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DESCRIPTION

TECHNICAL FIELD

[0001] The application relates the field of diagnostic methods of IgA nephropathy. The present invention is directed to methods for diagnosis and monitoring using urine proteins, in particular alpha-1B-glycoprotein (A1BG), orosomucoid 1 (ORM1) and Ig lambda-2 chain C regions (IGLC2), as markers in IgA nephropathy.

BACKGROUND OF THE INVENTION

[0002] IgA nephropathy (IgAN) is the most common primary glomerulonephritis worldwide that may lead to a chronic kidney disease (CKD). CKD represents an increasing worldwide public health problem, which causes an extensive socio-economic burden for the society. The prevalence of CKD is up to 14,2% in the USA, 10,2% in Norway, and 11,9% in Poland. It is estimated that over 4 million people in Poland suffer from CKD and the number of patients with end stage renal disease (ESRD) on dialysis in Poland exceeds 18 000, in addition to 13 300 renal transplant recipients. Both, early stages of CKD and ESRD are associated with high morbidity and increased healthcare utilization. For example, in England, according to a recent report published by NHS Kidney Care, chronic kidney disease costs more than breast, lung, colon and skin cancer combined. Therefore, the IgAN focuses attention of researchers, clinicians and healthcare providers. The individuals affected by IgAN develop characteristic IgA-containing antibody complexes that deposit in the kidney producing tissue injury. Up to date, kidney biopsy with histopathologic evaluation is the best available method to diagnose IgAN. IgAN is a genetically complex trait, and not much is known about its pathogenesis and pathophysiology. Therefore, the treatment options are presently limited and empirics-based.

[0003] It is hoped that with an early diagnosis and treatment, it's possible to slow or even halt the progression of kidney diseases, such as IgAN. A pressing need also exists for personalizing the medical care and finding new molecularly targeted therapies in this disease. A great potential for new findings regarding diagnostic procedures for kidney disease lies in the '-omics' technologies, which can provide new data in regard to IgAN biology. Notably, however, while genetics-based methods can provide information correlated with the pathogenesis of the disease, the most directly related to its pathophysiology are the expression and production of proteins. The most accurate screening information as to the presence of specific proteins provides proteomics. Therefore, one of the most promising diagnostic tools is urine proteomics, particularly because the biological material can be obtained easily and comes directly from the diseased organ, the kidney. Indeed, it was previously reported that the presence of urinary proteins is indicative of glomerular damage and interstitial fibrosis. During the last decade, several valuable studies have linked proteomics to IgAN, and a number of urine proteins considered IgAN-specific have been reported. The study published by Mucha K.

at al. (Pol Arch Med Wewn. 2014;124 (7-8):380-386) presents a systematic analysis of urine proteomics from renal disease patients, namely IgAN versus healthy controls. Notably, the discoveries of alpha-1B-glycoprotein (A1BG), alpha-1-acid glycoprotein 1 (ORM1), Ig lambda-2 chain C regions (IGLC2) and serotransferrin (TF) have not been disclosed in Mucha et al. Urinary proteomics as means to identify markers in patients with ANCA-associated vasculitis, IgA nephropathy, and Henoch-Schönlein purpura nephritis was also discussed by Siwy J. at al. (Nephrology Dialysis Transplantation 2015; 30 (Suppl. 3): iii40-iii41).

[0004] Methods for kidney disease detection by protein profiling are known in the prior art. For example, WO2003002757 (A1) relates to improved methods of detecting an early stage of renal disease and/or renal complications of a disease, particularly diabetes, and discloses α 1 acid glycoprotein (also known as orosomucoid) that is used in a method for diagnosing a renal disease and/or renal complications of a disease in a subject. The disease comprises a disease selected from the group consisting of diabetes insipidus, diabetes type I, diabetes II and renal disease, including IgA nephropathy. A urinary protein fragmentation profile, in terms of the size, and sequence of particular fragments derived from intact filtered proteins together with the position where enzymes scission occurs along the protein polypeptide chain is characteristic of the diseased state of the kidney.

[0005] CN102590491 (A) discloses a testing method and a testing kit for early screening or diagnosis of urinary system diseasing. The method disclosed therein relies on identification and quantification of the following protein markers: COL6A1, GC, RBP4, DNASE1, GAA, SERPING1, CTSD, KNG1, UMOD, PIGR, AHSG, A1BG, AMBP, AZGP1, PTGDS, HPX, CD14 and ORM1. In this document specific protein marker combinations are suggested for renal cell carcinoma, bladder cancer, acute kidney injury, type II diabetes, IgA nephropathy, interstitial cystitis, polycystic kidney disease, and acute kidney transplant rejection.

[0006] US20160061845 (A1) discloses a method of diagnosing and treating a subject having a nephrotic syndrome, comprising the step of determining the level of one or more biomarkers in a biofluid, wherein the biomarker indicates a level of a protein selected from Vitamin D-binding protein (VDBP), Neutrophil gelatinase-associated lipocalin (NGAL), Fetuin A, AGP1, AGP2, A2MCG, and prealbumin.

[0007] US8927220 (B2) relates to the development of a protein that can be used for diagnosing IgA nephropathy and thin-glomerular-basement-membrane (hereinafter, referred to as "TGBM") nephropathy, and used as a biomarker for diagnosing serious cases thereof, and more particularly to a biomarker protein that shows increased/decreased levels in urine of IgA nephropathy patients or TGBM nephropathy patients compared to those in urine of normal people, and a diagnostic kit using the biomarker protein, which can be used to diagnose IgA nephropathy and TGBM nephropathy early, and predict and determine the degree of progression of the disease in advance. The biomarker protein that shows increased/decreased levels in urine of IgA nephropathy patients or TGBM nephropathy patients is selected from a vast list of biomarkers including Ceruloplasmin precursor, Alpha-1-antitrypsin precursor, Serotransferrin precursor Transferrin variant Fragment and Alpha-2-macroglobulin precursor.

[0008] US20140038203 (A1) discloses a method of detecting or predicting the onset or magnitude of kidney disease, such as acute kidney disease (AKI), previously called acute renal failure 1ARF. In various aspects, methods and kits are provided to detect specific urinary proteins associated with AKI diagnosis or prognosis using (a) angiotensinogen, apolipoprotein A-IV, pigment epithelium-derived factor, thymosin J34, insulin-like growth factor-binding protein I, myoglobin, vitamin D binding protein, complement C4-B, profilin-1, alpha-i antitrypsin, fibrinogen alpha chain, glutathione peroxidase 3, superoxide dismutase [Cu Zn], complement C3, antithrombin neutrophil defensin I, and (b) non-secretory ribonuclease, secreted Ly-6/uPAR-related protein I, pro-epidermal growth factor precursor (pro-EGF protein), and CD59 glycoprotein. Also the following markers are disclosed: Serotransferrin (P02787), Alpha-1-acid glycoprotein 1 (P02763), Alpha-1-acid glycoprotein 2 (ORM2) (P19652), Alpha-1B-glycoprotein (P04217), Ig lambda-2 chain C regions (IGLC2) (POCG05), Platelet glycoprotein VI (GP6) (Q9HCN6), SERPINA1, SERPINA3, SERPINA5, SERPINA7 and Cytosolic non-specific dipeptidase (CNDP2).

[0009] WO2013152989 (A2) relates to a cancer diagnostic and/or therapeutic and/or prognostic and/or patient stratification biomarker assay for the prognosis and/or diagnosis and/or therapy of colorectal cancer and/or lung cancer and/or pancreatic cancer comprising the combined measurement of at least two, preferably at least three protein/peptide biomarkers and/or fragments of protein biomarkers selected from a first group consisting of: CP; SERPINA3; PON1; optionally in combination with at least one or both protein/peptide biomarkers and/or fragments of protein biomarkers selected from a second group consisting of: IGFBP3; ATRN; LR61; TIMP1. In this publication SERPINA6 marker is also disclosed.

[0010] WO2011035323 (A1) relates to methods and compositions for monitoring, diagnosis, prognosis, and determination of treatment regimens in subjects suffering from or suspected of having a renal injury. In particular, the methods disclosed therein utilize a plurality of assays, one or more of which is configured to detect a kidney injury marker as diagnostic and prognostic biomarkers in renal injuries. Additional clinical indicia may be combined with the kidney injury marker assay result(s). These include other biomarkers related to renal status. Examples include the following consisting of metalloproteinase inhibitor 2, soluble oxidized low-density lipoprotein receptor 1, interleukin-2, von Willebrand factor, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor receptor superfamily member 11B, neutrophil elastase, interleukin-1 beta, heart-type fatty acid-binding protein, beta-2-glycoprotein 1, soluble CD40 ligand, coagulation factor VII, C-C motif chemokine 2, IgM, CA 19-9, IL-10, TNF-01, and myoglobin. It also discloses Ferritin (light chain, P02793; heavy chain P02794) and Alpha-1-acid glycoprotein 1 (P02763).

[0011] US2014235503 A1 indicates CNDP1 (also known as carnosinase) as protein associated with kidney function/dysfunction and publication in Postepy Hig. Med. Dosw. (2012); vol. 66, pages 215-221 discloses results of studies concerning carnosinase's role in kidney diseases, particularly in ischemia/reperfusion induced acute renal failure, diabetic nephropathy, gentamicin-induced nephrotoxicity and also in blood pressure regulation.

[0012] Even though a number of different markers related to renal diseases is significant, there still exists a need for providing highly selective and sensitive diagnostic methods and tests that would enable diagnosis and monitoring of IgAN. Moreover, there is a need for methods that are suitable to differentiate IgAN from other chronic kidney diseases.

DISCLOSURE OF INVENTION

[0013] The present invention aims to solve the above identified problems. The present inventors have disclosed that the urine protein concentration of alpha-1B-glycoprotein (A1BG), alpha-1-acid glycoprotein 1 (ORM1), Ig lambda-2 chain C regions (IGLC2), and serotransferrin (TF) level is changed in urine samples collected from patients suffering from IgA nephropathy (IgAN), as compared to healthy individuals or individuals with renal diseases of a different etiology, in particular autosomal dominant polycystic kidney disease (ADPKD) and lupus nephritis (LN). In particular, the expression of said markers in IgAN patients is higher than in healthy individuals or individuals with renal diseases of other etiology, such as ADPKD and LN. The term "expression" as used herein corresponds to amounts of said markers or their concentration levels in a urine sample.

[0014] The above indicated markers are unique for IgAN and have been selected based on analysis of urine samples from patients suffering from IgAN, ADPKD, LN and healthy controls. It is herein disclosed that the levels of each protein and their non-full-length fragments, i.e. marker proteins truncated on one or both sides of the amino acid sequence of the complete protein, and/or the combination thereof correlate with the disease type, thus allowing for detection of IgAN in a patient, its differentiation from renal diseases having different etiology, and monitoring of the IgAN patient response to a treatment.

[0015] Accordingly, disclosed herein is a use of a combination of alpha-1B-glycoprotein (A1BG), alpha-1-acid glycoprotein 1 (ORM1), and Ig lambda-2 chain C regions (IGLC2), optionally further in combination with one or both serotransferrin (TF) and platelet glycoprotein VI (GP6), wherein said markers also comprise the non-full-length fragments thereof, as markers in diagnosis, monitoring and differentiation of IgAN. Table 1 below lists the Uniprot ID accession numbers and summarizes the known functions for A1BG, ORM1, IGLC2 and TF.

Table 1. The overview of the proteins used in accordance with the present invention

No.	Name (gene symbol)	Uniprot ID	Molecular functions	Biological process
1	Alpha-1B-glycoprotein (A1BG)	P04217	Not known	Not known
2	Alpha-1-acid glycoprotein 1 (ORM1)	P02763	transport protein in the blood stream	acute-phase response, inflammatory response, transport
3	Ig lambda-2 chain	P0CG05	antigen binding	Fc-epsilon receptor signaling

No.	Name (gene symbol)	Uniprot ID	Molecular functions	Biological process
	C regions (IGLC2)			pathway, complement activation, innate immune response
4	Serotransferrin (TF)	P02787	ferric iron binding	transport

[0016] Brief molecular and functional characteristics of each of the molecules reported are presented below:

1. Alpha-1B-glycoprotein (A1BG) is a 54.3 kDa protein in humans that is constituted of 495 amino acids and encoded by the A1BG gene localized in 19q13.43 (by Entrez Gene). The protein shows sequence similarity to the variable regions of some immunoglobulin supergene family member proteins and contains five Ig-like V-type (immunoglobulin-like) domains. The function of the protein in biological systems is yet to be established. It has been reported that A1BG protein can be a subject for fragmentation and that the 13.8 kDa A1BG fragment has a high discriminatory power for steroid resistance in pediatric nephrotic syndrome, but is only present in a subset of patients (see Piyaphanee N et al. Proteomics Clin Appl. 2011; 5:334-42).
2. Human Alpha-1-acid glycoprotein 1 (AGP1), also referred to as Orosomucoid 1 (ORM1), is a 41-43-kDa glycoprotein encoded by the gene localized in human genome at 9q32 (by Entrez Gene). In humans, the peptide moiety is a single chain of 201 amino acids of 23.5 kDa of molecular weight. Carbohydrates constitute approximately the remaining 45% of the molecular weight of the posttranslationally modified protein, attached in the form of five to six highly sialylated complex-type-N-linked glycans. AGP1 belongs to the family of acute phase proteins. Accordingly, its serum concentration increases in response to systemic tissue injury, inflammation or infection. This increase in serum concentration results primarily from an elevated protein production in liver, as a part of an acute phase response. Expression of the AGP1 gene is a subject of regulation by a combination of the major regulatory mediators of an acute phase response, i.e. a cytokine network containing mainly interleukin-1beta (IL-1beta), tumor necrosis factor-alpha (TNFalpha), interleukin-6 and a range of IL-6-related cytokines as well as glucocorticoids. The biological function of AGP1 is not clear. The main known ability of AGP1 is to bind and to carry numerous basic and neutral lipophilic drugs from endogenous (e.g. steroid hormones) and exogenous (such as phenobarbital) origin. The primary factor influencing the immunomodulatory or the binding activities of AGP1 is related to the composition of carbohydrates bound to AGP1 polypeptide.
3. Ig lambda-2 chain C regions (IGLC2) is encoded by a gene belonging to the gene family of the constant region of immunoglobulin lambda chains. The rearranged IGLC2 gene (localized at 22q11.2, by Entez gene) encodes the protein constituted of approximately 106 amino acids of a theoretical weight of approximately 11.2 kDa. The main function of IGLC2 protein in participation in antigen recognition and binding as well

as subsequent initiation and regulation of antigen-specific immune response.

4. 4. Serotransferrin (TF), also referred to as transferrin or siderophilin, is a ~80 kDa acute-phase serum glycoprotein responsible for transportation of Fe^{3+} ions from sites of absorption and heme degradation to the sites of storage or degradation. The main site of production is liver, but this protein can be also produced in peripheral tissues. Serotransferrin plays a role in multiple processes in human body. In nephrotic syndrome, urinary loss of transferrin can be one of the causative mechanisms for an iron-resistant microcytic anemia. Used as a urine biomarker, serotransferrin has been reported one of the predictors of renal functional decline in lupus nephritis (see Abulaban KM et al. Lupus. 2016, in press).
5. 5. Platelet glycoprotein VI (GP6) is a 58-kD platelet membrane protein playing a substantial function in the collagen-induced activation and aggregation of platelets. It acts as a major role player in vascular homeostasis and integrity. For instance, it has been shown that inhibition of platelet GP6 protects against myocardial ischemia-reperfusion injury (see Pachel C et al. Arterioscler Thromb Vase Biol. 2016;36(4):629-35). In relation to kidney diseases, platelet recruitment to the inflamed glomerulus, which is crucial in the pathogenesis of certain forms of glomerulonephritis, has been reported to occur via an $\alpha\text{IIb}\beta\text{3}/\text{GPVI}$ -dependent pathway (see Devi S, Am J Pathol. 2010;177(3): 1131-42).

[0017] In the first embodiment of the invention a method of diagnosis of IgA nephropathy in a subject is provided. This method of the invention comprises

1. (a) a step of identification of the of a combination of A1BG, ORM1 and IGLC2, wherein said markers also comprise the non-full-length fragments thereof, in a urine sample from said subject and
2. (b) a step of quantitative or semi-quantitative comparison of the markers identified in step (a) with the markers identified in a healthy individual.

Preferably, a TF or GP6 as a further marker is identified and compared in the method of the invention.

[0018] The term "a non-full length fragment" as used herein refers to marker proteins truncated on one or both sides of the amino acid sequence of the complete protein. A non-full length fragment of A1BG marker is any A1BG protein fragment having molecular weight lower than 85 kDa and preferably any protein having molecular weight of 13 - 60 kDa. More preferably, a non-full length fragment of A1BG marker is a middle range length fragment of 35 - 60 kDa and/or a bottom length fragment of 13 - 17 kDa. A non-full length fragment of TF marker is any TF protein fragment having molecular weight lower than 80 kDa and preferably any protein having molecular weight of 10 - 70 kDa. For other two markers no non-full length fragments were observed.

[0019] The term "quantitative comparison" refers to a comparison made using a quantitative

measurement technique, wherein absolute amounts are measured. An example of such a technique includes mass spectrometry and ELISA. The term "semi-quantitative comparison" refers to a comparison made using a semi-quantitative measurement technique, wherein relative amounts are determined. An example of such a technique includes Western blot.

[0020] In said method of the invention a urine sample collected from a subject is analyzed, wherein said analysis comprises a step of separating all the solid parts from the sample, for example by filtration, centrifuging, or any other suitable method, and subsequently a step of identification of a combination of A1BG, ORM1 and IGLC2, and optionally GP6 and TF, as well as their non-full length fragments, in said urine sample.

[0021] The presence of the markers in the urine sample in the method of the invention can be preferably determined by mass spectrometry (MS). In this aspect of the invention, the amino acid sequence can be identified based mass-to-charge ratio used to generate high-resolution mass spectra. An example of that method is presented in Example 1 below. In preferred aspect of this invention a tandem mass spectrometry (MS/MS) can be used as it was previously described, for example, in Aebersold R and Mann M, *Nature*, 2003, 422(6928), 198-207, and in Yates III J. R., *Annual Review of Biophysics and Biomolecular Structure*, 2004, 33, 297-316. Alternatively, different MS based technics can also be used to identify the above identified combinations of markers in urine samples (such as MALDI (matrix-assisted laser desorption) imaging mass spectrometry (MALDI-IMS), liquid chromatography-mass spectrometry (LC-MS), and electrospray ionization ESI MS and their combination),

[0022] In a more preferred embodiment the combination of A1BG, ORM1 and IGLC2, and optionally GP6 and TF, can be identified in said urine sample by ELISA-based methods, including microfluidic ELISA, protein electrophoresis and Western blotting, including microfluidic electrophoresis and Western blotting using capillary electrophoresis. These methods are well known in the art.

[0023] Ultrasensitive microfluidic solid-phase ELISA was reported and described, for example, in *Lab Chip* 2013; 13(21), 4190-4197. This method is useful in rapid and ultrasensitive quantitative detection of low abundance proteins. The microwell-based solid-phase ELISA strategy provides an expandable platform for developing the next-generation microfluidic immunoassay systems that integrate and automate digital and analog measurements to further improve the sensitivity, dynamic ranges, and reproducibility of proteomic analysis.

[0024] The other method, Microfluidic Electrophoresis Assays for Rapid Characterization of Protein, was characterized and discussed in Science/AAAS audio webinar (14.11.2012) by Dr. Joey Studts from Boehringer Ingelheim in Germany, Dr. Timothy Blanc from ImClone Systems in Branchburg, New Jersey, and Dr. Bahram Fathollahi from PerkinElmer in San Francisco, California. What was discussed there concerned the application of high throughput microfluidic technologies to the analysis of biotherapeutic proteins. These microfluidic-based assays provide a good solution because they address the limitations of SDS-PAGE, as well as other separation assays that depend on conventional capillary electrophoresis in particularly analysis

time, which can be reduced to a minute or less per sample. Advantages include miniaturization, integration, and automation, which enable labs to perform experiments at a rapid turnaround time, thus faster analytical analysis to reduce time and expense in the process development.

[0025] In publication Anal Chem. 2011; 83(4), 1350-1355 a microscale Western blotting system based on separating sodium-dodecyl sulfate protein complexes by capillary gel electrophoresis followed by deposition onto a blotting membrane for immunoassay was described by Anderson et al. In the system, the separation capillary is grounded through a sheath capillary to a mobile X-Y translation stage, which moves a blotting membrane past the capillary outlet for protein deposition. The obtained results demonstrate substantial reduction in time requirements and improvement in mass sensitivity compared to conventional Western blots. Western blotting using capillary electrophoresis shows promise to analyze low volume samples with reduced reagents and time, while retaining the information content of a typical Western blot.

[0026] The above described analysis method makes it possible to determine marker patterns useful in monitoring of a response of a patient to a treatment for IgAN. The solution of the invention may eliminate the need to perform biopsy to confirm diagnosis of IgAN.

[0027] In a further embodiment a method of monitoring a response to an IgAN treatment is provided, wherein (a) in a first point in time the quantitative or semi-quantitative analysis of a combination of A1BG, ORM1 and IGLC2, wherein said markers also comprise the non-full-length fragments thereof, in a urine sample of a subject is performed; (b) subsequently the same analysis is carried out at a later point of time, and (c) the response to the IgA nephropathy treatment is assessed based on comparison of the results obtained in step (a) and (b), wherein the lower marker expression is indicative of a response to the treatment response. Preferably, in steps (a) - (c) TF or GP6, or the non-full-length fragments thereof, is used as a further marker. More preferably steps (a) and (b) in the method of monitoring of the invention are repeated.

[0028] Disclosed herein is also a kit for diagnosis, differentiation and monitoring of IgA nephropathy in a subject, which comprises at least two antibodies that specifically bind to markers selected from a group consisting of A1BG, ORM1, IGLC2 and TF, wherein said markers also comprise the non-full-length fragments thereof, and wherein said kit is equipped with means of identification of markers that bind the antibodies in said kit. The term "antibody"/ "antibodies" includes also antibody fragments or derivatives that specifically bind the markers. The kit may determine or provide instructions for calculating a ratio or relationship between the markers. Further the antibody or antibodies in the kit disclosed herein may be conjugated to a label, such as a fluorophore or an enzyme or alternatively the kit can be provided with any other detection means known in the field, that enable identification of the markers bound to the antibodies. The antibody or antibodies in the kit of the invention may be comprised in a lateral flow device. In the most preferred embodiment the kit comprises a microfluidic chip. The kit may further comprise a package insert providing instructions for measuring the expression

levels of the markers in a urine sample. The kit may further comprise instructions for determining the likelihood of developing a progressing or worsening IgAN in the subject.

[0029] The invention also provides the use of a combination of the protein markers consisting of A1BG, ORM1 and IGLC2 for diagnosis and monitoring of IgA nephropathy. Preferably, in accordance with the use of the invention serotransferrin (TF) or a truncated fragment thereof having molecular weight of 10 - 70 kDa or platelet glycoprotein VI (GP6) is used as a further marker for diagnosis and monitoring of IgA nephropathy.

BRIEF DESCRIPTION OF DRAWINGS

[0030] The invention was described in relation to the following figures of drawings in which

Fig. 1 present sample results of Western blot analysis of A1BG, ORM1, IGLC2 and TF content in urine samples derived from patients with renal diseases versus healthy controls;

Fig. 2A shows evaluation by Western blotting of the A1BG protein in selected urine samples of study participants with renal diseases (left-hand side of the blots) as compared to healthy subjects (right hand side of the blots). The presence of several subforms of the protein within various molecular weight can be noticed. Densitometry measurements were done for the upper, middle and bottom ranges separately. As shown in Fig 2B, each of the molecular ranges correlated differently with the clinical diagnosis and also as compared to the cumulative assessment of all ranges (Fig. 2C);

Fig. 3 shows a summary of densitometry readings from Western blotting of the ORM1 protein in selected urine samples of study participants with renal diseases as compared to healthy subjects.

Fig. 4 shows a summary of densitometry readings from Western blotting of the IGLC2 protein (~30 kDa) in selected urine samples of study participants with renal diseases as compared to healthy subjects.

Fig. 5 shows a summary of densitometry readings from Western blotting of the TF protein in selected urine samples of study participants with renal diseases as compared to healthy subjects.

Fig. 6 (A) present sample results of Western blot analysis of GP6 content in urine samples derived from patients with renal diseases versus healthy controls, and (B) shows a summary of densitometry readings from Western blotting of the GP6 protein in selected urine samples of study participants with renal diseases as compared to healthy subjects.

EXAMPLES

Example 1

Discovery phase

[0031] The methodological approach of the discovery phase has been described in Mucha et al. Below, the most pertinent information is listed.

Patients characteristics

Groups of patients

[0032] The study included 30 patients with IgAN and 30 healthy age- and sex-matched volunteers serving as controls. Demographic and clinical data of both groups are presented in Mucha, et al. (Supplementary material online, Table S1). Briefly, patients with biopsy-proven IgAN at different stages of chronic kidney disease (CKD) and older than 18 years were included. The inclusion criteria for the control group were as follows: age older than 18 years and absence of any kidney disease or other chronic diseases requiring treatment. The exclusion criteria for both groups included: active infection, history of malignancy, previous organ transplantation, or current pregnancy. To estimate GFR, we used the Chronic Kidney Disease Epidemiology Collaboration equations, which are the most accurate, have been evaluated in large diverse populations, and are applicable for clinical use. The study protocol was approved by the local ethics committee and informed consent was obtained from all participants. The study was performed in accordance with the Declaration of Helsinki.

Urine collection

[0033] Samples were collected from all individuals according to a uniform study protocol, following the recommendations on urine proteomic sample collection. The second- or third-morning midstream urine was collected to sterile urine containers 1 to 3 h after previous urination. The pH of each sample was stabilized at 7.2 by addition of 1/10th vol. of 1M HEPES pH 7.2 immediately after collection. Then, samples were vortexed for 2 min, centrifuged at 3000×g at room temperature for 10 min to clear the debris, filtered (0.4-µm filter, Rotilabo-Spritzenfilter, Roth, Karlsruhe, Germany), and portioned into 1-ml aliquots that were stored at -80°C before further use.

Sample filtration

[0034] Membrane filters of the 10 kDa cut-off (Amicon Ultra-0.5, UFC501096, Millipore, Billerica, United States) were washed twice with MilliQ (MQ) water prior to use. Urine was centrifuged through the membrane at 14,000×g for 15 min. Next, 500 µl MQ was added to the retentate and centrifugation step was repeated. To recover the concentrated and desalted sample, the filter was placed upside down and centrifuged in a clean microcentrifuge tube for 2 min at 1000×g. The protein concentration was measured by the Bradford method. Aliquots of samples were stored at -80°C.

Sample preparation

[0035] 30 IgAN samples were divided into 3 disease pooled samples (DPSs I, II, and III), and similarly, 30 control samples were divided into 3 control pooled samples (CPSs I, II, and III). Age and sex matching was preserved within the 3 pairs of pooled sample groups. All DPSs and CPSs were obtained in 2 technical replicates (marked A and B) each, making a set of 12 pooled samples to be compared after isobaric tags for relative and absolute quantitation (iTRAQ) labeling. As 4-plex iTRAQ was used, 2 technical replications of DPSs and CPSs were compared in 1 isoelectric focusing/liquid chromatography-mass spectrometry/mass spectrometry (IEF/LC-MS/MS) experiment. To analyze 12 samples, we conducted a set of 3 independent IEF/LC-MS/MS experiments. Aliquots with extracted peptides were stored at -80°C for the IEF/LC-MS/MS analysis.

Mass spectrometry

[0036] Qualitative MS/MS data processing The MS/MS data were pre-processed with Mascot Distiller (v. 2.3.2.0, Matrix Science, London, United Kingdom). Data search using the MASCOT search engine was conducted on the Swiss-Prot database with the taxonomy restricted to Homo sapiens (20,236 sequences) in a 3-step procedure described elsewhere to calculate MS and MS/MS measurement errors and to recalibrate the data for the repeated MASCOT to remove systematic bias. Protein ratios were calculated as the median ratio of their peptide's ratios. The statistical significance of a single protein ratio was assessed by an in-house program, Diffprot. Calculated P values were adjusted for multiple testing using a false discovery rate-controlling procedure, yielding protein ratio q values.

Results of the discovery phase

[0037] As a result of qualitative analysis (peptide and protein identification) in each of the 3 IEF/LC-MS-MS/MS experiments, 761, 951, and 956 proteins were identified, respectively, each represented by 2 or more peptides. The results of this observations were partially presented in

Mucha et al. The discovery of alpha-1B-glycoprotein (A1BG), alpha-1-acid glycoprotein 1 (AGP1, ORM1), Ig lambda-2 chain C regions (IGLC2) and serotransferrin (TF) as markers for IgAN is being reported in the current invention (Table 2).

Table 2. Peptide read-outs of urine proteomics specific for alpha-1B-glycoprotein (A1BG), alpha-1-acid glycoprotein 1 (AGP1, ORM1), Ig lambda-2 chain C regions (IGLC2) and serotransferrin (TF) obtained in a discovery phase of the invention.

<i>ID 1</i>	<i>Protein name</i>	<i>Gene</i>	<i>Number of peptides</i>
P04217	Alpha-1B-glycoprotein	A1BG	51
	Peptides	q-value	
	SLPAPW	4,26E-03	
	ITPGLK	4,66E-03	
	GVTFLLR	2,84E-03	
	SWPHTF	5,71E-03	
	LLELTGPK	0,00E+00	
	SWITPGLK	4,57E-04	
	ATWSGAVLAGR	0,00E+00	
	LELHVDGPPPR	7,51E-04	
	SLPAPWLSMAPV	0,00E+00	
	VAPLSGVDFQLR	0,00E+00	
	IFVGPQHAGNYR	6,31E-05	
	HQFLLTGDTQGR	0,00E+00	
	LETPDFQLFK	0,00E+00	
	SGLSTGWTQLSK	0,00E+00	
	SVIAPVSWITPGLK	1,67E-03	
	HGESSQVLHPGNK	0,00E+00	
	SLPAPWLSMAPVSW	6,55E-03	
	LELHVDGPPPRPQL	3,58E-04	
	HHGESSQVLHPGNK	7,56E-05	
	HQFLLTGDTQGRR	7,25E-03	
	GVAQEPVHLDSPAIK	0,00E+00	
	LELIFVGPQHAGNYR	0,00E+00	
	LELHVDGPPPRPQLR	2,78E-04	
	IFFHLNAVALGDGGHY	0,00E+00	
	NLELIFVGPQHAGNYR	0,00E+00	
	TFESELSDPVELLVAES	7,70E-04	
	GAAANLELIFVGPQHAGNYR	0,00E+00	

ID 1	Protein name	Gene	Number of peptides
P04217	Alpha-1B-glycoprotein	A1BG	51
	SLPAPWLSMAPVSWITPGLK	0,00E+00	
	TPGAAANLELIFVGPQHAGNYR	0,00E+00	
	SWVPHTFESELSDPVELLVAES	0,00E+00	
	TVRTPGAAANLELIFVGPQHAGNYR	0,00E+00	
	LHDNQNGWSGDSAPVELILSDETLPAPEFSPEPES GR	1,74E-03	
	TDGEGALSEPSATVTIEELAAPPPVLMHHGESS QVLHPGNK	0,00E+00	
	SWVPHTFE	6,44E-03	
	VGPQHAGNYR	6,31E-05	
	STGWTQLSK	9,51E-03	
	HQFLLTGDTQ	0,00E+00	
	PVSWITPGLK	0,00E+00	
	HVDGPPPRPQLR	1,02E-03	
	LSMAPVSWITPGLK	6,31E-05	
	MHHGESSQVLHPGNK	3,73E-04	
	SGLSTGWTQLSKLLELTGPK	3,87E-05	
	GPPPRPQLR	7,28E-03	
	SLPAPWLSMA	5,85E-04	
	LELHVDGPPPRPQ	4,34E-04	
	IFFHLNAVALGDGGH	0,00E+00	
	NGVAQEPVHLDSPAİK	3,87E-05	
	TPGAAANLELIFVGPQHAGN	0,00E+00	
	LPAPWLSMAPVSWITPGLK	3,87E-05	
ID 2	Protein name	Gene	Number of peptides
P02763	Alpha-1-acid glycoprotein 1	ORM1	29
	<i>Peptides</i>	<i>q-value</i>	
	TYMLAF	1,964E-03	
	AHLLILR	0,000E+00	
	NWGLSVY	6,310E-05	

<i>ID 2</i>	<i>Protein name</i>	<i>Gene</i>	<i>Number of peptides</i>
P02763	Alpha-1-acid glycoprotein 1	ORM1	29
	TYLNVQR	5,215E-03	
	YVGGQEHF	6,264E-03	
	FAHLLILR	1,789E-04	
	TTYLNVQR	4,565E-04	
	YVGGQEHFA	5,451E-04	
	YVGGQEHFAH	0,000E+00	
	EHFAHLLILR	6,704E-03	
	SDVYTDWK	0,000E+00	
	YVGGQEHFAHL	0,000E+00	
	MLAFDVNDEK	0,000E+00	
	YVGGQEHFAHLL	3,866E-05	
	GQEHFAHLLILR	0,000E+00	
	SVYADKPETTK	0,000E+00	
	TYMLAFDVNDEK	0,000E+00	
	GLSVYADKPETTK	3,866E-05	
	EQLGEFYEALDCLR	0,000E+00	
	YVGGQEHFAHLLILR	0,000E+00	
	WGLSVYADKPETTK	3,866E-05	
	NWGLSVYADKPETTK	0,000E+00	
	DVNDEKNWGLSVYADKPETTK	0,000E+00	
	TYMLAFDVNDEKNWGLSVYADKPETTK	0,000E+00	
	VVYTDWK	7,322E-03	
	VYADKPETTK	1,877E-03	
	VGGQEHFAHLLILR	2,390E-04	
	IPKSDVVYTDWK	6,846E-03	
	GGQEHFAHLLILR	3,901E-03	
<i>ID 3</i>	<i>Protein name</i>	<i>Gene</i>	<i>Number of peptides</i>
P0CG05	Ig lambda-2 chain C regions	IGLC2	4
	Peptides	q-value	
	ADSSPVK	5,85E-04	
	GVETTTPSK	1,61E-04	
	AGVETTTPSK	0,00E+00	

ID 3	Protein name	Gene	Number of peptides
P0CG05	Ig lambda-2 chain C regions	IGLC2	4
	KAGVETTTPSK	8,03E-04	
ID 4	Protein name	Gene	Number of peptides
P02787	Serotransferrin	TF	76
	Peptides	q-value	
	VYIAGK	4,49E-03	
	DSGFQMN	1,79E-04	
	HSTIFEN	1,05E-03	
	GLLYNK	3,85E-03	
	SAGWNIPI	9,31E-03	
	PDPWAK	5,33E-03	
	MYLGYEY	4,17E-04	
	NPDPWAK	1,79E-04	
	DSAHGFLK	0,00E+00	
	FGYSGAFK	2,78E-04	
	VAEFYGSK	0,00E+00	
	KDSGFQMN	9,23E-04	
	EFQLFSSPH	2,43E-04	
	KPVEEYAN	6,13E-04	
	DGAGDVAFVK	0,00E+00	
	SAGWNIPIGLL	0,00E+00	
	EDLIWELLN	3,73E-04	
	YLGEEYVK	3,87E-05	
	HSTIFENLAN	0,00E+00	
	GYGYTGAFR	0,00E+00	
	KPVDEYK	4,57E-04	
	IPMGLLYNK	3,87E-05	
	DSGFQMNQLR	0,00E+00	
	PVVAEFYGSK	0,00E+00	
	LAQVPSHTVVAR	0,00E+00	
	KPVDEYKD	8,27E-04	
	EGYYGYTGAFR	3,87E-05	
	SAGWNIPIGLLY	6,31E-05	

<i>ID 4</i>	<i>Protein name</i>	<i>Gene</i>	<i>Number of peptides</i>
P02787	Serotransferrin	TF	76
	NIPMGLLYNK	2,78E-04	
	HQTVPQNTGGK	0,00E+00	
	QYFGYSGAFK	6,31E-05	
	TAGWNIPMGLLY	6,31E-05	
	SLDGGFVYIAGK	0,00E+00	
	SASDLTWDNLK	0,00E+00	
	HSTIFENLANK	0,00E+00	
	EFQLFSSPHGK	0,00E+00	
	TAGWNIPMGLLYN	3,87E-05	
	SKEFQLFSSPH	2,29E-03	
	KDSGFQMNQLR	0,00E+00	
	EDLIWELLNQAQ	1,61E-04	
	MYLGYEYVTAIR	0,00E+00	
	KEGYGYTGAFR	0,00E+00	
	LKPVVAEFYGSK	0,00E+00	
	KSASDLTWDNLK	0,00E+00	
	TAGWNIPMGLLYNK	0,00E+00	
	STLNQYFGYSGAFK	6,31E-05	
	NLKPVVAEFYGSK	0,00E+00	
	EDPQTFYYAVAVVK	0,00E+00	
	SKEFQLFSSPHGK	0,00E+00	
	AIAANEADAVTL DAGLVYDAY	0,00E+00	
	LAPNNLKPVVAEFYGSK	0,00E+00	
	EDLIWELLNQAQEHFGK	0,00E+00	
	IMNGEADAMS LDGGFVYIAGK	3,87E-05	
	AIAANEADAVTL DAGLVYDAY LAPN	0,00E+00	
	GKEDLIWELLNQAQEHFGK	0,00E+00	
	GGKEDLIWELLNQAQEHFGK	0,00E+00	
	EDLIWELLNQAQEHFGKDK	0,00E+00	
	SVIGGKEDLIWELLNQAQEHFGK	0,00E+00	
	AIAANEADAVTL DAGLVYDAY LAPNNLKPVVA EFYGSK	0,00E+00	

ID 4	Protein name	Gene	Number of peptides
P02787	Serotransferrin	TF	76
	APNHAVVT	8,70E-03	
	APNHAVVTR	6,31E-05	
	SAGWNIPIGL	8,24E-03	
	QVPSHTVVAR	1,06E-03	
	STIFENLANK	0,00E+00	
	HLAQVPSHTVVAR	0,00E+00	
	GWNIPMGLLYNK	6,31E-05	
	MYLGYEYVTAIRNLR	3,86E-03	
	PNNLKPVVAEFYGSK	5,33E-03	
	HSTIFENLA	5,85E-04	
	ADRDQYELL	1,81E-03	
	QLFSSPHGK	1,75E-03	
	LGYEYVTAIR	2,69E-03	
	HSTIFENLANKADR	5,85E-04	
	HQTVPQNTGGKNPDPWAK	3,87E-05	
	KEDLIWELLNQAQEHFGK	1,52E-03	
	GLVYDAYLAPNNLKPVVAEFYGSK	3,87E-05	

Example 2

Validation phase

[0038] The primary difference between the discovery and validation phases is the transition from the assessment of the pooled urine samples (i.e. the discovery phase) to the individual evaluation of each protein in a given patient or a healthy person and a direct correlation of these results with the known clinical parameters in each case (i.e. the validation phase).

Patient characteristics

[0039] The study included 133 renal disease patients and 19 healthy controls. Renal disease included IgAN (77 cases), ADPKD (29) and LN (27).

Sample collection

[0040] Urinary samples were collected according to the protocol standardized in the Transplantation Institute, Medical University of Warsaw.

SDS-PAGE

[0041] Samples were defrozen to room temperature (~23°C), then suspended by intensive pipetting or mixing using a vortex. Leammli buffer was added to urine samples to achieve final concentrations as follows: 2% SDS; 10% glycerol; 5% β -mercaptoethanol; 0,002% bromophenol blue; 0,125 M Tris-HCl; pH 6,8. Samples were boiled at 95 ° C for 2 min. 10 μ l of each sample was loaded on the Mini-PROTEAN TGX 4-15% gradient gel.

Western blotting analysis

[0042] The method developed by the Department of Molecular Biology, Institute of Biochemistry and Biophysics, Polish Academy of Sciences (patent application no P.415033) allows to assay all the proteins of interest in the urine samples. It permits the analysis of all the selected protein biomarkers with the accuracy not reachable by classical methods. To date, the proteome analysis of urine in medicine starts from centrifugation of the sample (in line with the European Confederacy of Laboratory Medicine guidelines), which result losing of protein which are insoluble, and exist as aggregates or degradants. This solid fraction is crucial because proteins progress into insoluble forms randomly, depending of protein, state of patient, his diet and properties of urine. For that reason, in the current study we used the whole urine in form of suspension, which next were analyzed by Western blotting technique. That gives an opportunity to examine all of proteins present in a given in urine sample. Advantages of this method are important for medicinal diagnostics. Method is noninvasive for patients, it allows collecting the samples from the patients even few times per day, and it is relatively little time consuming. For Western blotting analysis, the urine protein samples were separated on SDS-PAGE gels, as described above, and transferred to a nitrocellulose membrane. Membranes were incubated in appropriate blocking buffer (either 5% low-fat dry milk or bovine serum albumin in TBS-Tween 20 (TBST)). After an incubation in the primary antibody (A1BG (F-9); catalog number sc-374415; Santa Cruz) the cells were washed in TBST, and incubated with a horseradish peroxidase-conjugated secondary antibody. The chemiluminescence reaction for HRP was developed using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) and visualized with Stella 8300 bioimager. Densitometry read-outs were carried out for each of the bands in the blots. Eight randomly chosen patients samples were pooled together and used on each Western blot as a benchmark. Densitometry read-outs from other band on a given Western blot membrane were divided by the read-out of the respective benchmark. The results were referred to as a "relative band density".

[0043] List of antibodies used for Western blotting: A1BG (cat # sc-374415, Santa Cruz), ORM1 (sc-69753, Santa Cruz), IGLC2 (sc-33134, Santa Cruz), TF (sc-21011, Santa Cruz), GP6 (sc-20149, Santa Cruz).

Results

[0044] The results of representative Western blotting analysis for A1BG, ORM1, IGLC2 and TF presence in urine samples are presented in Figure 1.

[0045] A1BG Based on the Western blotting analysis, it becomes evident that study participants with kidney diseases exhibit the presence of different forms of the protein (for the purpose of this invention segregated into within the high [~80 kDa], medium [~45 kDa] and low [~15 kDa] molecular weight range), occurring in different proportions. A direct comparison of selected samples from kidney disease versus healthy patients is presented in Figure 2A. Mutual relations between the visible forms may be important in the pathophysiology of the given disease. Indeed, as presented in Figure 2B, various subforms of A1BG correlate differently with the type of kidney disease and also differently as compared to the cumulative assessment of the protein (Figure 2C). Notably, the bottom-range bands tend to be most prominently elevated in IgAN patients.

[0046] Validation phase as described above was also performed for the other markers, in particular ORM1, IGLC2 and TF and the results are presented in Figs. 3-5. The results are also presented for GP6 (Fig. 6). Although GP6 is on average expressed at higher levels in LN than IgA, on the basis of our results and data, the compilation of 4 proteins reported in the GP6 compilation may increase the sensitivity and specificity of the test.

REFERENCES CITED IN THE DESCRIPTION

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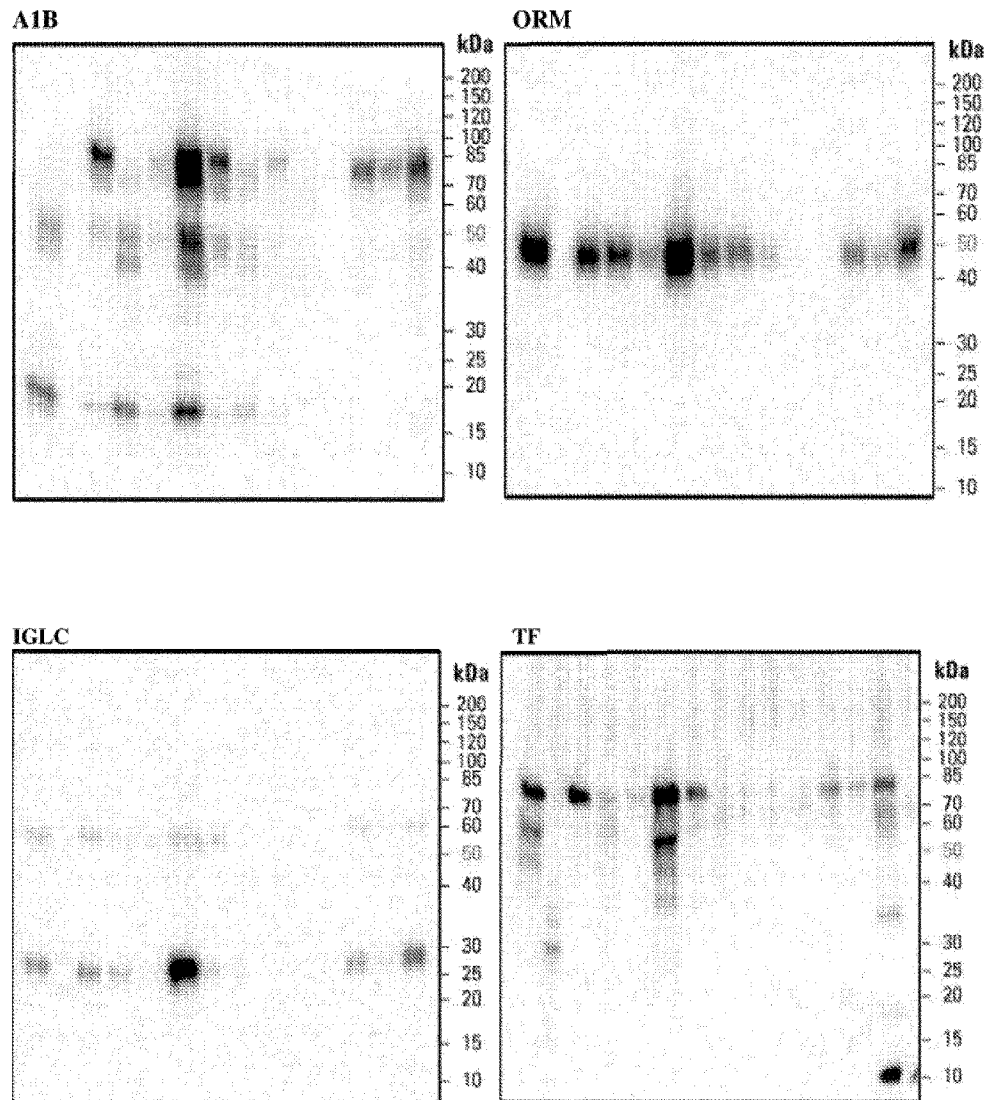
Patentkrav

1. Fremgangsmåde til diagnose af IgA-nefropati hos et forsøgsobjekt, omfattende
5
(a) et trin til identifikation af en kombination af alfa-1B-glykoprotein (A1BG) eller et afkortet fragment deraf, som har en molekylvægt på 13-60 kDa, orosomucoid 1 (ORM1) og Ig lambda-2-kæde C-områder (IGLC2) som proteinmarkører i en urinprøve fra forsøgsobjektet og
(b) et trin til kvantitativ eller semi-kvantitativ sammenligning af de markører, der blev
10 identificeret i trin (a), med de markører, der blev identificeret hos en sund person.
2. Fremgangsmåden ifølge krav 1, hvor en serotransferrin (TF) eller et afkortet fragment deraf, som har en molekylvægt på 10-70 kDa, eller blodpladeglykoprotein VI (GP6) som en yderligere markør, identificeres og sammenlignes.
15
3. Fremgangsmåden ifølge krav 1 eller 2, hvor markørerne identificeres ved hjælp af teknikker, der omfatter massespektrometri eller en antistofbaseret test, eller en kombination af teknikkerne.
- 20 4. Fremgangsmåden ifølge krav 3, hvor den antistofbaserede test er Western-afttryk.
5. Fremgangsmåde til overvågning af en reaktion på en IgA-nefropatibehandling, hvor
(a) på et første tidspunkt den kvantitative eller semi-kvantitative analyse af en kombination af alfa-1B-glykoprotein (A1BG) eller et afkortet fragment deraf, som har en molekylvægt på 13-
25 60 kDa, orosomucoid 1 (ORM1) og Ig lambda-2-kæde C-områder (IGLC2) som proteinmarkører i en urinprøve fra et forsøgsobjekt udføres;
(b) efterfølgende den samme analyse udføres på et senere tidspunkt, og
(c) reaktionen på IgA-nefropatibehandlingen evalueres på grundlag af sammenligning af de resultater, der blev opnået i trin (a) og (b), hvor det lavere markørudtryk er indikativ for en
30 reaktion på behandlingen.
6. Fremgangsmåden ifølge krav 5, hvor i trin (a) til (c) serotransferrin (TF) eller et afkortet fragment deraf, som har en molekylvægt på 10-70 kDa eller blodpladeglykoprotein VI (GP6) anvendes som en yderligere markør.

7. Fremgangsmåden ifølge krav 5 eller 6, hvor (a), (b) og (c) gentages.
- 5 8. Anvendelse af en kombination af proteinmarkører, der består af alfa-1B-glykoprotein (A1BG), orosomucoid 1 (ORM1) og Ig lambda-2-kæde C-områder (IGLC2) til diagnose og overvågning af IgA-nefropati.
- 10 9. Anvendelsen ifølge krav 8, hvor serotransferrin (TF) eller et afkortet fragment deraf, som har en molekylvægt på 10-70 kDa, eller blodpladeglykoprotein VI (GP6) anvendes som en yderligere markør til diagnose og overvågning af IgA-nefropati.

DRAWINGS

Fig. 1



WB: A1BG

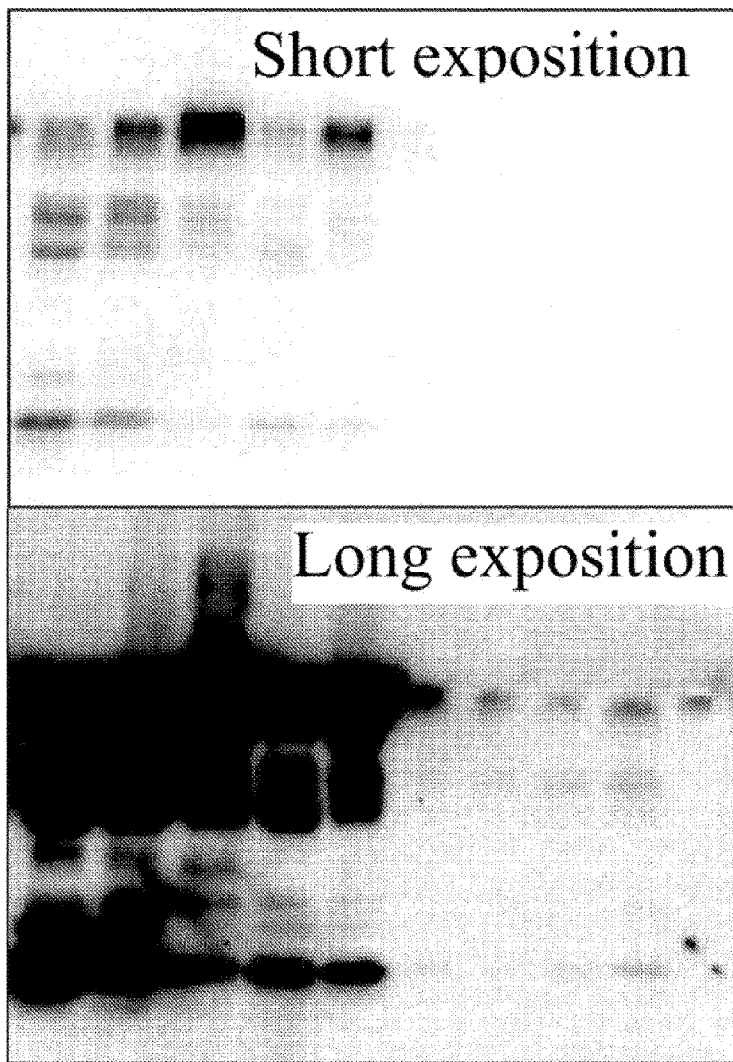


Fig. 2 A

<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>	<u>8</u>	<u>9</u>	<u>10</u>
Patients with					Healthy				
kidney					controls				

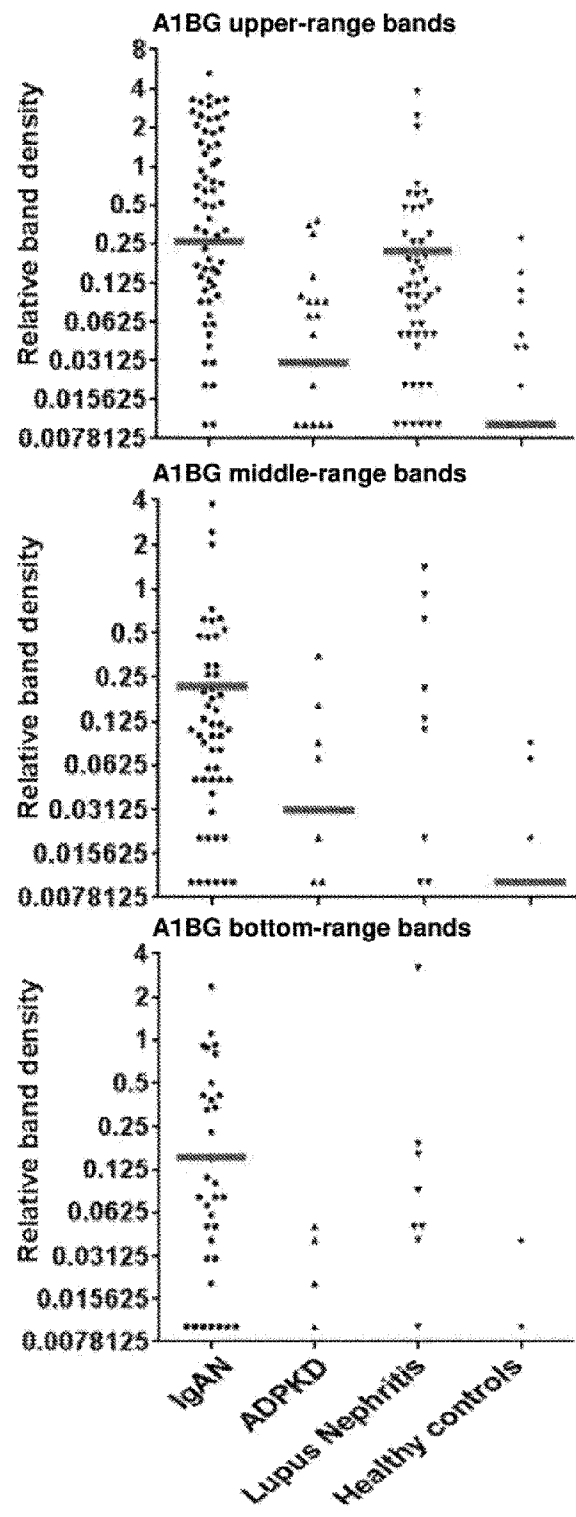


Fig. 2 B

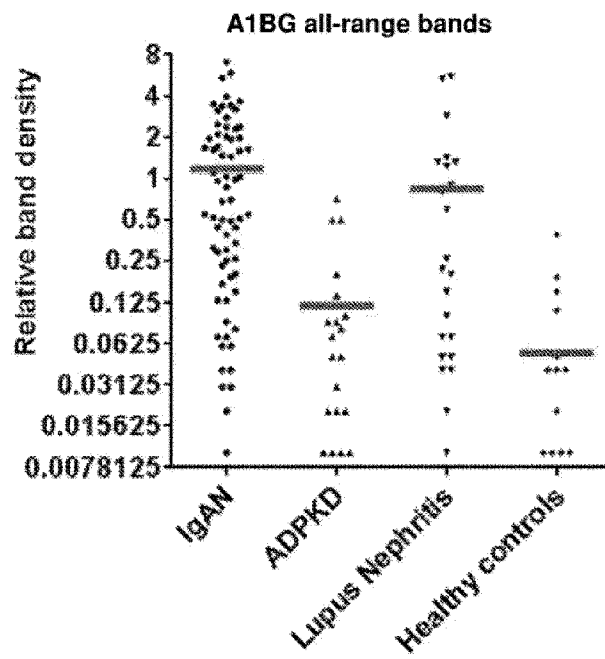


Fig. 2 C

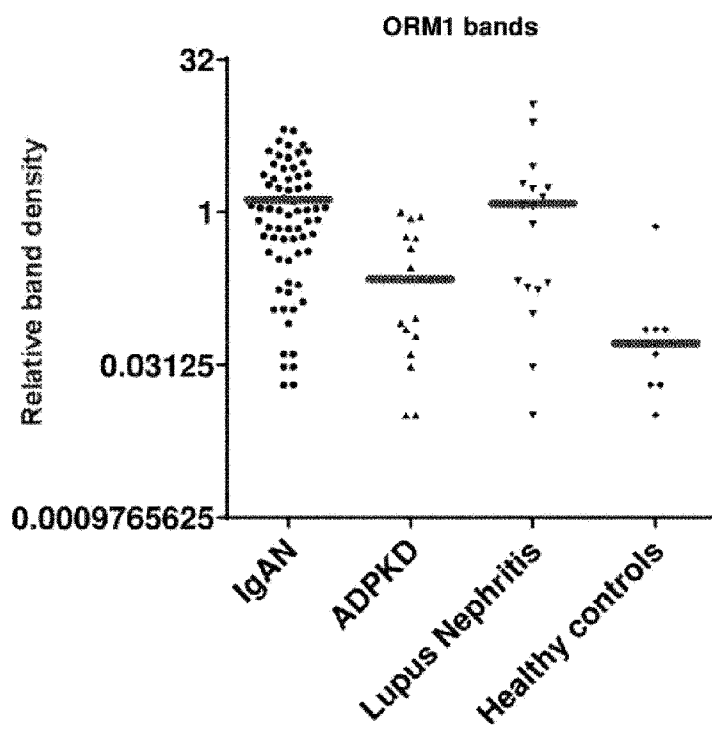
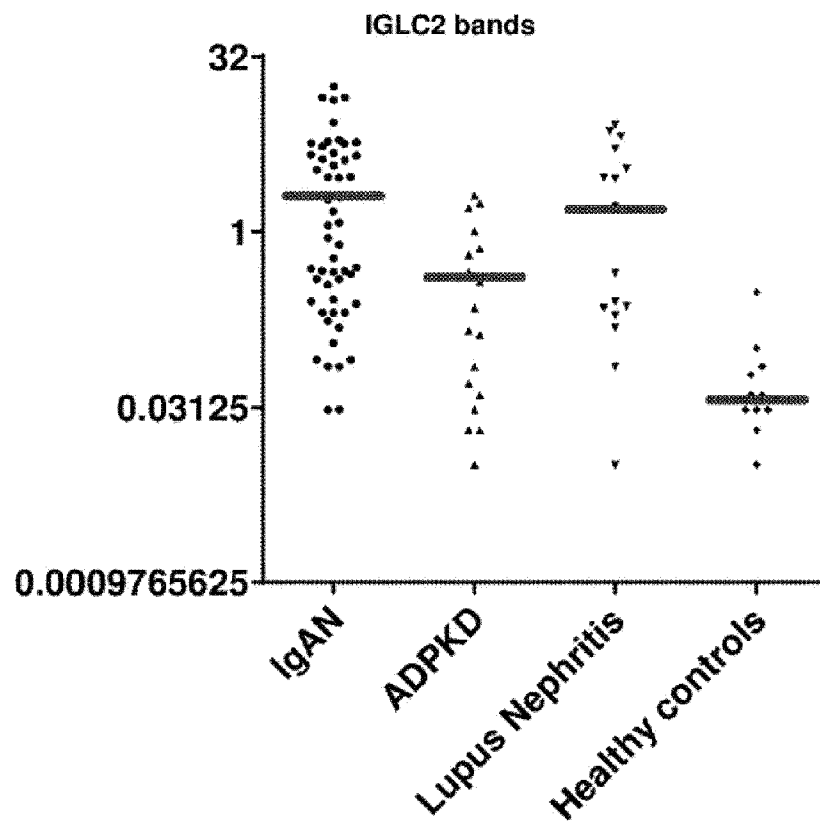


Fig. 3

Fig. 4



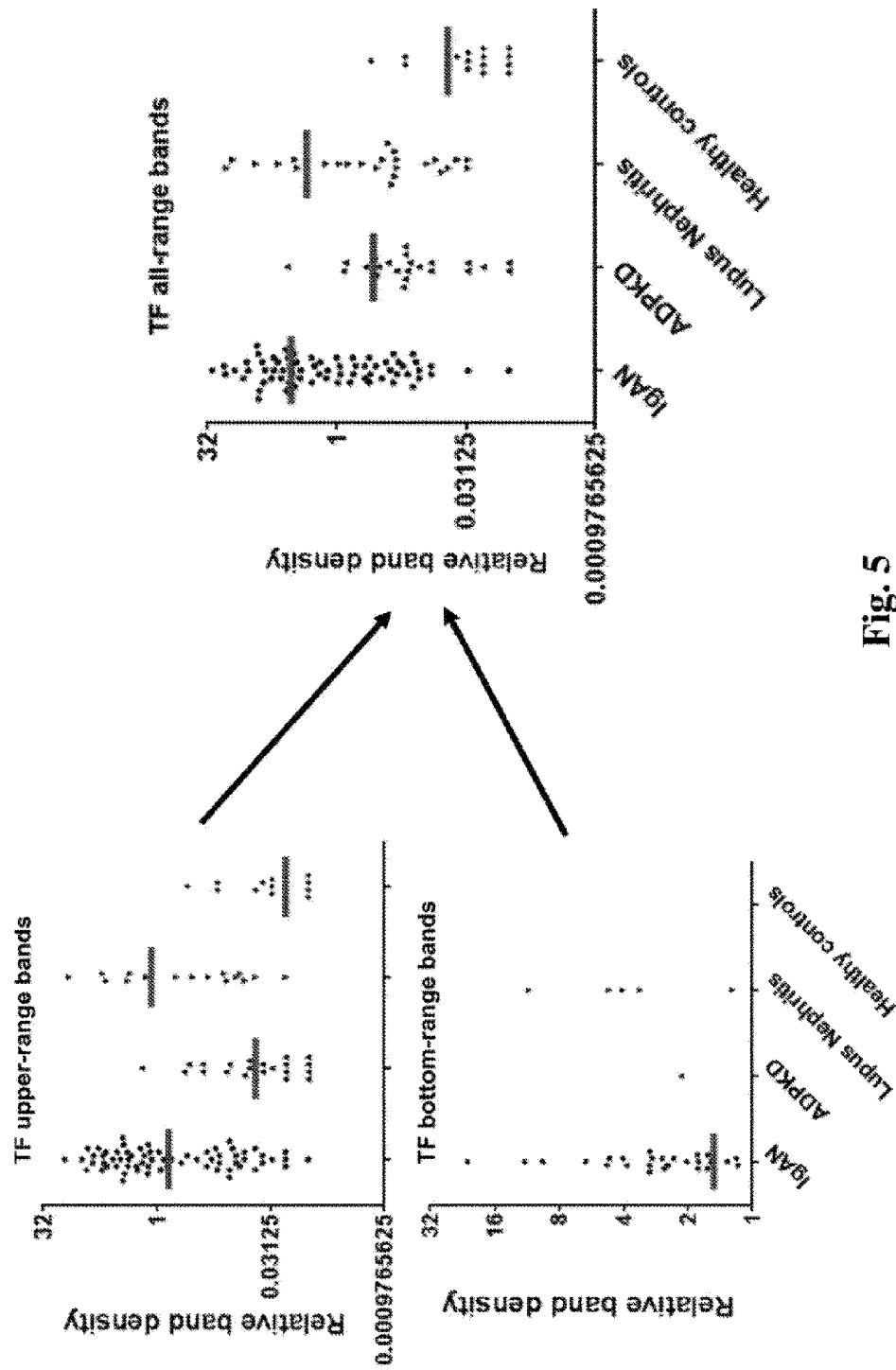
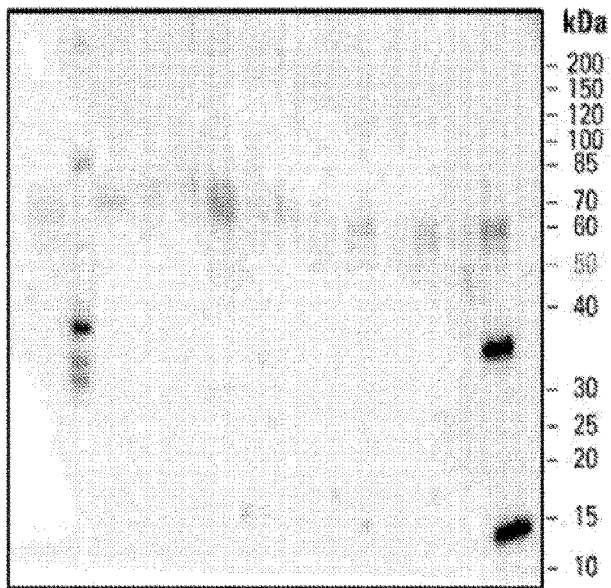


Fig. 5

Fig. 6 A

GP6



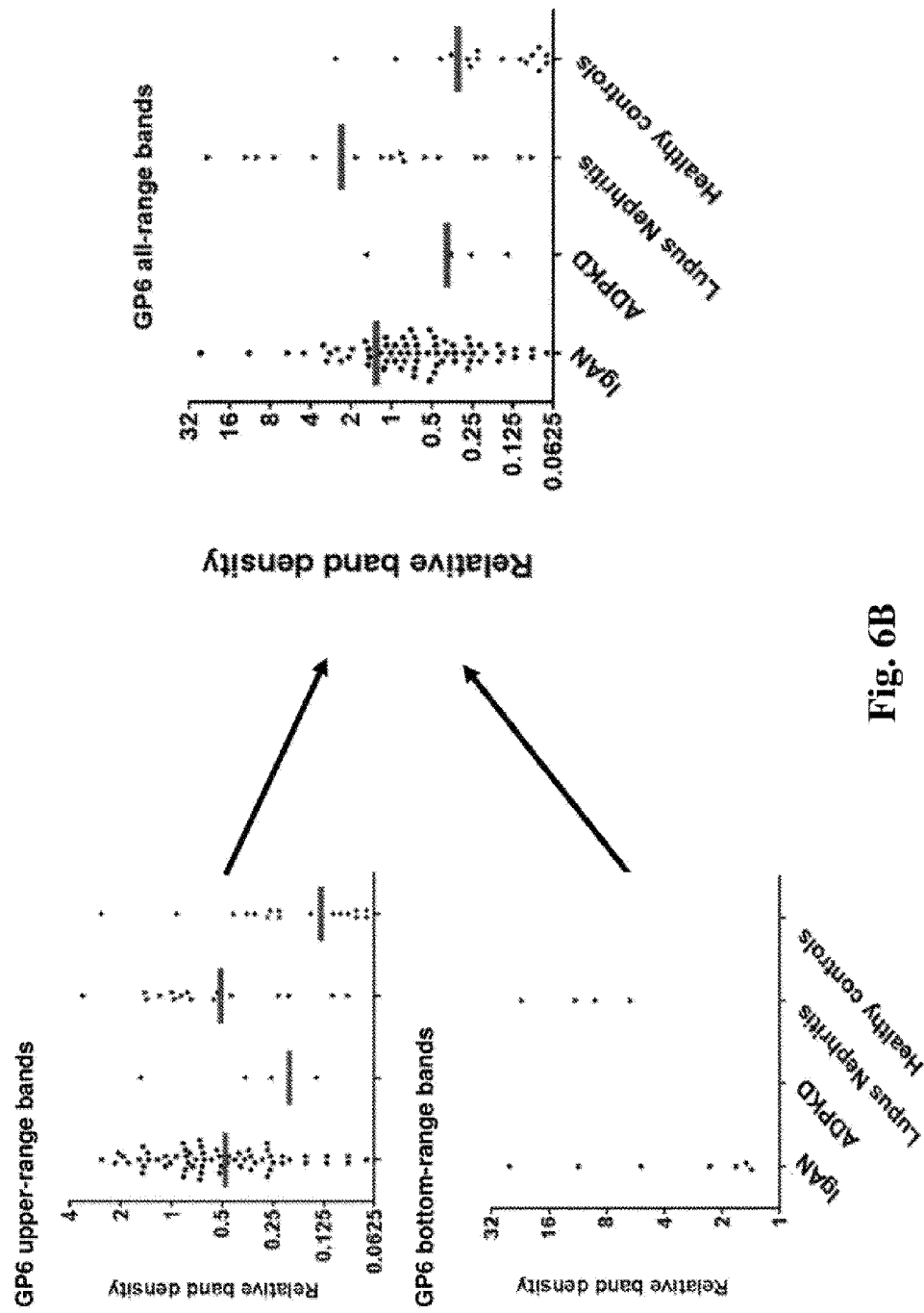


Fig. 6B