritis. These proteins are also normally found in limbal epithelial cells and elevated levels have been detected in patients with localized ocular surface conditions such as dry eyes and pterygia. Other proteins within the S100 family include the A4 and A11 which are related to osmotic stress and apoptosis.

[0003] Graves' orbitopathy [Bartley et al., AJO, 1995, 119(6), 792-5; Douglas RS et al., ITEDS Arch Ophthalmol, 2009, 127(9), 1155-60] is an automimmune condition which is clinically apparent in 25-50% of individuals with Graves' disease. Cell-mediated mechanisms target and activate fibroblast populations through recognition of an orbital antigen. Most of the cells involved are CD2⁺/CD3⁺ T lymphocytes and macrophages. The cytokine production from the orbital T cell repertoire shows Th1-typical cytokine production in early disease and Th2 typical cytokine patterns in advanced disease. Over-production of extracellular matrix by orbital fibroblast and adipogenesis then may lead to increased intraorbital volume. Limitation of fixed bony orbit to the expansion of orbital connective and fatty tissues gradually produce signs and symptoms caused by compression of orbital blood vessels, nerves, anterior displacement of globe and enlargement of extra-ocular muscles. Contracted fibrosis of extraocular muscles at later stage explain the development of potential blinding complications such as compressive optic neuropathy, severe exophthalmos, exposure keratopathy, functional debilitating diplopia and disfiguring outcome in some patients. Early disease recognition may be possible through detection of inflammatory cascade proteins in the tears.

[0004] Previous studies on tear protein analysis for patients with thyroid orbitopathy (TO) have failed to identify the types of proteins or peptides in the tear films of said patients [Okrojek R et al., Horm Metab Res, 2009, 41, 265-470; Piera Vet et al., Current Opinion in Allergy & Clinical Immunology, 2010, 10, 486-92; Nina M et al., Thyroid, 2012, 22, 10, 1039-45; Khalil HA et al., AJO, 1988, 106, 190; Baker GRC et al., Arch Ophthal, 2006, 24, 451-56; Khalil HA et al., Doc Ophthal, 1989; 72, 329-94; Yoon JS et al., Graefes Arch Clin Exp Ophthalmol, 2010, 248, 271-76; Huang D et al., Graefes Arch Clin Exp Ophthal, 2012, 250, 619-25]. Knowledge of the tear protein profile of patients with thyroid orbitopathy is useful for the understanding of underlying disease mechanisms and to develop diagnostic tools for screening, staging, treatment response and prediction of the development of thyroid orbitopathy. Hence,

there is need in the art for the identification of tear proteins related to thyroid orbitopathy and methods of diagnosis using said thyroid disease marker proteins.

SUMMARY OF THE INVENTION

[0005] It is an object of the present invention to meet the above need by providing methods of diagnosing thyroid disease or diagnosing the risk of developing thyroid disease using tear protein markers. Surprisingly, the present inventors have found that significant changes in tear protein expression profiles can be identified between patients having thyroid disease without orbitopathy (TD) or patients having thyroid eye disease (TED) when compared with the protein expressions of healthy control individuals. Further, by using these protein expression profiles it was also possible to differentiate between TD and TED patients and between two subgroups of TED, namely patients having a mild form of TED and patients having a severe form of TED.

[0006] In a first aspect, the present invention is thus directed to a method of diagnosing thyroid disease or diagnosing the risk of developing thyroid disease, said method comprising: (a) determining the level of at least one tear protein or the amount of expression of at least one gene encoding a tear protein in a sample obtained from a patient; (b) comparing the data obtained in step a) to a predetermined level of at least one tear protein or the amount of expression of at least one gene encoding a tear protein observed in a healthy individual, wherein when the level of the at least one tear protein or the amount of expression of the at least one gene encoding a tear protein is changed compared to the predetermined levels in a healthy individual said patient is diagnosed with thyroid disease or an increased risk to develop thyroid disease.

[0007] In various embodiments of the invention, the thyroid disease is thyroid disease without thyroid orbitopathy and the tear protein is at least one protein set forth in SEQ ID Nos. 3, 7, 21, 38, 54, 63, 79, 99 or 108-124; or the thyroid disease is thyroid eye disease with mild or severe thyroid orbitopathy and the tear protein is at least one protein set forth in SEQ ID Nos. 1-107.

[0008] In various embodiments of the invention, when the level of at least one protein set forth in SEQ ID Nos. 7, 38, 99, 108 or 109 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 7, 38, 99, 108 or 109 is increased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid disease without thyroid orbitopathy or an increased risk to develop thyroid disease without thyroid orbitopathy. In other various embodiments, when the level of at least one protein set forth in SEQ ID Nos. 3, 21, 54, 63, 79 or 110-124 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 3, 21, 54, 63, 79 or 110-124 is decreased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid disease without thyroid orbitopathy or an increased risk to develop thyroid disease without thyroid orbitopathy.

[0009] In various embodiments of the invention, when the level of at least one protein set forth in SEQ ID Nos. 13, 14, 15, 16, 22, 24, 28, 29, 36, 43, 45, 47, 48, 49, 50, 51, 52, 53, 56 58, 59, 70, 71, 73, 81, 89 or 96 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 13, 14, 15, 16, 22, 24, 28, 29, 36, 43, 45, 47, 48, 49, 50, 51, 52, 53, 56 58, 59, 70, 71, 73, 81, 89 or 96 is increased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid eye disease with mild or severe thyroid orbitopathy or an increased risk to develop thyroid eye disease with mild or severe thyroid orbitopathy. In other various embodiments, when the level of at least one protein set forth in SEQ ID Nos. 1-12, 17-21, 23, 25, 26, 27, 30-35, 37-42, 44, 46, 54, 55, 57, 60-69, 72, 75-80, 82-88, 90-95 or 97-107 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 1-12, 17-21, 23, 25, 26, 27, 30-35, 37-42, 44, 46, 54, 55, 57, 60-69, 72, 75-80, 82-88, 90-95 or 97-107 is decreased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid eye disease with mild or severe thyroid orbitopathy or an increased risk to develop thyroid eye disease with mild or severe thyroid orbitopathy.

[00010] In various embodiments of the invention, the thyroid disease is thyroid eye disease with mild thyroid orbitopathy and the tear protein is at least one protein set forth in SEQ ID Nos. 1-5, 7-11, 14, 17-19, 21, 23-26, 30-32, 34, 35, 37, 38, 40-42, 53-55, 58, 59, 62, 63, 65, 66, 68, 69, 72, 75, 77, 78, 80, 83, 86, 88, 90, 91, 93, 95 or 97-106; or the thyroid disease is thyroid eye disease with severe thyroid orbitopathy and the tear-specific protein is at least one protein set

forth in SEQ ID Nos. 2-6, 8, 10, 12-16, 19, 20, 22-24, 27-29, 31-33, 36, 39-41, 43-61, 64-67, 69-71, 73, 74, 76, 77, 79, 81-85, 87-90, 92-96, 98 or 103-107.

[00011] In various embodiments of the invention, when the level of at least one protein set forth in SEQ ID Nos. 14, 24, 53, 58 or 59 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 14, 24, 53, 58 or 59 is increased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid eye disease with mild thyroid orbitopathy or an increased risk to develop thyroid eye disease with mild thyroid orbitopathy. In other various embodiments, when the level of at least one protein set forth in SEQ ID Nos. 1-5, 7-11, 17-19, 21, 23, 25, 26, 30-32, 34, 35, 37, 38, 40-42, 54, 55, 62, 63, 65, 66, 68, 69, 72, 75, 77, 78, 80, 83, 86, 88, 90, 91, 93, 95 or 97-106 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 1-5, 7-11, 17-19, 21, 23, 25, 26, 30-32, 34, 35, 37, 38, 40-42, 54, 55, 62, 63, 65, 66, 68, 69, 72, 75, 77, 78, 80, 83, 86, 88, 90, 91, 93, 95 or 97-106 is decreased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid eye disease with mild thyroid orbitopathy or an increased risk to develop thyroid eye disease with mild thyroid orbitopathy.

[00012] In various embodiments of the invention, when the level of at least one protein set forth in SEQ ID Nos. 13-16, 22, 24, 28, 29, 36, 43, 45, 47-53, 56, 58, 59, 70, 71, 73, 81, 89 or 96 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 13-16, 22, 24, 28, 29, 36, 43, 45, 47-53, 56, 58, 59, 70, 71, 73, 81, 89 or 96 is increased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid eye disease with severe thyroid orbitopathy or an increased risk to develop thyroid eye disease with severe thyroid orbitopathy. In other various embodiments, when the level of at least one protein set forth in SEQ ID Nos. 2-6, 8, 10, 12, 19, 20, 23, 27, 31-33, 39-41, 44, 46, 54, 55, 57, 60, 61, 64-67, 69, 74, 76, 77, 79, 82-85, 87, 88, 90, 92-95, 98 or 103-107 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 2-6, 8, 10, 12, 19, 20, 23, 27, 31-33, 39-41, 44, 46, 54, 55, 57, 60, 61, 64-67, 69, 74, 76, 77, 79, 82-85, 87, 88, 90, 92-95, 98 or 103-107 is decreased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid eye disease with severe thyroid orbitopathy or an increased risk to develop thyroid eye disease with severe thyroid orbitopathy.

[00013] In various embodiments of the invention, the thyroid disease is Graves' disease.

[00014] In various embodiments of the invention, the sample is a body fluid sample, preferably the body fluid is tear fluid. In still further various embodiments of the invention, the level of said at least one tear protein is determined.

[00015] In various embodiments of the invention, the level of said at least one tear protein is determined by using quantitative proteomics, preferably the at least one tear protein is labeled with an iTRAQ marker before being exposed to quantitative proteomics.

[00016] In various embodiments of the invention, the patient is a mammal, preferably the mammal is a human.

[00017] In another aspect, the present invention relates to a kit for detecting at least one of the proteins set forth in SEQ ID Nos. 1-124 or gene encoding a protein set forth in SEQ ID Nos. 1-124.

BRIEF DESCRIPTION OF THE DRAWINGS

[00018] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings.

[00019] **Figure 1** shows the expression of the indicated proteins in patients having thyroid disease without orbitopathy (TD) compared to their expression in healthy controls. (A) SEQ ID Nos. 7, 38, 99, 108 and 109 relate to proteins whose expression is increased in patients with TD compared to the control group. (B) SEQ ID Nos. 3, 21, 54, 63, 79 and 110-124 relate to proteins whose expression is decreased in patients with TD compared to the control group.

[00020] **Figure 2** shows the expression of the indicated proteins in patients having thyroid eye disease with mild orbitopathy (mild TED) compared to their expression in healthy controls. (A) SEQ ID Nos. 14, 24, 53, 58 and 59 relate to proteins whose expression is increased in patients with mild TED compared to the control group. (B) SEQ ID Nos. 1-5, 7-11, 17-19, 21, 23, 25, 26, 30-32, 34, 35, 37, 38, 40-42, 54, 55, 62, 63, 65, 66, 68, 69, 72, 75, 77, 78, 80, 83, 86, 88, 90, 91, 93, 95 or 97-106 relate to proteins whose expression is decreased in patients with mild TED compared to the control group.

[00021] **Figure 3** shows the expression of the indicated proteins in patients having thyroid eye disease with severe orbitopathy (mild TED) compared to their expression in healthy controls. (A) SEQ ID Nos. 13-16, 22, 24, 28, 29, 36, 43, 45, 47-53, 56, 58, 59, 70, 71, 73, 81, 89 or 96 relate to proteins whose expression is increased in patients with severe TED compared to the control group. (B) SEQ ID Nos. 2-6, 8, 10, 12, 19, 20, 23, 27, 31-33, 39-41, 44, 46, 54, 55, 57, 60, 61, 64-67, 69, 74, 76, 77, 79, 82-85, 87, 88, 90, 92-95, 98 or 103-107 relate to proteins whose expression is decreased in patients with severe TED compared to the control group.

[00022] **Figure 4** shows a list of proteins that are commonly up or down-regulated in mild and severe TED compared to the expression in healthy controls.

[00023] **Figure 5** shows the expression profile of clusterin in patients having TD, mild TED or severe TED. The expression of clusterin was measured in tear samples. It is demonstrated that this tear protein can be used to differentiate between TD and TED. The levels of clusterin can also be used to differentiate between mild TED and severe TED.

[00024] **Figure 6** shows the expression profile of mesothelin in patients having TD, mild TED or severe TED. The expression of mesothelin was measured in tear samples. It is demonstrated that this tear protein can be used to differentiate between TD and TED. The levels of mesothelin can also be used to differentiate between mild TED and severe TED.

DETAILED DESCRIPTION OF THE INVENTION

[00025] The present inventors surprisingly found that the expression profile of a specific group of tear proteins allows distinguishing healthy individuals from patients having thyroid disease without orbitopathy (TD) and patients having a mild or severe form of thyroid eye disease (TED).

[00026] Thus, in a first aspect, the present invention is thus directed to a method of diagnosing thyroid disease or diagnosing the risk of developing thyroid disease, said method comprising: (a) determining the level of at least one tear protein or the amount of expression of at least one gene encoding a tear protein in a sample obtained from a patient; (b) comparing the

data obtained in step a) to a predetermined level of at least one tear protein or the amount of expression of at least one gene encoding a tear protein observed in a healthy individual, wherein when the level of the at least one tear protein or the amount of expression of the at least one gene encoding a tear protein is changed compared to the predetermined levels in a healthy individual said patient is diagnosed with thyroid disease or an increased risk to develop thyroid disease.

[00027] In various embodiments of the invention, the thyroid disease is thyroid disease without thyroid orbitopathy and the tear protein is at least one protein set forth in SEQ ID Nos. 3, 7, 21, 38, 54, 63, 79, 99 or 108-124; or the thyroid disease is thyroid eye disease with mild or severe thyroid orbitopathy and the tear protein is at least one protein set forth in SEQ ID Nos. 1-107.

[00028] In various embodiments of the invention, when the level of at least one protein set forth in SEQ ID Nos. 7, 38, 99, 108 or 109 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 7, 38, 99, 108 or 109 is increased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid disease without thyroid orbitopathy or an increased risk to develop thyroid disease without thyroid orbitopathy. In other various embodiments, when the level of at least one protein set forth in SEQ ID Nos. 3, 21, 54, 63, 79 or 110-124 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 3, 21, 54, 63, 79 or 110-124 is decreased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid disease without thyroid orbitopathy or an increased risk to develop thyroid disease without thyroid orbitopathy.

[00029] In various embodiments of the invention, when the level of at least one protein set forth in SEQ ID Nos. 13, 14, 15, 16, 22, 24, 28, 29, 36, 43, 45, 47, 48, 49, 50, 51, 52, 53, 56 58, 59, 70, 71, 73, 81, 89 or 96 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 13, 14, 15, 16, 22, 24, 28, 29, 36, 43, 45, 47, 48, 49, 50, 51, 52, 53, 56 58, 59, 70, 71, 73, 81, 89 or 96 is increased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid eye disease with mild or severe thyroid orbitopathy or an increased risk to develop thyroid eye disease with mild or severe thyroid orbitopathy. In other various embodiments, when the level of at least one protein set forth in SEQ ID Nos. 1-12, 17-21, 23, 25, 26, 27, 30-35, 37-42, 44, 46, 54, 55, 57, 60-69, 72, 75-80, 82-88, 90-95 or 97-107 or the amount of expression of at least one gene encoding a protein set forth

in SEQ ID Nos. 1-12, 17-21, 23, 25, 26, 27, 30-35, 37-42, 44, 46, 54, 55, 57, 60-69, 72, 75-80, 82-88, 90-95 or 97-107 is decreased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid eye disease with mild or severe thyroid orbitopathy or an increased risk to develop thyroid eye disease with mild or severe thyroid orbitopathy.

[00030] In various embodiments of the invention, the thyroid disease is thyroid eye disease with mild thyroid orbitopathy and the tear protein is at least one protein set forth in SEQ ID Nos. 1-5, 7-11, 14, 17-19, 21, 23-26, 30-32, 34, 35, 37, 38, 40-42, 53-55, 58, 59, 62, 63, 65, 66, 68, 69, 72, 75, 77, 78, 80, 83, 86, 88, 90, 91, 93, 95 or 97-106; or the thyroid disease is thyroid eye disease with severe thyroid orbitopathy and the tear-specific protein is at least one protein set forth in SEQ ID Nos. 2-6, 8, 10, 12-16, 19, 20, 22-24, 27-29, 31-33, 36, 39-41, 43-61, 64-67, 69-71, 73, 74, 76, 77, 79, 81-85, 87-90, 92-96, 98 or 103-107.

[00031] In various embodiments of the invention, when the level of at least one protein set forth in SEQ ID Nos. 14, 24, 53, 58 or 59 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 14, 24, 53, 58 or 59 is increased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid eye disease with mild thyroid orbitopathy or an increased risk to develop thyroid eye disease with mild thyroid orbitopathy. In other various embodiments, when the level of at least one protein set forth in SEQ ID Nos. 1-5, 7-11, 17-19, 21, 23, 25, 26, 30-32, 34, 35, 37, 38, 40-42, 54, 55, 62, 63, 65, 66, 68, 69, 72, 75, 77, 78, 80, 83, 86, 88, 90, 91, 93, 95 or 97-106 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 1-5, 7-11, 17-19, 21, 23, 25, 26, 30-32, 34, 35, 37, 38, 40-42, 54, 55, 62, 63, 65, 66, 68, 69, 72, 75, 77, 78, 80, 83, 86, 88, 90, 91, 93, 95 or 97-106 is decreased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid eye disease with mild thyroid orbitopathy or an increased risk to develop thyroid eye disease with mild thyroid orbitopathy.

[00032] In various embodiments of the invention, when the level of at least one protein set forth in SEQ ID Nos. 13-16, 22, 24, 28, 29, 36, 43, 45, 47-53, 56, 58, 59, 70, 71, 73, 81, 89 or 96 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 13-16, 22, 24, 28, 29, 36, 43, 45, 47-53, 56, 58, 59, 70, 71, 73, 81, 89 or 96 is increased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid eye disease with severe thyroid orbitopathy or an increased risk to develop thyroid eye disease with severe

thyroid orbitopathy. In other various embodiments, when the level of at least one protein set forth in SEQ ID Nos. 2-6, 8, 10, 12, 19, 20, 23, 27, 31-33, 39-41, 44, 46, 54, 55, 57, 60, 61, 64-67, 69, 74, 76, 77, 79, 82-85, 87, 88, 90, 92-95, 98 or 103-107 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 2-6, 8, 10, 12, 19, 20, 23, 27, 31-33, 39-41, 44, 46, 54, 55, 57, 60, 61, 64-67, 69, 74, 76, 77, 79, 82-85, 87, 88, 90, 92-95, 98 or 103-107 is decreased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid eye disease with severe thyroid orbitopathy or an increased risk to develop thyroid eye disease with severe thyroid orbitopathy.

[00033] In various embodiments of the invention, the thyroid disease is Graves' disease.

[00034] In various embodiments of the invention, the sample is a body fluid sample, preferably the body fluid is tear fluid. In still further various embodiments of the invention, the level of said at least one tear protein is determined.

[00035] In various embodiments of the invention, the level of said at least one tear protein is determined by using quantitative proteomics, preferably the at least one tear protein is labeled with an iTRAQ marker before being exposed to quantitative proteomics.

[00036] In various embodiments of the invention, the patient is a mammal, preferably the mammal is a human.

[00037] In another aspect, the present invention relates to a kit for detecting at least one of the proteins set forth in SEQ ID Nos. 1-124 or gene encoding a protein set forth in SEQ ID Nos. 1-124.

[00038] The term "diagnosing thyroid disease", as used herein, relates to the identification of any thyroid disease in an individual. The diagnosis may be based on the use of different sample types and/or markers. The term "thyroid disease", as used herein, relates to any thyroid disease such Graves' disease, Hashimoto's thyroiditis/thyroiditis, thyroid cancer, Ord's thyroiditis, Postoperative hypothyroidism, Postpartum thyroiditis, Silent thyroiditis, Acute thyroiditis, Iatrogenic hypothyroidism, Thyroid hormone resistance, Euthyroid sick syndrome, Thyroid storm, Toxic thyroid nodule, Toxic nodular struma (Plummer's disease), Hashitoxicosis. In preferred embodiments of the invention, the thyroid disease is Graves' disease. "Risk to develop thyroid disease", as used in the present application, refers to the chance a person has,

over the course of his or her lifetime (from birth to death), of being diagnosed with thyroid disease. The term “prognosis” relates to a medical term for predicting the likely outcome of one’s current standing, e.g. the chance of developing a thyroid disease or for having complications including birth defects in pregnancy, increased risk of a miscarriage, and in extreme cases, death. Besides the survival rate, a prognosis may also relate to the chance of developing a specific type of thyroid disease, the survival time or the grade of a specific type of thyroid disease. In preferred embodiments of the invention, the methods of the present invention are able to determine a risk to develop thyroid disease that is at least 5%, at least 10%, at least 20%, at least 35%, at least 50%, at least 80%, at least 95% or 100% compared to a group of healthy controls.

[00039] The term “marker”, as used herein, relates to a protein or a gene/gene product whose level of expression is different in patients with a given disease compared to the levels in healthy control individuals and thus allows the detection of said disease. In various embodiments of the present invention, the disease that can be detected by the markers of the present invention is thyroid disease, preferably Graves’ disease. In other various embodiments of the invention, the marker is at least one protein set forth in SEQ ID Nos. 1-124 or at least one gene encoding a protein set forth in SEQ ID Nos. 1-124.

[00040] A “changed level” of a marker means that its concentration is increased or decreased relative to a normal state, i.e. a healthy individual not afflicted by thyroid disease. This term includes the situation that in the normal healthy state the marker is not detectable, e.g. is present in levels below the detection limit, but can be detected in thyroid disease patients. It is also possible to define a threshold level, where when the determined level is above this level, it is defined as increased or when the determined level is below this level, it is defined as decreased. In a preferred embodiment of the invention, the increased or decreased marker levels are at least 1.5-fold, at least 2-fold, at least 3-fold, at least 4-fold or at least 5-fold higher/lower compared to the above described control/normal state.

[00041] “At least one”, as used herein, relates to one or more, in particular 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more. Therefore, in various embodiments of the invention, at least 2, 3, 4, 5, 6, 7, 8, 9, 10 or more different markers set forth in SEQ ID Nos. 1-124 may be used for diagnosis or to determine the risk of developing thyroid disease.

[00042] The term “tear protein”, as used herein, relates to proteins that are found in tear fluid under healthy and/or pathological conditions. Tear proteins are not limited to proteins that are specifically expressed in tear fluid but may include such proteins.

[00043] The term “expression”, as used herein, relates to a process in which information from a gene is used for the synthesis of a gene product. In cell-based expression systems the expression comprises transcription and translation steps.

[00044] The term “protein”, as used herein, relates to one or more associated polypeptides, wherein the polypeptides consist of amino acids coupled by peptide (amide) bonds. The term polypeptide refers to a polymeric compound comprised of covalently linked amino acid residues. The amino acids are preferably the 20 naturally occurring amino acids glycine, alanine, valine, leucine, isoleucine, phenylalanine, cysteine, methionine, proline, serine, threonine, glutamine, asparagine, aspartic acid, glutamic acid, histidine, lysine, arginine, tyrosine and tryptophan.

[00045] The term “nucleic acid molecule” or “nucleic acid sequence”, as used herein, relates to DNA (deoxyribonucleic acid) or RNA (ribonucleic acid) molecules. Said molecules may appear independent of their natural genetic context and/or background. The term “nucleic acid molecule/sequence” further refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; “RNA molecules”) or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; “DNA molecules”), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms.

[00046] The term “gene”, as used in the present invention, relates to a nucleic acid sequence consisting either of deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) that code for a polypeptide or for an RNA chain that has a function in an organism. A gene is the basic physical unit of heredity. Further, a gene may be transcribed into RNA, so that the amount of expression of a given gene may be measured by detecting and quantifying the amount of the corresponding RNA. “RNA” or “ribonucleic acid”, as interchangeably used herein, relates to a

chain of nucleotides wherein the nucleotides contain the sugar ribose and bases selected from the group of adenine (A), cytosine (C), guanine (G), or uracil (U). "DNA" or "deoxyribonucleic acid", as interchangeably used herein, relates to a chain of nucleotides wherein the nucleotides contain the sugar 2'-deoxyribose and bases selected from adenine (A), guanine (G), cytosine (C) and thymine (T). The term "mRNA" refers to messenger RNA.

[00047] For the detection of the markers of the present invention specific binding partners may be employed. In some embodiments, the specific binding partners are useful to detect the presence of a marker in a sample, wherein the marker is a protein or RNA. The marker and its binding partner represent a binding pair of molecules, which interact with each other through any of a variety of molecular forces including, for example, ionic, covalent, hydrophobic, van der Waals, and hydrogen bonding. Preferably, this binding is specific. "Specific binding" means that the members of a binding pair bind preferentially to each other, i.e. usually with a significant higher affinity than to non-specific binding partners. The binding affinity for specific binding partners is thus usually at least 10-fold, preferably at least 100-fold higher than that for non-specific binding partners.

[00048] Exemplary binding partners for the markers of the invention are selected from the group consisting of antibodies, antibody fragments and variants, molecules with antibody-like properties, such as lipocalin muteins or Spiegelmers or aptamers. Antibody fragments and variants include Fv fragments, linear single chain antibodies and the like all of which are known to those skilled in the art.

[00049] Accordingly, in some embodiments the level of at least one or more markers is determined on mRNA level. In further embodiments, the level of at least one or more markers is determined on protein level. In various embodiments, at least one or more markers are determined at mRNA level and at least one or more markers are determined at protein level.

[00050] If a marker is determined on mRNA level, the mRNA may be the mRNA transcript, a 5'- and/or 3'-truncated mRNA or spliced mRNA forms. Such marker may be detected by PCR technologies well-known in the art.

[00051] If a marker is determined on protein level, the protein may be the full length protein or a fragment thereof. The protein fragment may be a truncated protein, i.e. lack one or

more amino acids at the N-terminus or C-terminus or both. This may be due to post-translational processing or due to the action of proteases present in the cell or the sample. The markers determined in the methods of the invention thus also include naturally occurring fragments, preferably immunogenic fragments. Also, the protein may be posttranslationally modified, e.g., phosphorylated, hydroxylated, glycosylated, N-glycosylated, O-glycosylated, ubiquitinated, sumoylated, acetylated, methylated, prenylated or sulphated.

[00052] In certain embodiments of the methods of the invention, in addition to the level of proteins set forth in SEQ ID Nos. 1-124 the levels of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50 or 100 or more markers additional are determined.

[00053] The methods detailed above, wherein the level of at least one or more markers is determined on protein level comprise in some embodiments the determination of the protein level by mass spectrometry, immunoassay, chromatography, Western Blot, or gel electrophoresis.

[00054] In some embodiments, the immunoassay may be, but are not limited to an Enzyme-linked Immunosorbent Assay (ELISA), Western blot, agglutination test, biotin/avidin type assays, radioimmunoassays, immunoelectrophoresis and immunoprecipitation. The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, or other methods for detecting the formation of a complex between the antigen and the antibody or antibodies reacted therewith. These and further immunoassays are well known in the art (David Wild (Ed.): The Immunoassay Handbook. 3rd ed. Elsevier Science Publishing Company, Amsterdam 2005).

[00055] The aforementioned assays may involve separation of unbound protein in a liquid phase from a solid phase support to which antigen-antibody complexes are bound. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e. g., in membrane or microtiter well form); polyvinylchloride (e. g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidene fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like.

[00056] More particularly, an ELISA method can be used, wherein the wells of a microtiter plate are coated with an antibody against the protein to be tested. A biological sample

containing or suspected of containing the marker is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate(s) can be washed to remove unbound moieties and a detectably labelled secondary binding molecule added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate washed and the presence of the secondary binding molecule detected using methods well known in the art.

[00057] In certain embodiments of the above detailed methods, if the determination is via mass spectrometry, the mass spectrometry may be selected from the group comprising MS measurements using EI, CI, ESI, APLI, APPI and APCI. In preferred embodiments of the invention, the at least one target protein/marker is labeled with an iTRAQ marker before being exposed to mass spectrometry.

[00058] The marker determination on protein level employing chromatography may be selected from the group comprising liquid chromatography, HPLC, FPLC, Smart chromatography, gel chromatography, size exclusion chromatography, reverse phase chromatography and ion-exchange chromatography (Introduction to Modern Liquid Chromatography, Lloyd R. Snyder, Wiley, 2009).

[00059] In various embodiments, if the marker is detected via gel electrophoresis, the gel electrophoresis may be selected from the group, but not limited to agarose gel electrophoresis, sodium dodecyl sulfate poly acryl amide gel electrophoresis (SDS-PAGE), 2D-gel electrophoresis, native gel electrophoresis and quantitative preparative native continuous polyacrylamide gel electrophoresis (QPNC-PAGE).

[00060] Of course, in certain embodiments of the methods of the present invention at least two determination methods may be coupled to each other in a subsequent manner. In a variant, a gel electrophoresis may be followed by a mass spectroscopic analysis. Alternatively, a gel electrophoresis may be followed by a Western Blot, a chromatography may be followed by a mass spectroscopic analysis, a chromatography may be followed by an immune assay, e.g. an ELISA.

[00061] Where in the methods detailed above a marker is determined on mRNA level, the RNA level may be determined by PCR, gel electrophoresis and/or Northern Blot.

[00062] In case the marker level is determined on the RNA level, the detection reagent may be a nucleic acid molecule, such as an oligonucleotide. The oligonucleotide may be a nucleic acid probe that may be labelled to allow detection or may be an oligonucleotide primer that allows amplification of the target molecule.

[00063] The term “comparing”, as used herein, relates to examination of two or more samples in order to note similarities or differences in the level of proteins SEQ ID Nos. 1-124 and/or gene encoding these proteins. Typically, one or more samples obtained from patients being tested (e.g. for their thyroid disease status) are “compared” to samples obtained from one or more control individuals. “Control individual” or “healthy individual”, as used interchangeably herein, means that any symptoms and/or complications that may relate to a specific disease that is investigated by the methods of the present application such as thyroid disease are absent in said individual. The health status of the control individual may have been determined by tests known in the art.

[00064] In various embodiments of the invention, the sample is a biological sample, for example a body fluid, cell or tissue sample. Body fluids comprise, but are not limited to tear fluid, blood, blood plasma, blood serum, breast milk, cerebrospinal fluid, cerumen (earwax), endolymph and perilymph, gastric juice, mucus (including nasal drainage and phlegm), peritoneal fluid, pleural fluid, saliva, sebum (skin oil), semen, sweat, vaginal secretion, nipple aspirate fluid, vomit and urine. The cell or tissue sample may comprise material originated from any part of the body such as connective tissue, muscle tissue, nervous tissue, and epithelial tissue. In preferred embodiments, the sample is tear fluid. The term “obtaining a sample”, as used herein, relates to different methods known in the art that comprise, but not limited to, filter based absorption, biopsy, sentinel node biopsy or removal of blood, bone marrow, sputum or bronchial fluids.

[00065] The term “tear fluid”, as used herein, relates to an eye fluid that is composed of a lipid, aqueous and mucin layer and has many functions including defending the ocular surface. The tear film covering the ocular surface presents a mechanical and antimicrobial barrier and ensures an optical refractive surface. The lipid component originates from the meibomian glands of the tarsus and forms the superficial layer of the tear film. The aqueous component contains electrolytes, water, and a large variety of proteins, peptides and glycoproteins, and is primarily

secreted by the lacrimal gland. Mucins are glycoproteins expressed by epithelial tissues of mucous surfaces. They protect tissues by functioning as antioxidants, providing lubrication, and inhibiting bacterial adherence.

[00066] In some embodiments the patient is a mammal, preferably a human. Generally, the term "mammal", as used herein, comprises humans, monkeys, pigs, cows, cats, dogs, guinea pigs, rabbits, mice, sheeps, goats and horses.

[00067] The term "Graves' disease", as used herein, relates to an autoimmune disease. It most commonly affects the thyroid, frequently causing it to enlarge to twice its size or more (goiter), become overactive, with related hyperthyroid symptoms such as increased heartbeat, muscle weakness, disturbed sleep, and irritability. In some forms of Graves' disease the eyes are also affected, causing bulging eyes (exophthalmos). In addition, Graves' disease may affect other systems of the body, including the skin, heart, circulation and nervous system. Thyroid-associated orbitopathy is one of the most typical symptoms of Graves' disease. It is known by a variety of terms, for example, Graves' ophthalmopathy, thyroid eye disease (TED), dysthyroid/thyroid-associated orbitopathy (TAO), Graves' orbitopathy. Thyroid eye disease is an inflammatory condition, which affects the orbital contents including the extraocular muscles and orbital fat. It is almost always associated with Graves' disease but may rarely be seen in Hashimoto's thyroiditis, primary hypothyroidism, or thyroid cancer. In general, thyroid eye disease causes the muscles and soft tissues within the eye socket to swell. This pushes the eyeball forward and causes various eye symptoms. Treatment involves steps to protect the eye as the disease runs its course. This may involve artificial tears, medicines and, in some cases, surgery. Thyroid eye disease is usually associated with an abnormality of thyroid gland function. The term "orbitopathy", as used herein, relates to diseases affecting the orbit and its contents.

[00068] The term "kit", as used herein, relates to packaged reagents for the detection of the presence of the marker proteins of SEQ ID Nos. 1-124 and/or genes encoding these proteins. Accordingly, the kits of the invention comprise in various embodiments detection reagents that allow determining the transcription levels of one or more marker proteins or genes identified according to the methods of the present invention. Additionally, such a kit may comprise instructions for use as well as typical reagents known to those skilled in the art. For example, the

exact number of reaction tubes, their holders, PCR-primers and/or antibodies etc. can be determined by the skilled person.

EXAMPLES

Materials and Methods

Patients and tear sample collection

[00069] Patients are diagnosed with Graves' orbitopathy according to the criteria proposed by Bartley et al. [Bartley et al., Am J Ophthalmol, 1995, 120(4), 511-7]. The activity and severity of the orbitopathy is graded according to the clinical classification system from the International Thyroid Eye Disease (TED) Society. The following clinical data is documented for all patients:

1. Age, gender, ethnic group, past medical history, smoking, any past ocular co-morbidities;
2. BCVA, pupil reflexes, colour vision, IOP, exophthalmometry, range of extraocular motility, TED inflammatory index (using the VISA classification 10), optic disc appearance and presence of maculopathy.

[00070] Human tear samples were obtained from the following groups of patients:

- 1) Thyroid disease patients with no orbitopathy;
- 2) Thyroid disease patients with inactive mild thyroid orbitopathy (lid signs, mild proptosis, mild myopathy);
- 3) Thyroid disease patients with active severe thyroid orbitopathy (activity score 5 or >5/10 using ITED classification plus at least one of the following: exophthalmometry > 25mm in one or two eyes, restrictive myopathy resulting in loss of central 20 degree field, exposure keratopathy or dysthyroid optic neuropathy);
- 4) Normal/healthy subjects (control group)

[00071] Tears are collected from each eye using Schirmer's filter paper strips placed for 5 minutes in the inferior cul-de-sac, with special care not to touch the ocular surface. After collection, the Schirmer's strips are immediately frozen at -80 degree C until analysis.

Sample preparation

[00072] The wet region of the strip was cut into small pieces and soaked in 150 μ L of phosphate-buffered saline (PBS) for 3 hours to elute tear proteins. Total tear protein concentrations were measured after elution using Micro BCA Protein Assay Kit (Pierce Biotechnology). Quantitative proteomics using iTRAQ technology coupled with 1D-nanoLC-MS/MS and a statistical model were used to uncover the potential tear protein biomarkers. The experimental design using iTRAQ relative quantitative proteomics technology is known in the art. Briefly, 50 μ g of tear proteins were reduced with by Tris-(2-carboxyethyl) phosphine (TCEP). After 1.5 h of incubation at 60 °C, the cysteine residue was blocked by the addition of methyl methanethiosulfonate (MMTS) at room temperature for 20min. The protein sample was then digested with trypsin for 16 h at 37 °C. The samples were subsequently labeled with iTRAQ reagent (iTRAQ kit, MA, USA). The samples were then combined and analyzed by one dimensional nanoLC-MS/MS.

One dimensional nanoLC-MS/MS proteomic analysis

[00073] The LC-MS/MS was performed using Dionex UltiMate 3000 (Dionex/Thermo Fisher Scientific, Waltham, MA, USA) coupled with the AB Sciex Triple TOF 5600. For the one dimension separation of peptides, sample was first loaded onto the trap column (Acclaim PepMap 75mm x 2cm C18 3 μ m x 100 Å by Dionex/Thermo Fisher Scientific, Waltham, MA, USA) for 5 minutes, at a flow rate of 5 μ L/min. The flow was then directed in line with the Acclaim PepMap RSLC column 75mm x 50cm C18 2 μ m x 100 Å (Dionex/Thermo Fisher Scientific, Waltham, MA, USA) at a flow rate of 0.3 μ L/min which is connected to the spray tip (PicoTip Emitter Silica Tip™ by New Objective, Woburn, MA, USA). The total step gradient time was set at 104 minutes. Mobile phases A (0.1% FA, 2% ACN in water) and B (0.1% FA, 2% water in ACN) were used to create the separation gradient for eluting peptides in the column at an increasing concentration of solvent B as follows: 5% to 30% for 69 minutes; 30% to 40% for 27 minutes; 40% to 60% for 7 minutes followed by 60% to 95% for 1 minute.

[00074] For IDA (Information dependent acquisition) experiment, all data were acquired from the Triple TOF MS using Analyst TF 1.5 software by AB Sciex in the Information-dependent acquisition (IDA) mode. Peptide profiling was performed using a mass range of 350 to 1250 Da mass range, followed by a MS/MS product ion scan from 100 to 1500 Da, with the abundance threshold set at more than 120 cps. The accumulation time for ions was set at 50ms. Target ions were excluded from the scan for 12s after being detected and former ions were excluded from the scan after one repetition. The IDA advanced 'rolling collision energy (CE)' option was required to automatically ramp up the CE value in the collision cell as the m/z value was increased. A maximum of 30 spectra were collected from candidate ions per cycle.

Example 1: Identification of tear proteins that are differentially expressed in the tear fluid of control groups, patients having thyroid disease without orbitopathy (TD) and patients having a mild or severe form of thyroid eye disease (TED)

[00075] In total, 1167 tear proteins were identified. Quantitative proteomics results showed that the tear protein profiles between normal control and patients having thyroid disease without orbitopathy (TD) are different with 5 up regulated and 20 down regulated proteins (SEQ ID Nos. 3, 7, 21, 38, 54, 63, 79, 99 and 108-124; Figure 1). Further, the tear protein profiles between normal control and patients having mild TED are different with 5 up regulated and 57 down regulated proteins (SEQ ID Nos. 1-5, 7-11, 14, 17-19, 21, 23-26, 30-32, 34, 35, 37, 38, 40-42, 53-55, 58, 59, 62, 63, 65, 66, 68, 69, 72, 75, 77, 78, 80, 83, 86, 88, 90, 91, 93, 95 and 97-106, Figure 2). In addition, the tear protein profiles between normal control and patients having severe TED are different with 27 up regulated and 51 down regulated proteins (SEQ ID Nos. 2-6, 8, 10, 12-16, 19, 20, 22-24, 27-29, 31-33, 36, 39-41, 43-61, 64-67, 69-71, 73, 74, 76, 77, 79, 81-85, 87-90, 92-96, 98 and 103-107, Figure 3). Common tear protein markers are present for mild and severe TED with 5 up regulated and 28 down regulated proteins (Figure 4).

[00076] Figures 5 and 6 indicate that two tear proteins (clusterin and mesothelin) can be used to differentiate between patients having TD without orbitopathy and TED. The levels of these two proteins can also be used to differentiate between mild TED and severe TED.

[00077] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of

the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject-matter from the genus, regardless of whether or not the excised material is specifically recited herein. Other embodiments are within the following claims. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

[00078] One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. Further, it will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The compositions, methods, procedures, treatments, molecules and specific compounds described herein are presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims. The listing or discussion of a previously published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

[00079] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including," "containing", etc. shall be read expansively and without limitation. The word "comprise" or variations such as "comprises" or "comprising" will accordingly be understood to imply the inclusion of a stated integer or groups of integers but not the exclusion of any other integer or group of integers. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by exemplary embodiments and optional features, modification and variation of the inventions embodied

therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[00080] The content of all documents and patent documents cited herein is incorporated by reference in their entirety.

CLAIMS

1. Method of diagnosing thyroid disease or diagnosing the risk of developing thyroid disease, said method comprising:
 - (a) determining the level of at least one tear protein or the amount of expression of at least one gene encoding a tear protein in a sample obtained from a patient,
 - (b) comparing the data obtained in step a) to a predetermined level of at least one tear protein or the amount of expression of at least one gene encoding a tear protein observed in a healthy individual, wherein when the level of the at least one tear protein or the amount of expression of the at least one gene encoding a tear protein is changed compared to the predetermined levels in a healthy individual said patient is diagnosed with thyroid disease or an increased risk to develop thyroid disease.
2. The method of claim 1, wherein
 - (a) the thyroid disease is thyroid disease without thyroid orbitopathy and the tear protein is at least one protein set forth in SEQ ID Nos. 3, 7, 21, 38, 54, 63, 79, 99 or 108-124; or
 - (b) the thyroid disease is thyroid eye disease with mild or severe thyroid orbitopathy and the tear protein is at least one protein set forth in SEQ ID Nos. 1-107.
3. The method of claim 2(a), wherein
 - (a) when the level of at least one protein set forth in SEQ ID Nos. 7, 38, 99, 108 or 109 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 7, 38, 99, 108 or 109 is increased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid disease without thyroid orbitopathy or an increased risk to develop thyroid disease without thyroid orbitopathy; or
 - (b) when the level of at least one protein set forth in SEQ ID Nos. 3, 21, 54, 63, 79 or 110-124 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 3, 21, 54, 63, 79 or 110-124 is decreased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid disease without thyroid orbitopathy or an increased risk to develop thyroid disease without thyroid orbitopathy.

4. The method of claim 2(b), wherein

(a) when the level of at least one protein set forth in SEQ ID Nos. 13, 14, 15, 16, 22, 24, 28, 29, 36, 43, 45, 47, 48, 49, 50, 51, 52, 53, 56 58, 59, 70, 71, 73, 81, 89 or 96 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 13, 14, 15, 16, 22, 24, 28, 29, 36, 43, 45, 47, 48, 49, 50, 51, 52, 53, 56 58, 59, 70, 71, 73, 81, 89 or 96 is increased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid eye disease with mild or severe thyroid orbitopathy or an increased risk to develop thyroid eye disease with mild or severe thyroid orbitopathy; or

(b) when the level of at least one protein set forth in SEQ ID Nos. 1-12, 17-21, 23, 25, 26, 27, 30-35, 37-42, 44, 46, 54, 55, 57, 60-69, 72, 75-80, 82-88, 90-95 or 97-107 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 1-12, 17-21, 23, 25, 26, 27, 30-35, 37-42, 44, 46, 54, 55, 57, 60-69, 72, 75-80, 82-88, 90-95 or 97-107 is decreased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid eye disease with mild or severe thyroid orbitopathy or an increased risk to develop thyroid eye disease with mild or severe thyroid orbitopathy.

5. The method of claim 2(b), wherein

(a) the thyroid eye disease is thyroid eye disease with mild thyroid orbitopathy and the tear protein is at least one protein set forth in SEQ ID Nos. 1-5, 7-11, 14, 17-19, 21, 23-26, 30-32, 34, 35, 37, 38, 40-42, 53-55, 58, 59, 62, 63, 65, 66, 68, 69, 72, 75, 77, 78, 80, 83, 86, 88, 90, 91, 93, 95 or 97-106; or

(b) the thyroid eye disease is thyroid eye disease with severe thyroid orbitopathy and the tear-specific protein is at least one protein set forth in SEQ ID Nos. 2-6, 8, 10, 12-16, 19, 20, 22-24, 27-29, 31-33, 36, 39-41, 43-61, 64-67, 69-71, 73, 74, 76, 77, 79, 81-85, 87-90, 92-96, 98 or 103-107.

6. The method of claim 5, wherein

(a) when the level of at least one protein set forth in SEQ ID Nos. 14, 24, 53, 58 or 59 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 14, 24, 53,

58 or 59 is increased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid eye disease with mild thyroid orbitopathy or an increased risk to develop thyroid eye disease with mild thyroid orbitopathy; or

(b) when the level of at least one protein set forth in SEQ ID Nos. 1-5, 7-11, 17-19, 21, 23, 25, 26, 30-32, 34, 35, 37, 38, 40-42, 54, 55, 62, 63, 65, 66, 68, 69, 72, 75, 77, 78, 80, 83, 86, 88, 90, 91, 93, 95 or 97-106 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 1-5, 7-11, 17-19, 21, 23, 25, 26, 30-32, 34, 35, 37, 38, 40-42, 54, 55, 62, 63, 65, 66, 68, 69, 72, 75, 77, 78, 80, 83, 86, 88, 90, 91, 93, 95 or 97-106 is decreased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid eye disease with mild thyroid orbitopathy or an increased risk to develop thyroid eye disease with mild thyroid orbitopathy.

7. The method of claim 5, wherein

(a) when the level of at least one protein set forth in SEQ ID Nos. 13-16, 22, 24, 28, 29, 36, 43, 45, 47-53, 56, 58, 59, 70, 71, 73, 81, 89 or 96 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 13-16, 22, 24, 28, 29, 36, 43, 45, 47-53, 56, 58, 59, 70, 71, 73, 81, 89 or 96 is increased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid eye disease with mild thyroid orbitopathy or an increased risk to develop thyroid eye disease with severe thyroid orbitopathy; or

(b) when the level of at least one protein set forth in SEQ ID Nos. 2-6, 8, 10, 12, 19, 20, 23, 27, 31-33, 39-41, 44, 46, 54, 55, 57, 60, 61, 64-67, 69, 74, 76, 77, 79, 82-85, 87, 88, 90, 92-95, 98 or 103-107 or the amount of expression of at least one gene encoding a protein set forth in SEQ ID Nos. 2-6, 8, 10, 12, 19, 20, 23, 27, 31-33, 39-41, 44, 46, 54, 55, 57, 60, 61, 64-67, 69, 74, 76, 77, 79, 82-85, 87, 88, 90, 92-95, 98 or 103-107 is decreased compared to the predetermined levels in a healthy individual the patient is diagnosed with thyroid eye disease with mild thyroid orbitopathy or an increased risk to develop thyroid eye disease with severe thyroid orbitopathy.

8. The method according to any one of claims 1 to 7, wherein the thyroid disease is Graves' disease.

9. The method according to any one of claims 1 to 8, wherein the sample is a body fluid sample.
10. The method according to any one of claims 1 to 9, wherein the body fluid is tear fluid.
11. The method according to any one of claims 1 to 10, wherein the level of said at least one tear protein is determined.
12. The method of claim 11, wherein the level of said at least one tear protein is determined by using quantitative proteomics.
13. The method of claim 12, wherein said at least one tear protein is labeled with an iTRAQ marker before being exposed to quantitative proteomics.
14. The method according to any one of claims 1 to 13, wherein the patient is a mammal.
15. The method of claim 14, wherein the mammal is a human.
16. A kit for detecting at least one of the proteins set forth in SEQ ID Nos. 1-124 or gene encoding a protein set forth in SEQ ID Nos. 1-124.

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Figure 1(A)

SEQ ID NO:	GENE ID	GEOMETRIC MEAN	P VALUE
7	ALDH3A1	3.972	0.020
108	CHMP4B	1.705	0.013
38	GSTO1	1.342	0.029
109	RARRES1	2.193	0.019
99	TXNDC17	1.337	0.045

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Figure 1(B)

SEQ ID NO:	GENE ID	GEOMETRIC MEAN	P VALUE
110	A1BG	0.164	0.035
3	AGR2	0.184	0.030
111	AHSG	0.53	0.031
112	ALB	0.12	0.014
113	APOA1	0.173	0.043
114	APOA2	0.128	0.046
21	CCT8	0.681	0.037
115	GC	0.216	0.019
116	HPX	0.206	0.015
117	IGHG4	0.095	0.013
118	IGHV1OR15-1	0.737	0.037
119	ITIH1	0.398	0.046
54	LAP3	0.447	0.043
120	LTF	0.126	0.003
63	ORM1	0.188	0.027
121	PLA2G2A	0.178	0.040
79	PRDX5	0.271	0.036
122	SERPINA1	0.139	0.006
123	SERPINC1	0.468	0.050
124	TF	0.092	0.003

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Figure 2(A)

SEQ ID NO:	GS	Geometric mean	Pvalue
14	AZGP1	3.089	0.046
24	CLU	2.415	0.03
53	LACRT	4.605	0.031
58	LYZ	3.363	0.045
59	MSLN	3.285	0.028

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Figure 2(B)

SEQ ID NO:	GS	Geometric mean	Pvalue
1	A2M	0.375	0.01
2	ADH1C	0.077	0.007
3	AGR2	0.678	0.024
4	AKR1A1	0.45	0.043
5	AKR1C1	0.303	0.036
7	ALDH1A3	0.417	0.005
8	ALDOA	0.442	0.01
9	ANXA3	0.388	0.011
10	ANXA5	0.505	0.029
11	APRT	0.397	0.026
17	CALML3	0.401	0.034
18	CAP1	0.469	0.047
19	CAPG	0.346	0.033
21	CCT8	0.469	0.031
23	CLIC1	0.819	0.026
25	CMPK1	0.625	0.005
26	CNDP2	0.72	0.049
30	EIF5A2	0.69	0.03
31	ENO1	0.336	0.045
32	FBP1	0.179	0.001
34	GBE1	0.56	0.037
35	GMDS	0.739	0.04
37	GSS	0.608	0.02
38	GSTO1	0.534	0.019
40	HSPA1B;HSPA1A	0.354	0.01
41	HSPA8	0.593	0.032
42	HSPB1	0.448	0.034
54	LAP3	0.332	0.004
55	LDHA	0.404	0.033
62	NQO1	0.404	0.044
63	ORM1	0.544	0.043
65	PARK7	0.511	0.034
66	PCBD1	0.703	0.041
68	PFN2	0.305	0.022
69	PGK1	0.594	0.03

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Figure 2(B) (continued)

72	PKM2	0.436	0.026
75	PPIA	0.284	0.021
77	PRDX1	0.357	0.021
78	PRDX2	0.506	0.044
80	PRR4	0.098	0.027
83	PSMB9	0.306	0.046
86	RAN;RANP1	0.723	0.012
88	S100A6	0.432	0.007
90	SELENBP1	0.39	0.013
91	SFN	0.636	0.025
93	SORD	0.575	0.007
95	TALDO1	0.683	0.033
97	TKT	0.447	0.008
98	TP11;TP11P1	0.399	0.011
99	TXNDC17	0.922	0.034
100	TYMP	0.563	0.045
101	UGP2	0.495	0.04
102	VCL	0.548	0.014
103	YWHAB	0.522	0.005
104	YWHAE	0.68	0.021
105	YWHAG	0.735	0.03
106	YWHAZ	0.508	0.003

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Figure 3(A)

SEQ ID NO:	GS	Geometric mean	Pvalue
13	ATP5B	1.223	0.021
14	AZGP1	5.535	0.007
15	BBS1	1.363	0.031
16	C4orf40	2.152	0.027
22	CHI3L2	3.078	0.007
24	CLU	4.622	0.002
28	CST3	1.965	0.038
29	CST4	3.929	0.005
36	GOLM1	1.831	0.036
43	HSPG2	2.626	0.003
45	IGHA1	4.813	0.017
47	IGHG3	1.412	0.038
48	IGJ	2.251	0.049
49	IGKV2-30	1.994	0.032
50	IGKV3D-15	1.731	0.023
51	IGLV1-47	1.614	0.021
52	IGLV3-19	1.895	0.049
53	LACRT	7.122	0.002
56	LGALS3BP	2.658	0.005
58	LYZ	4.891	0.029
59	MSLN	4.406	0.023
70	PIGR	3.316	0.041
71	PIP	4.188	0.023
73	PLTP	2.173	0.026
81	PRR4;PRH2;PRH1	13.996	0
89	SCGB2A1	2.012	0.041
96	TCN1	2.01	0.048

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Figure 3(B)

SEQ ID NO:	GS	Geometric mean	Pvalue
2	ADH1C	0.362	0.026
3	AGR2	0.411	0.014
4	AKR1A1	0.504	0
5	AKR1C1	0.26	0.037
6	ALDH1A1	0.287	0.015
8	ALDOA	0.38	0.012
10	ANXA5	0.189	0.005
12	ASS1	0.321	0.018
19	CAPG	0.284	0.004
20	CAPN1	0.687	0.031
23	CLIC1	0.745	0.029
27	CRYZ	0.707	0.019
31	ENO1	0.231	0.005
32	FBP1	0.568	0.032
33	FGG	0.493	0.005
39	HSP90AA1	0.532	0.023
40	HSPA1B;HSPA1A	0.291	0.006
41	HSPA8	0.518	0.024
44	IDH1	0.576	0.019
46	IGHG2	0.21	0.035
54	LAP3	0.431	0.021
55	LDHA	0.355	0.007
57	LXN	0.424	0.011
60	NAPRT1	0.453	0.003
61	NME2;NME1;NME1-NME2	0.733	0.021
64	ORM2	0.442	0.02
65	PARK7	0.383	0.017
66	PCBD1	0.54	0.025
67	PEBP1	0.4	0
69	PGK1	0.251	0.002
74	PPA1	0.738	0.01
76	PPP1R7	0.434	0.044
77	PRDX1	0.288	0.003
107	PRDX5*	0.277	0.01
79	PRDX5**	0.337	0.015

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Figure 3(B) (continued)

82	PSMA1	0.319	0.028
83	PSMB9	0.391	0.038
84	PYGL	0.662	0.023
85	RAB1B	0.654	0.037
87	S100A4	0.332	0.029
88	S100A6	0.327	0.001
90	SELENBP1	0.37	0.014
92	SLC9A3R1	0.643	0.049
93	SORD	0.385	0.023
94	TAGLN2	0.352	0.006
95	TALDO1	0.503	0.016
98	TPI1;TPI1P1	0.242	0.018
103	YWHAB	0.534	0.02
104	YWHAE	0.722	0.014
105	YWHAG	0.784	0.008
106	YWHAZ	0.46	0.024

PRDX5* : PRDX5 Isoform Cytoplasmic+peroxisomal of Peroxiredoxin-5, mitochondrial

PRDX5** : PRDX5 Isoform Mitochondrial of Peroxiredoxin-5, mitochondrial

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Figure 4

mild TED

severe TED

SEQ ID NO:	GENE ID	GEOMETRIC MEAN	P VALUE	GEOMETRIC MEAN	P VALUE
14	AZGP1	3.089	0.046	5.535	0.007
24	CLU	2.415	0.030	4.622	0.002
53	LACRT	4.605	0.031	7.122	0.002
58	LYZ	3.363	0.045	4.891	0.029
59	MSLN	3.285	0.028	4.406	0.023
2	ADH1C	0.077	0.007	0.362	0.026
3	AGR2	0.678	0.024	0.411	0.014
4	AKR1A1	0.450	0.043	0.504	0.000
5	AKR1C1	0.303	0.036	0.260	0.037
8	ALDOA	0.442	0.010	0.380	0.012
10	ANXA5	0.505	0.029	0.189	0.005
19	CAPG	0.346	0.033	0.284	0.004
23	CLIC1	0.819	0.026	0.745	0.029
31	ENO1	0.336	0.045	0.231	0.005
32	FBP1	0.179	0.001	0.568	0.032
40	HSPA1B;HSPA1A	0.354	0.010	0.291	0.006
41	HSPA8	0.593	0.032	0.518	0.024
54	LAP3	0.332	0.004	0.431	0.021

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Figure 4 (continued)

mild TED

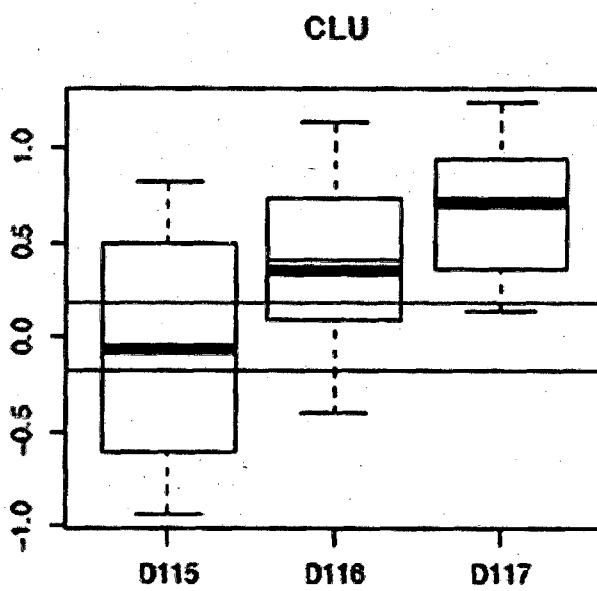
severe TED

55	LDHA	0.404	0.033	0.355	0.007
65	PARK7	0.511	0.034	0.383	0.017
66	PCBD1	0.703	0.041	0.540	0.025
69	PGK1	0.594	0.030	0.251	0.002
77	PRDX1	0.357	0.021	0.288	0.003
83	PSMB9	0.306	0.046	0.391	0.038
88	S100A6	0.432	0.007	0.327	0.001
90	SELENBP1	0.390	0.013	0.370	0.014
93	SORD	0.575	0.007	0.385	0.023
95	TALDO1	0.683	0.033	0.503	0.016
98	TPI1;TPI1P1	0.399	0.011	0.242	0.018
103	YWHAB	0.522	0.005	0.534	0.02
104	YWHAE	0.680	0.021	0.722	0.014
105	YWHAG	0.735	0.030	0.784	0.008
106	YWHAZ	0.508	0.003	0.460	0.024

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Figure 5

D115, Group2: TD alone
D116, Group3: TED, mild
D117, Group4: TED, severe
(Ratio vs control)



Boxplot



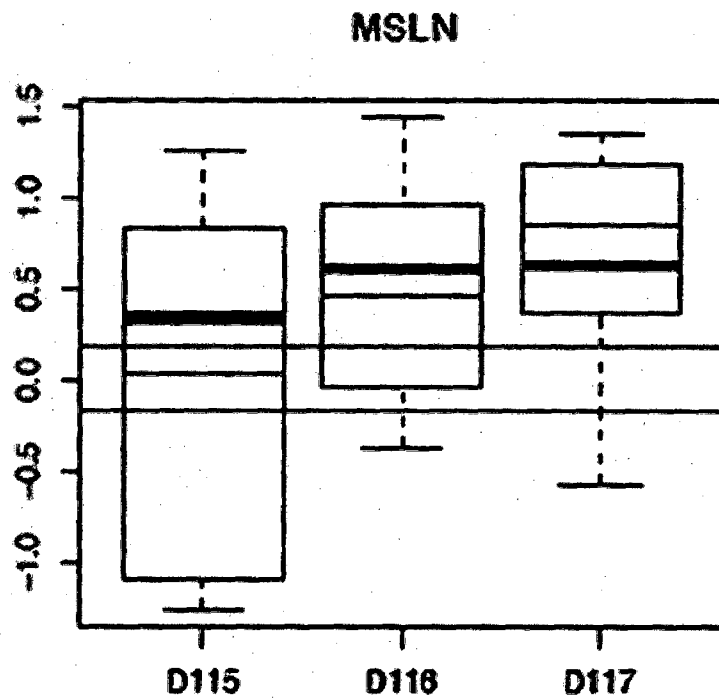
median

geometric mean

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Figure 6

D115, Group2: TD alone
D116, Group3: TED, mild
D117, Group4: TED, severe
(Ratio vs control)



Boxplot

— median
— geometric mean

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2014/000219

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/68 (2006.01) G01N 33/58 (2006.01)

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)

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

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

EPODOC, WPI, MEDLINE, HCAPLUS, BIOSIS, EMBASE: thyroid, graves' disease, thyroid eye disease, thyroid orbitopathy, grave's ophthalmopathy, tear, AGR2, AZGP1, CLU, LACRT, LYZ, MSLN, PARK7, PGK1, SORD, TPI1 and like terms

Google Scholar, Espacenet: Inventor/applicant name and thyroid

GenomeQuest: SEQ ID NOs: 3, 14, 24, 53, 58, 59, 65, 69, 93, 98

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 31 July 2014		Date of mailing of the international search report 31 July 2014	
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au		Authorised officer Jonathan Konrad AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61 2 6283 2601	

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/SG2014/000219
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Matheis, N. et al., 'Proteomics of tear fluid in thyroid-associated orbitopathy', Thyroid, 2012, Vol. 22, No. 10, pages 1039-1045 See the whole document, particularly the abstract, Tables 1-2 and page 1042 right column third paragraph	1-2, 4, 8-16
X	Baker, G. R. C. et al., 'Altered tear composition in smokers and patients with Graves ophthalmopathy', Archives of Ophthalmology, 2006, Vol. 124, pages 1451-1456 See the whole document, particularly the abstract and Table 2	1-2, 4, 8-16
X	US 2011/0275065 A1 (Walfish et al.) 10 November 2011 See the whole document, particularly Table 1, [0168]-[0173] and claim 11	16
L	Ujhelyi, B. et al., 'Graves' orbitopathy results in profound changes in tear composition: a study of plasminogen activator inhibitor-1 and seven cytokines', Thyroid, 2012, Vol. 22, No. 4, pages 407-414 To support lack of unity	
A	Tandon, N. et al., 'Expression and function of multiple regulators of complement activation in autoimmune thyroid disease', Immunology, 1994, Vol. 81, pages 643-647	

Form PCT/ISA/210 (fifth sheet) (July 2009)

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Supplemental Box**Continuation of: Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

This Authority has found that there are different inventions based on the following features that separate the claims into distinct groups:

- Invention 1: claims 1-16 (all in part). The feature of SEQ ID NO: 3 and its use as a tear protein marker for diagnosing thyroid disease is specific to this group of claims.
- Inventions 2-124: claims 1-16 (all in part). The feature of each different SEQ ID NO and its use as a tear protein marker for diagnosing thyroid disease is a separate invention.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. The only feature common to all of the claimed inventions and which provides a technical relationship among them is a tear protein marker for diagnosing thyroid disease. However this feature does not make a contribution over the prior art because it is disclosed in:

Ujhelyi, B. et al., 'Graves' orbitopathy results in profound changes in tear composition: a study of plasminogen activator inhibitor-1 and seven cytokines', *Thyroid*, 2012, Vol. 22, No. 4, pages 407-414

This document discloses plasminogen activator inhibitor (PAI) as a tear protein marker capable of distinguishing between healthy controls, Graves' disease without orbitopathy and Graves' orbitopathy (see the abstract, Table 1 and Fig. 1).

Therefore in the light of this document this common feature cannot be a special technical feature. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a posteriori*.

As a courtesy to the applicant for this case only, the Searching Authority decided to search 10 sequences of the applicant's choice and provide a search and opinion on these only. These sequences, as chosen by the applicant, are: SEQ ID NOs: 3, 14, 24, 53, 58, 59, 65, 69, 93 and 98 (AGR2, AZGP1, CLU, LACRT, LYZ, MSLN, PARK7, PGK1, SORD and TPI1).

INTERNATIONAL SEARCH REPORT Information on patent family members		International application No. PCT/SG2014/000219	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
US 2011/0275065 A1	10 November 2011	CA 2798562 A1	10 Nov 2011
		WO 2011137519 A1	10 Nov 2011
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2009)			