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Urinary Protein-Biomarkers Reliably Indicate Very Early Kidney Damage in Children With Alport Syndrome Independently of Albuminuria and Inflammation

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Introduction: Alport syndrome (AS) is a hereditary type IV collagen disease. It starts shortly after birth, without clinical symptoms, and progresses to end-stage kidney disease early in life. The earlier therapy starts, the more effectively end-stage kidney disease can be delayed. Clearly then, to ensure preemptive therapy, early diagnosis is an essential prerequisite.

Methods: To provide early diagnosis, we searched for protein biomarkers (BMs) by mass spectrometry in dogs with AS stage 0. At this very early stage, we identified 74 candidate BMs. Of these, using commercial enzyme-linked immunosorbent assays (ELISAs), we evaluated 27 in dogs and 28 in children, 50 with AS and 104 healthy controls.

Results: Most BMs from blood appeared as fractions of multiple variants of the same protein, as shown by their chromatographic distribution before mass spectrometry. Blood samples showed only minor differences because ELISAs rarely detect disease-specific variants. However, in urine , several proteins, individually or in combination, were promising indicators of very early and preclinical kidney injury. The BMs with the highest sensitivity and specificity were collagen type XIII, hyaluronan binding protein 2 (HABP2), and complement C4 binding protein (C4BP).

Conclusion: We generated very strong candidate BMs by our approach of first examining preclinical AS in dogs and then validating these BMs in children at early stages of disease. These BMs might serve for screening purposes for AS before the onset of kidney damage and therefore allow preemptive therapy.

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AS is a hereditary type IV collagen disease and is the most common monogenetic cause of chronic kidney disease (CKD). In its classical form, AS leads to end-stage kidney disease early in life. Angiotensin-converting-enzyme-inhibitors are able to delay disease progression if nephroprotective therapy is initiated preemptively. Therefore, it is unfortunate that early diagnosis of AS[1] is rare and only available where the diagnosis has already been made in relatives. This is because there are no preclinical or screening diagnostic parameters before the onset of proteinuria despite nonspecific microhematuria. Therefore, there is no practicable BM yet in use for the preclinical detection of AS despite several early cellular alterations recently being claimed as potential "early" BMs.[2-7] Histopathological changes occur only late in disease progression. Genetic testing is recommended in symptomatic children.[8] However, many children with AS are diagnosed too late to benefit very much from preemptive therapy.

AS develops only slowly in early childhood because of the maturation of the glomerular basement membrane (GBM). This delay opens a "window of opportunity" for early diagnosis and therapy. Thus, there is

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an urgent need for preclinical BMs to act as GBM alarm signals before proteinuria. The earlier angiotensin-converting-enzyme-inhibition starts in an affected child, the better the chances are of improving life quality and life expectancy.[9-11]

Early diagnosis is therefore very important and was the ultimate target of our research into early BMs. However, finding BMs is also important for monitoring responses to therapy and for finding new therapeutic strategies.

Therefore, because early diagnosis is so important, we restricted our search for BMs to early stages of AS: stage 0 (isolated microhematuria) and stage I (micro-albuminuria).[12] We defined stage 0 as "preclinical," because, despite microhematuria, there is no other specific sign of ongoing kidney disease. Searching for preclinical BMs is very challenging and not possible in toddlers. In addition, different time courses must be considered.

Our investigation spanned 10 years. It fell naturally into 4 parts, each building on the other as follows:

1. Screening in animal models: Our discovery phase started in animal models of AS with the goal of uncovering BMs of type A and B for glomerular injury (Figure 1). Mice and dogs with AS are ideal subjects for such an investigation because, given that type IV collagens are highly conserved, AS develops in all mammals.[13-19] Our search started in preclinical mice,[13] and was improved by data from dogs.
2. Validation in animal models: We validated some BMs in AS animals at preclinical stages of disease thereby demonstrating the reliability of the proteomic search principle.
3. Confirmation in children with AS: In order to see whether our BM candidates occurred in humans, we tested samples from children. These children were in the early stages of AS and were part of the EARLY PRO-TECT Alport trial.[20] The samples were taken 4 years apart. We were therefore able to estimate the time course of the BMs and the BM type (Figure 1).
4. Verification of "real-world" practicality: Finally, we measured the BM candidates in an additional group of children with early stages of AS and similar diseases. Other nephropathies were also analyzed (in preparation).

In the proof-of-concept phase, we evaluated our BM candidates in dogs and humans by immunoassays. Our goals were to determine the ability of our proteomic strategy, to estimate the BM time courses, and the strength of their ability to discriminate between healthy and affected individuals.

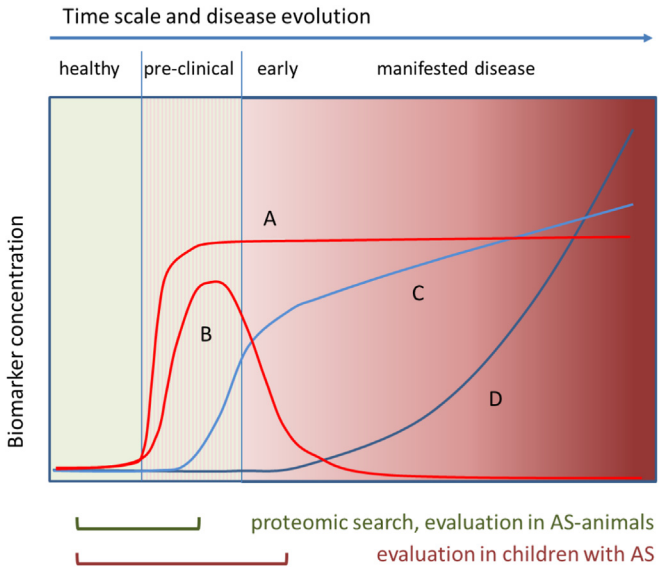

Figure 1. General time courses of disease biomarkers. There are several hypothetical BM types that are differently targeted by our approach. Type A: BMs applicable preclinically; increase indicates alterations starting in a preclinical stage and persisting under progression. Type B: truly preclinical BMs; indicating only preclinical alterations. Type C: early BMs; increase indicates alterations simultaneous with the development of early clinical signs, often overlooked or neglected (e.g., in AS, hematuria). Type D: late BMs; increase indicates subsequent alterations in manifested disease. This type (e.g., in AS, (micro)-albuminuria, decline of eGFR) is already in clinical use and was not part of our search. Type A and B were searched for in juvenile AS-mice (4 weeks[13]) and AS-dogs (7 weeks). This search might also detect type C. Human samples usually come from different points of individual disease development. Their evaluation might therefore miss type B BMs. However, all BMs detected (A, B, or C) are likely to be more suitable for early diagnosis of kidney injury than those currently applied. AS, Alport syndrome; BM, biomarker.

METHODS

Animals and Sampling Protocol

The study protocol was reviewed and approved by the Texas A&M University Laboratory Animal Care Committee. One author, MN sampled from 7-week old mixed-breed dogs with X-linked AS[17] and unaffected littermates. Texas A&M University Animal Rearing Center raised the dogs. These dogs were not under any treatment. Collected EDTA-blood was placed on ice. For serum, whole blood was allowed to clot for at least 30 minutes. Urine was refrigerated. We centrifuged the plasma at 3220 g for 20 minutes at 4 °C, serum at this rate and at 20 °C, and urine at 453 g for 15 minutes at 4 °C. We then stored aliquots at −80 °C. We measured the protein-to-creatinine ratio and blood chemistry values using a Vitros 250 analyzer (Johnson & Johnson Co, Rochester, NY). And we measured urine values using the dipstick Multistix (Bayer Corp, Elkhart, IN).

Table 1. Data of pooled dog samples used for mass spectrometry

		Pooled EDTA-plasma samples			Laboratory data			
		Input for chromatographic pre-fractionation			Urine		Serum	
Pool	Litter	Vol. (ml)	Protein (mg/ml)	Protein (mg)	UPC	Dipstick Blood	Creatinine mg/dl	Albumin g/dl
NM	1, 2, 2	0.78	78.85	61.5	0.48 ± 0.41	Tr, N, N	0.39 ± 0.01	2.80 ± 0.08
NF	1, 3, 3	0.78	80.97	63.2	0.36 ± 0.34	N, N, Tr	0.38 ± 0.05	2.50 ± 0.14
CF	1, 3, 3	0.78	83.58	65.2	0.14 ± 0.11	N, N, 1+	0.36 ± 0.03	2.50 ± 0.00
AM	1, 3, 3	0.78	79.27	61.8	0.92 ± 0.53	1+, 2+, Tr	0.35 ± 0.04	2.53 ± 0.09

AM, affected male; CF, carrier female; N, negative; NF, unaffected female; NM, unaffected male; Vol., volume; Tr, trace; UPC, urinary protein-to-creatinine ratio.
Samples were obtained at week 7 of age.

Samples Used for Proteomic Search

Equal volumes of 1 plasma sample from each of 3 dogs were combined for each group (affected males [AM], carrier females [CF)], and unaffected siblings [NM, NF]). Each pool was prefractionated and subsequently analyzed (Table 1).

Sample Preparation for Mass Spectrometry

Our native prefractionation workflow followed[21] (Supplementary Methods). The chromatographic distributions of proteins are shown in Supplementary Figures S1 to S3.

Selection of BM Candidates

BM candidates were selected from all identified protein chains after pairwise comparison of data of 2 dimensional-(2D-)subfractions from affected and nonaffected dogs. Peptides supporting proteins identified in 2D-subfractions from AM were compared with those of the homologous 2D-subfractions from NM by Sieve, as well as those from CF with those from NF. The quantifier "ratio" is related to the concentration quotient (AM/NM, CF/NF) and "hits" to the amount of the respective protein in these subfractions. Sieve data on each protein identified were sorted to our separation matrix by in-house macros to visualize the chromatographic distribution of altered protein variants according.[21,22] Therefore, a BM was defined either by a >2-fold higher concentration based on the "overall trend" (Supplementary Methods, Formula 1) or by mean ratios, when we saw exclusive synergistic alterations. Alternatively, we defined a BM as a fraction out of an entire protein family by a >5-fold higher mean ratio in distinct clusters of subfractions, that is, a distinct strongly elevated variant (Figure 2).

Analysis of Posttranslational Modifications

Kidney disease induces several metabolic changes. Therefore, we additionally analyzed a few posttranslational modifications (PTM) of highly abundant dog proteins to determine whether proteins were already metabolically altered in preclinical AS. The Proteome Discoverer (Thermo Fisher Scientific GmbH, Karlsruhe, Germany) performed this search[21] (Supplementary Methods).

Proof-of-Concept

For proof-of-concept, it was also necessary to evaluate the differences in BM concentrations and to assess their sensitivity and specificity. We therefore measured several BMs by ELISA in samples from dogs and humans.

Dog Samples

We evaluated these features of BMs using samples from mixed-breed dogs. These dogs were of the same cohort as those used for proteomic search but not the same animals. However, the number of samples was insufficient for statistical analysis because the human COVID-19 pandemic during our research restricted access.

Human Samples

We used 3 sets of human samples. All storage and transport were at below −80°C.

Set 1 was of prospective longitudinal real-world clinical serum and urine samples from the multicenter trial "EARLY PRO-TECT Alport"[20] (ClinicalTrials.gov Identifier NCT01485978, Table 2). Samples from each patient were obtained at 2 time points. The first point was during the very early stages of AS and the second about 4 years later.

Set 2 was obtained from the Jena University Hospital, Germany, with serum and urine samples from pediatric patients suffering from AS, including the Alport spectrum of thin basement membrane nephropathy, or benign familial hematuria. These diseases were diagnosed clinically by family history, ultrasound, histology, genetic analysis, or by established renal parameters. For controls, we took serum and urine samples using standard methods from children with no known nephropathy (ethical approval JUH: 2020-1800) (details in Table 3).

Set 3 was of pediatric control samples independently obtained from the Leipzig Medical Biobank (Leipzig Research Center for Civilization Diseases, LIFE-child) (details in Table 3).

Altered proteins in dogs (7. wk)		Males		Females		BM candidates				Verified		
		Mean ratio								∅: no, ●: yes		
ID	Annotation	higher in AM		higher in CF		mice wk		dogs wk 7		mice	dogs	human
		in sub-fractions	overall trend	in sub-fractions	overall trend	4	6-7.5	AM	CF			
Plasma proteins												
F1PNV5	Kallikrein B1	DIV/0	114.84	0	0		x	x			∅	
E2R886	Kininogen 1	56.2	2.08	20.3	1.35	x	x	x	o			
E2QUV3	α2-HS glycoprotein	326	16.25	n.a.		x	x	x				
F1P6E1	Complement C1s	0	0	3.11	DIV/0				x			
F1PNG7	Complement C1r	n.a.		115	9.99				x			
J9P4B4	Complement C1q A chain	DIV/0	35.13	n.f.				x				
J9P1G2	Complement C1q B chain	DIV/0	107.34	4.09	0.16			x				
E2RJC2	Complement C1q C chain	DIV/0	78.88	3.21	0.07			x				●
E2RS79	Complement C2 (human homolog)	3.44	0.13	0	0		x					
F1PIX8	Complement C3	32.2	0.46	34.77	1.54	o	x	o	o			
J9PAD1	Complement C4 (human homolog)	29.6	0.22	150	0.93		x	o	o			
F1PWR2	Complement C4 (human homolog)	6.09	0.20	37.3	0.48		x	o	o			
F1P7J4	Complement C5	21.3	0.12	14.9	0.13			o	o			
E2RGT6	Complement C6	DIV/0	34.84	0	0			x				
E2RG01	Complement C7	DIV/0	34.21	0	0			x				
E2R109	Complement C8 alpha chain	DIV/0	44.49	2.26	0			x				
E2R141	Complement C8 beta chain	2.41	0.02	n.a.								
J9P8Z6	Complement C9	5.79	DIV/0	n.a.			x	x				●
F1PY40	Complement factor I	66.2	1.13	49.5	0.60		x	o	o			●
J9NTL7	Complement factor H (homolog in mammals)	69.5	0.47	47.6	0.08		x	o	o			●
E2RS80	Complement factor B (homolog in primates)	39	0.91	25	0.29			o	o			
F1PGM9	Complement component 4 binding protein alpha	170.4	13.87	20.2	0.47			x	o			●
G1K2D9	Haptoglobin	114	4.88	0	0	x	x	x				
F1PGM9	Complement component 4 binding protein alpha	170.4	13.87	20.2	0.47			x	o			●
G1K2D9	Haptoglobin	114	4.88	0	0	x	x	x				
F6UME0	α2-macroglobulin	77.55	2.48	179	1.08		x	x	o			
F6Y713	α1-acid glycoprotein	23	9.31	13.6	0.07			x	o			●
P33703	APOH, Beta-2-glycoprotein 1	42.2	3.76	15.7	0.41	x	x	x	o			
T2KEN6	Pentraxin (CRP)	3.53	0.06	0	0	x						●
A1ILJ0	Alpha-1-antitrypsin	6.2	1.33	4.08	0.10	x	x	o				
F1PAX2	Ceruloplasmin	4.38	0.07	6.28	0.07		x	o	o			
F1PCE5	Serpin family A member 1	114	5.04	53.1	2.93	x		x	x			
F1PH86	Serpin family A member 3	n.f.		2.4	0.11							
E2RMF9	Serpin family A member 5	0	0	34	1.33				o			
F1PB85	Serpin family A member 7	8.55	2.48	0	0			x				
F1PYX9	Serpin family G member 1	11.3	0.14	12.9	0.08			o	o			
J9P8Q8	Serpin family D member 1	2.17	0.02	2.53	0.28							
E2R833	Leucine rich alpha-2-glycoprotein 1	22	13.15	10.6	0.43	x	x	x	o		●	●
J9NVE9	Serum amyloid A	86.4	DIV/0	0	0	x	x	x			●	∅
F1PAL5	Angiotensinogen	19.4	3.59	6.4	0.83	x	x	x	o			●
F1PN98	Coagulation factor V	3.35	DIV/0	0	0							
J9NSF9	Prothrombin	51	3.18	11.9	0.25			x	o			
F1PZR4	Hemopexin	44.9	0.90	68.7	1.41		o	o	o			∅
F1Q421	Plasminogen	n.a.		52.6	3.18	x	x		x			
J9P430	Transferrin	20.4	0.56	29.1	0.17	x	o	o	o			
F6V1W9	Transferrin	4.12	3.04	3.95	0.91			x	o			
J9JHX7	Serotransferrin (homolog, several species)	9.23	1.45	20.2	0.90			o	o			
E2QZQ1	Serotransferrin (homolog, several species)	6.75	0.53	DIV/0	40.50			o	x			
F1PKX3	Coagulation factor XIII A chain	2.76	0.02	41.2	44.06				x			
F1Q041	Coagulation factor XIII B chain	DIV/0	3.95	DIV/0	21.47			x	x			∅
F1PSS8	Protein S	2.98	0.06	0	0							
F1Q4A3	Coagulation factor X	0	0	20.5	2.40				x			
E2R4E7	Afamin	6.76	0.59	14	0.23		x	o	o			
F6Y3P9	Gelsolin	34.5	3.68	20	3.67	x		x	x	●	●	●
E2R5U8	Transthyretin	8.76	0.11	n.a.			x	o				
A0A1K0FUG1	Globin A1	109	42.85	DIV/0	6.56			x	x			
A0A1K0GGH0	Globin A2	103	16.80	0	0		x	x				
J9NXL3	Hemoglobin subunit alpha (dog homolog)	115	34.62	2.8	0.01		x	x				
E2R9B6	Fetuin B	30.4	4.69	2	0.03			x				
F1PYM4	Insulin like growth factor binding protein	31	0.82	3.15	1.59			o				
B5U1S6	Adiponectin (fragment)	DIV/0	51.86	DIV/0	1.93			x	o		●	●
F1PDJ7	Alpha-2-glycoprotein 1	3.75	1.99	n.a.								
E2RPW3	Paraoxonase 1	3.55	0.08	DIV/0	264.38				x			
J9P8B0	Dipeptidyl peptidase 4	2.31	DIV/0	n.a.								
F1PCK2	α1-B glycoprotein	21.9	0.67	25.2	1.85			o	o			
F1Q4D9	Retinol binding protein 4	8.97	0.20	25.06	4.95	x		o	x			∅
E2RPB8	C-type lectin domain family 3 member B	n.a.		2.59	0.06							
E2RKQ6	Galectin 3 binding protein	2.24	1.70	3.3	0.08							

Figure 2. Biomarker candidates identified by mass spectrometry. Criteria defining a biomarker: Biomarker selection in mice, see reference.[13] BM candidates in dogs were by weighted mass spectrometric data (Supplementary Methods, Formula 1). Peptides supporting the proteins in a 2D-sub-fraction from AM were compared by Sieve with those of the homologous 2D-sub-fraction from NM as well as those from CF with those from NF. The quantifier "ratio" is related to the concentration quotient (AM/NM, CF/NF) of each specific protein in the sub-fractions compared. n.a., not altered; n.f., not found; 0, no cluster in this direction; x, overall trend higher in AM than in NM and in CF than in NF; o, increased parts/protein variants in AM and CF; DIV/0: no protein detected in healthy controls (no ratio can be given); mean ratio, mean of all ratios with (continued)

Apolipoproteins												
J9P843	Apo AI	325	12.75	41.1	0.60	x	x	x	o			
E2RE76	Apo AIV	6.76	0.44	21.5	5.07	x	x	o	x			
P56595	Apo CI	4.72	0.05	3.97	DIV/0							
9NWJ6	Apo CII	15.8	0.18	50.3	12.99			o	x			
D5G334	Apo E4 (fragment)	4.8	0.16	37.4	4.13				o			
F1PJ74	Apo E	15	0.34	33	2.93	x	x	o	x			
E2RQ71	Apo M	6.86	0.09	2.57	1.16			o				
F1P8Z5	Apo B	87	0.68	19.3	0.08		x	o	o			
E2RNM1	Apo D	11.7	0.74	108.2	30.31			o	x			
Intracellular proteins												
F1PZK8	Dynein heavy chain domain 1	DIV/0	182.61	3.24	1.35			x			∅	
E2RB62	Myosin IB	6.19	0.23	n.f.				o				
E2R1Q3	Myosin XVIIIB	n.a.		3.02	DIV/0				x			
J9P187	Myosin IXA	894	66.05	328	77.51			x	x		∅	
J9P007	Kinesin-like protein	3.43	DIV/0	3.46	1.01			x				
F1PM26	CD109 molecule	17.3	6.15	n.f.				x				
J9P216	Formin 1	DIV/0	204.62	4.95	DIV/0			x	o		∅	●
J9P7F7	Ficolin 1	DIV/0	22.66	4.1	0.12		x	x				∅
F1PAQ3	Maltase-glucoamylase	6.49	0.70	2.18	0			o				
J9P5V6	Talin 1	17.6	1.19	128	7.90			o	x		∅	∅
E2RFS6	Sacsin molecular chaperone	12	2.87	12.7	2.54			x	x			
E2QV33	Mediator complex subunit 22 (RNA-Pol)	98.4	DIV/0	0	0			x				
E2R926	Fukutin	29.5	3.72	n.f.				x			∅	
F1PRY8	Dispatched RND transporter family member 2	n.a.		DIV/0	DIV/0				x			
F6Y1C9	DNA Polymerase	n.a.		4.14	4.80				x			
A0A0A0MPC5	Trafficking protein particle complex subunit 2	n.f.		DIV/0	DIV/0				x			
J9P514	RB associated KRAB zinc finger	6.05	0.26	9.04	DIV/0			o	x			
J9P653	Ciliogenesis-associated TTC17-interacting protein	10.1	DIV/0	2.9	0.16			x				
J9NYQ5	Growth arrest specific 2 like 3	6.17	4.07	11.5	35.18			x	x			
J9NYS3	Regulating synaptic membrane exocytosis 2	2.18	1.00	0	0							
E2RF52	Proteasome subunit alpha type	n.f.		171	DIV/0				x			
Extracellular matrix proteins												
J9P8M2	Fibronectin	28.9	0.46	32.6	0.11	x	x	o	o			∅
J9NV47	Hyaluronan binding protein 2	31.6	DIV/0	2.18	0.20			x			∅	●
F1Q3I5	Collagen α1(I) chain	2.87	DIV/0	0	0	x		x		∅		
E5G723	Collagen α1 (I) (Fragment)	31.05	1.49	DIV/0	137.80			o	x		●	●
F1PHY1	Collagen α2 (I) chain	3.26	9.11	0	0			x				
F1PJH3	Collagen type XIII alpha 1 chain	455	DIV/0	0	0			x			●	●
J9NRV7	Fibrinogen alpha chain	265	9.74	0	0			x				
F1PW65	Fibrinogen beta chain	96.9	3.28	37.4	0.42			x	o			
F1P8G0	Fibrinogen gamma chain	399	22.27	53.4	0.62			x	o			●
F1PG39	Inter-alpha-trypsin inhibitor heavy chain 2	3.09	0.06	42	0.27	x			o			
J9P7I3	Inter-alpha-trypsin inhibitor heavy chain 3	0	0	25.7	1.38		x		o			
H9GWY3	Inter-alpha-trypsin inhibitor heavy chain 4	59.9	1.78	5.51	0.08		x	o	o			∅
J9NUI6	Alpha-1-microglobulin/bikunin precursor	33.6	0.20	75	1.24	x	x	o	o			
E2RQF8	Extracellular matrix protein 1 (human homolog)	24.1	1.88	0	0	x	x	o				
J9P669	Cadherin 5	3.3	0.30	5.54	1.45				o			
J9PAA4	Carboxypeptidase N subunit 2	5.81	0.72	177	0.19		x	o	o			
F1PLT8	Sulfhydryl oxidase	2.92	DIV/0	0	0	x	x	x				
F1PV43	Capping actin protein of muscle Z-line alpha subunit 3	11.1	DIV/0	n.f.	n.f.			x				
F1Q1P0	Coiled-coil domain containing 178	39.7	22.95	17.2	13.97			x	x			
E2R416	Lumican	44.5	2.11	11.9	0.30			x	o		∅	●
F6Y120	Serine/arginine repetitive matrix protein 3 (homolog)	20.90	9.22	3.78	0.45			x				
Q2YF02	Vitronectin (fragment),	68.7	14.64	2.38	0.44			x			∅	●
H2BF45	Attractin (fragment)	n.a.		4.14	0							
F1Q117	Attractin	n.a.		2.75	0							

Figure 2. (continued) synergistic alteration of the protein (AM/NM or CF/NF). Red: candidate BMs; blue: some proteoforms might serve as candidate BMs; green: candidate BMs in mice, criteria not fulfilled in dogs. Grey filling: positive APR; black filling: negative APR. Altogether, 124 protein chains were more abundant in affected than in unaffected dogs, either in males or females or both, and 74 proteins fulfilled the definition for a BM candidate. AM, affected male; APR, acute phase protein; BM, biomarker; CF, carrier female; NF, unaffected female; NM, unaffected male.

Evaluation by Immune Assays

All measurements were carried out in duplicates using commercial ELISA test kits (Supplementary Table S1). We initially tested a few samples to determine the measurable range and the dilutional linearity of each assay. We were unable to measure any promising BM candidates due to the limited availability or specificity of the test kits. Furthermore, it was not possible to apply all the immune assays to all samples because of the limited sample volumes.

Analysis of ELISA Results

The data were analyzed by SPSS version 27 (IBM Corp., Armonk, NY). Because the data distribution were non-normal, we tested the similarity of medians using Kruskal-Wallis tests. We followed these by *post hoc* analyses using Mann-Whitney-U-test pairwise comparisons. The

statistical significance was $P < 0.05$ (95% confidence interval). We correlated the immune reactivity of BMs with each other using SPSS (v. 27).

Moreover, we used correlation analyses with several clinical data and receiver operating characteristic curve analyses to assess the diagnostic values of each BM and BM combination. Accuracy was judged moderate for area under the curve 0.7 to 0.9 and high for area under the curve >0.9.[26]

RESULTS

Discovery Phase: Proteomic Search in Blood Plasma From Dogs With Preclinical Stage AS

The global protein distribution after prefractionation is given (Supplementary Figures S1 and S2). The high precision of our method[21] allowed reliable comparisons between the constituents of subfractions from different samples.[27] In total, using 2 analysis methods, we found 3115 protein chains using Proteome Discoverer, but 918 with Sieve (Supplementary Tables S2 and S3). The 3115 chains represented approximately 600 unique proteins and the 918 chains represented approximately 300 unique proteins. The majority of proteins showed several chromatographic clusters indicating the existence of diverse proteoforms with different degrees and directions of alteration in AS. Only a few were uniformly increased or reduced in affected versus nonaffected dogs (Supplementary Figures S3–S6, Supplementary Table S4).

In order to define BM candidates out of the list of altered proteins, we either selected spots with a mean ratio of ≥5.0 (Sieve) or, in the case of miscellaneous clusters, we applied formula 1 (Supplementary Methods) and accepted ≥2.0 as sufficient to define a BM candidate. Therefore, all clusters, both increased and reduced, were included in estimating a value for the global alteration of the protein family. By this procedure, we identified a total of 124 protein chains determining 74 proteins as BM candidates (Figure 2, Supplementary Table S4).

Several BM candidates were almost exclusively increased in AM, such as serum amyloid A, complement component C9, HABP2, collagen type XIII α1 chain (ColXIII), and the capping actin protein of the muscle Z-line alpha subunit. All other BM candidates showed miscellaneous alterations (Supplementary Figures S4–S6, Supplementary Table S4).

Adiponectin and complement C1q were higher in male dogs with AS (AM) than in controls, in overlapping chromatographic clusters (Figure 2, Supplementary Figure S7). The α1 chain of type I collagen was more abundant in affected males and females, the α2 chain only in males than in controls. By sequence analysis, we detected only the c-terminal propeptides of both chains. ColXIII was more abundant in AM in several subfractions than in unaffected animals (Supplementary Figure S8). The sequence detected was located within the collagenous ectodomain. The unprocessed angiotensinogen was also higher in AM than in controls (Figure 2).

We next investigated PTMs. It is possible that disease alters these PTMs, thereby altering protein chromatographic profiles. We therefore analyzed PTMs of some of the highly abundant proteins. The major albumin and transferrin clusters did not differ between groups. However, some of their minor clusters differed in amount and in deamidation and carbamyolation rates. It is possible therefore that these minor cluster differences are early indications of disease (Supplementary Results, Supplementary Figures S10 and S11).

Validation in Dogs

We then validated the BMs by examining whether they were present in representative dogs with AS. However, it was only possible by ELISA, to quantify some of the BM candidates in dog samples. Of those we were able to quantify, the results of 7 were consistent with the mass spectrometric data. These 7 were leucine-rich alpha 2-glycoprotein 1 (LRG1), gelsolin (GS), procollagen type I carboxy-terminal propeptide (PICP), ColXIII, serum amyloid A, and adiponectin (Supplementary Table S5). Although not comprehensive, these results confirm the reliability of our proteomic results.

Confirmation of Candidate BMs for Early Screening in Children With AS

Thirteen BMs in the blood and 14 BMs in urine were significantly higher from children at early stage AS than in healthy children (Table 4, Figure 3, Supplementary Figure S9). Of these 14 in urine, 3 allowed the identification of all the disease groups included. These were HABP2, complement factor H (CFH), and C4BP. A further 5 differed for 3 disease groups. These 5 were adiponectin, ColXIII, LRGP1, formin 1 (FMN), and fibrinogen γ-chain. Furthermore, it is notable that 8 urinary BMs even allowed discrimination between preclinical AS (less severe, compare Table 2) and controls. Because several BMs correlate with other BMs (Supplementary Table S6), we multiplied the correlated BMs in order to improve the significant differences between patients and controls. Indeed, the products of urinary BMs increased the overall discriminatory power (Table 5). As a consequence of this procedure, we were able to identify 19 combinations of BMs and 54 times when patients had significantly higher values than controls, and 11

Table 2. Data from set 1; prospective samples (EARLY PRO-TECT Alport) from 35 male Alport patients

Sampling	Age (yr, mean ± SD)	Age (yr, range)	ACI therapy (*n*)	Serum creatinine (mg/dl, mean ± SD)	Urine albumin (mg/g creatinine, mean ± SD)	Values
All individuals ($N = 35$)						*n*
First	8.9 ± 4.1	3–17	20	0.44 ± 0.15	293.0 ± 601.9	32
Second	12.5 ± 4.1	6–21	24	0.58 ± 0.19	578.9 ± 1107.7	33
Genetic variants[23]						
Less severe ($n = 7$)						
First	10.3 ± 3.7	4–17	5	0.51 ± 0.10	16.2 ± 7.3	7
Second	13.6 ± 3.8	7–20	5	0.58 ± 0.13	14.8 ± 11.8	6
Intermediate ($n = 17$)						
First	9.5 ± 4.3	3–17	6	0.43 ± 0.13	390.3 ± 789.8	15
Second	13.4 ± 4.3	6–21	10	0.62 ± 0.22	647.5 ± 1314.6	16
Severe ($n = 11$)						
First	7.0 ± 3.0	3–13	9	0.38 ± 0.14	245.3 ± 391.8	10
Second	10.6 ± 3.0	7–17	9	0.51 ± 0.16	638.1 ± 683.9	11

ACI, angiotensin-converting-enzyme-inhibition.

combinations that distinguished preclinical AS (less severe, compare Table 2) from controls.

Verification of "Real-World" Practicality

To verify our BM candidates for "real-world" practicality, we also examined whether BMs occurred in an additional group of children with early stage AS or other causes of microhematuria (Table 3). The receiver operating characteristic curve analyses indicated the diagnostic value of our BMs. For this analysis, the area under the curve were >0.800 for serum TGF-ß1, for complement factor I (CFI), for urinary ColXIII, HABP2, CFH, C4BP, and combinations thereof. They also exhibited reliable sensitivity and false positive rate, either alone or in combination (Figure 4 and Table 6) like with set 1 (Supplementary Table S10). Albuminuria did not correlate with any of our BM candidates, only urinary lumican (LUM) did (Supplementary Tables S7–S9). Urinary LUM also correlated with urine albumin-to-creatinine ratio at progression of disease (4 years later). Our early BM candidates did not correlate with the parameters of late stage kidney failure, such as glomerular filtration rate, or with indicators of inflammation, that is, C-reactive protein (CRP). We are therefore able to confirm the real world practicality of several BMs for diagnostic purposes.

DISCUSSION

Our search included preclinical mice and dogs with AS, a unique cohort of children from the EARLY PRO-TECT Alport trial, and from children with early stages of AS and similar glomerular diseases.[28,29]

After nondenaturing 2D-prefractionation, the majority of proteins appeared as a natural multitude of chromatographic clusters including disease specific proteoforms (Supplementary Figures S5–S7).[13,21] Thus, one BM-cluster did not reflect the general deviation of a particular protein-family, rather the most meaningful variant out of many. Different clusters reflect heterogeneity, likely due to diverse PTM.[21] Unfortunately, most commercial ELISAs are not variant-selective, as is required. Thus, out of all altered proteins, we selected only BM candidates with considerable global elevation derived from weighted mass spectrometric data, and for which an ELISA was available.

Because the glomerulus has close contact with both blood and urine, components are able to escape from the glomerulus into both fluids.[13] Although identified in blood, the plurality of proteoforms might mask strongly altered glomerular protein variants (as discussed above). In urine, however, we were able to detect, immunologically, the proteoform that, very probably, leaks from the injured glomerulus. This was even possible using nonselective ELISA. Therefore, urine, particularly useful in pediatrics, might better mirror glomerular disturbances without accompanying variants. This was confirmed by our ELISA results.

Our study uncovered several preclinical BMs for AS (type A and B, Figure 1). We identified a variety of extracellular matrix (ECM) components, acute phase reactants, apo-lipoproteins, cellular and cytoskeletal proteins (Figure 2). For example, α2-macroglobulin, α1-acid glycoprotein and LRG1 are similarly increased in both males and females, as well as several serpins, adiponectin, GS, and myosin IXA. Others were differently altered reflecting either gender or stage related differences. In accordance with preclinical mice with AS,[13] preclinical dogs with AS show neither elevated markers of advanced CKD nor of acute kidney injury.[30-33]

C-reactive protein (pentraxin), the main acute phase reactants in dogs,[32] was only minimally elevated, supporting a lack of systemic inflammation at this early stage of the disease. Several other acute phase reactants were less in affected dogs than in controls or unaltered. Some negative acute phase reactants (e.g., transferrin),

Table 3. Basic and clinical characteristics in the study population and controls, i.e., sample sets 2 and 3

Group	*n*	Gender	Age (yr)	BMI (SDS, z)	eGFR (ml/min per 1.73 m²)	Cystatin CGFR (ml/min per 1.73 m²)	Cystatin C (serum) mg/l	Blood pressure syst. (SDS, z)	Blood pressure diast (SDS, z)	Serum creatinine (µM)	Urine albumin (mg/g creatinine)
Controls	48	Male	10.3 (4.1, 3.1 to 17.1)	0.220 (1.28, −2.50 to 3.68)	145.6 (25.6)	113.2 (23.1)	0.87 (0.12)	0.490 (0.997)	0.274 (0.866)	51.0 (15.8)	6.9 (5.9)
Controls	56	Female	10.3 (4.0, 3.5 to 16.7	−0.064 (1.25, −3.44 to 2.90)	153.4 (30.0)	121.5 (22.5)	0.81 (0.11)	0.325 (1.00)	0.409 (1.01)	45.5 (12.7)	11.1 (12.0)
Controls	104	48 m/56f	10.3 (4.0, 3.1 to 17.1)	0.069 (1.264, −3.44 to 3.68)	149.8 (28.2)	117.7 (23.0)	0.84 (0.12)	0.404 (0.999)	0.344 (0.943)	48.1 (14.4)	9.2 (9.9)
AS[c], TBMN, BFH	15	8 m/7 f	12.8 (3.1)	0.318 (1.131, −1.81 to 2.41)	154.6 (22.9)	117.6 (22.8)	0.82 (0.11)	0.658[d] (0.983)	0.137 (0.536)	52.6 (13.2)	106.1[b] (285.2)

AS, Alport syndrome; BMI, body mass index; BFH, benign familial hematuria; CGFR, calculated glomerular filtration rate; eGFR, estimated glomerular filtration rate; SDS, standard deviation scores (SDS; also known as z-scores); TBMN, thin basement membrane nephropathy.
The data are presented as means, SD and ranges (age, BMI).
[a] $P < 0.05$ is considered significant.
[b] $P < 0.01$ highly significant, Mann-Whitney, U-test.
[c]Autosomal Alport syndrome (AS) and histologically determined AS without genetic information.
Unmarked values are not significantly different from the corresponding controls (patients *versus* healthy controls and females (f) *versus* males (m)). Healthy pediatric controls included 70 samples from the Leipzig Medical Biobank (Leipzig Research Center for Civilisation Diseases. LIFE-child (collection: 02/2014-11/2014)) and 34 pediatric controls from Jena (09/2020-09/2021). We SDS transformed BMI[24] and blood pressure.[25]

Table 4. Significance levels (*P*-values) of BM concentrations in blood and urine different in patients and healthy controls

BM	Concentration range	AS[a], TBMN, BFH (set 2)	AS[b] severity (set 1)		
			Less severe	intermediate	severe
Serum					
		n = 4–10	*n* = 6–8	*n* = 5–17	*n* = 8–11
ADP	ng/ml				
α1AGP	mg/ml	0.023	<0.001	0.004	0.005
AGT	ng/ml				0.067
ColXIII	ng/ml			<0.001	0.085
GS	µg/ml		0.020	0.024	0.009
LRGP1	pg/ml			<0.001	
HABP2	ng/ml			0.046	0.008
PICP	ng/ml			<0.001	
TGFß	ng/ml		0.004	<0.001	<0.001
VTN	µg/ml		0.043		
C9	ng/ml			0.074	
C4BP	ng/ml			0.011	0.040
CRP	ng/ml		0.084	<0.001	0.005
LUM	ng/ml				
FMN	pg/ml				
CFH	ng/ml		0.024	0.036	0.006
CFI	ng/ml		<0.001	0.007	<0.001
FGG	ng/ml				
C1q	ng/ml		0.003	0.006	0.073
Urine					
		n = 10–13	*n* = 8	*n* = 14–16	*n* = 8–11
ADP	ng/mg c		<0.001	<.001	0.016
α1AGP	ng/mg c	0.093	0.093	<0.001	<0.001
AGT	ng/mg c	0.068			
ColXIII	ng/mg c	0.015		<0.001	<0.001
GS	ng/mg c				
LRGP1	ng/mg c		0.012	<0.001	<0.001
HABP2	ng/mg c	0.001	0.010	<0.001	<0.001
PICP	pg/mg c	0.003	0.007	0.072	
TGFß	pg/mg c				
VTN	ng/mg c				
C9	pg/mg c		0.016		
C4BP	ng/mg c	<0.001	0.037	<0.001	<0.001
CRP	pg/mg c	0.062	<0.001	<0.001	0.004
CFH	ng/mg c	0.033	<0.001	<0.001	<0.001
CFI	ng/mg c	0.098		<0.001	<0.001
FMN	pg/mg c	0.013	0.057	<0.001	0.002
LUM	ng/mg c				
FGG	ng/mg c	0.001	0.001	<0.001	
C1q	ng/mg c			<0.001	<0.001

ADP, adiponectin; AGT, angiotensinogen; AS, Alport syndrome; BMI, body mass index; BFH, benign familial hematuria; CFH, complement factor H; CFI, complement factor I; CGFR, calculated glomerular filtration rate; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; FGG, fibrinogen γ-chain; FMN, complement factor I; LUM, lumican; PICP, procollagen type I carboxy-terminal propeptide; SDS, standard deviation scores (SDS; also known as z-scores); TBMN, thin basement membrane nephropathy; VTN, vitronectin.
[a]Autosomal Alport syndrome (AS) and histologically determined AS without genetic information.
[b]Genetic characterized AS (set 1, first samples).
The number of patients differs from BM parameter to parameter, because we were unable to measure all parameters in all patients. Insufficient volumes of some samples caused this. n: counts of patient values (min-max) included. $P < 0.05$ is considered significant, $P < 0.01$ highly significant, (Mann-Whitney U-test). Some *P*-values of 0.05 to 0.10 included to show tendencies. All other comparisons yield nonsignificant differences. Bold: favored BMs. Urinary concentrations are normalized by creatinine (c). Healthy controls: $n = 11$–25 for serum; $n = 86$–101 for urine.

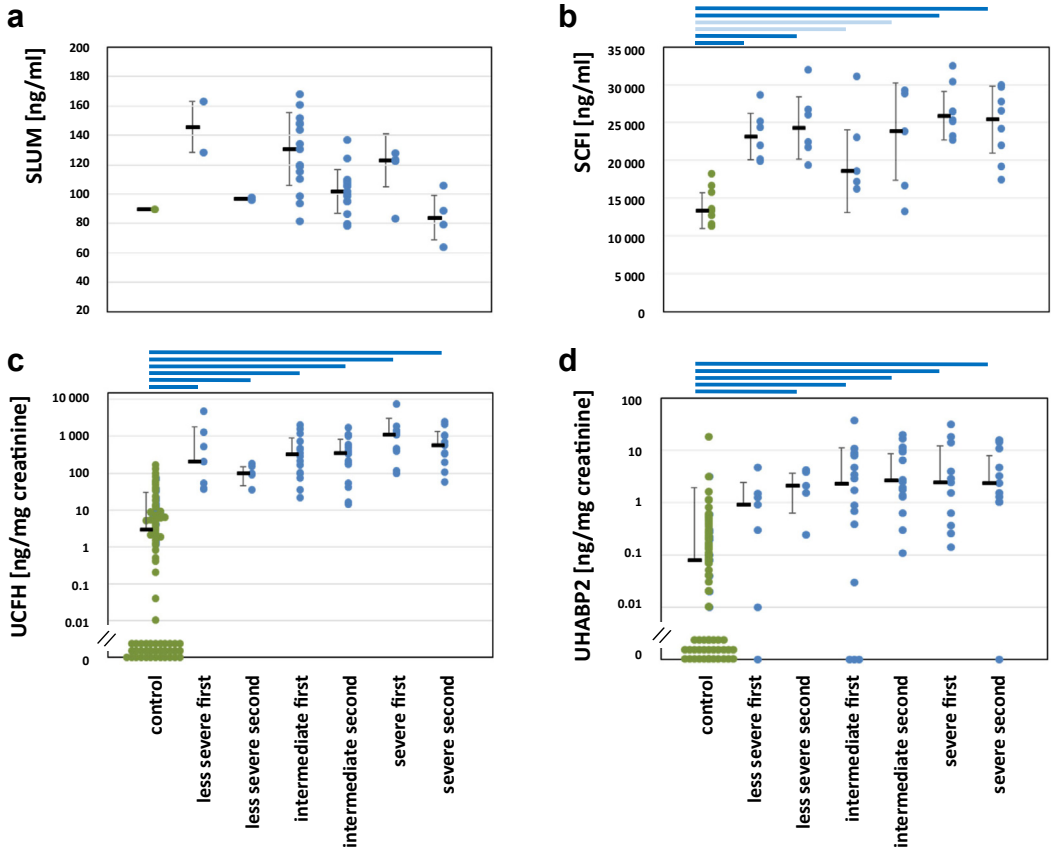

Figure 3. Comparison of BM concentrations in sample set 1 from patients with AS and healthy controls. Second samples were taken about 4 years after the first. Significances: $P < 0.05$ (light blue bars); $P < 0.01$ (dark blue bars); all other not significant. c, d: Values below the detection limit (= 0) are arranged close to the abscissa for visualization. (a) Lumican (LUM) in serum. Due to the small number of samples, dots indicate tendency. (b) Complement factor I (CFI) in serum. (c) Complement factor H (CFH) in urine; (d) Hyaluronan binding protein 2 (HABP2) in urine.

however, appeared to be greater. Therefore, the situation in dogs is similar to that in mice.[13] Typical GBM components,[34–36] however, were greater in AS (e.g., fibrinogen γ-chain, fibronectin, LUM, vitronectin [VTN], and HABP2). There were no peptides from type IV collagens. Our findings accord with the elevated expression of type I collagen in dogs and mice with AS.[13,37] During collagen processing, the fragment PICP is removed by ADAMTS. PICP is indicative for early ECM remodeling and fibrosis.[38–42] We retroactively identified 5 peptides in AS mice that align as precisely with murine PICP just as the canine sequences align with canine PICP. Moreover, several members of the ADAMTS-family are very homologous to ADAMs, including ADAM8,[43] which was identified in humans with later stages of AS.[44,45] Despite the significant deviation (Tables 4 and 5, and Supplementary Table S10), the receiver operating characteristic curve analysis shows urinary PICP appear insufficient for clinical use.

The transmembrane protein ColXIII is upregulated in AS.[46] Its ectodomain interacts with several matrix components and is released by proteases.[47–49] Therefore, this BM candidate appeared as one of the most promising BMs (Tables 4–6).

Hyaluronan is an important component of the filtration barrier and interstitial renal water reabsorption mechanism.[50–52] HABP2 is crucial for hyaluronan metabolism, in tissue homeostasis, and inflammation,[50,53] and has enzymatic and regulatory activities.[54] In children with AS, we verified HABP2 as one of the most promising early BMs.

The plurality of ECM components indicate early remodeling activity probably driven by TGF-ß1.[55] Due to the renal increase of TGF-ß1 mRNA in dogs at late stages of AS,[7,56–59] of urinary TGF-ß1 in children with AS,[31] and of TGF-ß1 in blood (Table 4), this mediator indicates progression.

Complement C1q and adiponectin are soluble defense collagens and are involved in repair and tissue homeostasis, with adiponectin also having antifibrotic effects.[60,61] There are increased amounts of adiponectin in a variety of CKDs, including focal segmental glomerulosclerosis and lupus nephritis.[62–65] These increased amounts of adiponectin seem to indicate progression of CKD, rather than being disease specific.[66] Our data are the first report of early alterations of adiponectin (Tables 4 and 5). However, in the preclinical stages of AS it has insufficient discriminatory power.

Table 5. Significance levels (*P*-values) of combined BM concentrations in urine that are different in patients and healthy controls

	AS[a], TBMN, BFH (set 2)	AS[b] (set 1) less severe	Intermediate	Severe
Product of BM	***n* = 5–10**	***n* = 8**	***n* = 14–16**	***n* = 7–11**
ADP × PICP	0.012	<0.001	<0.001	0.094
ADP × C9		<0.001	0.003	
ADP × C4BP	<0.001		0.040	<0.001
AGT × GS				
AGT × C9				
ColXIII × C4BP	0.002	0.076	<0.001	<0.001
ColXIII × FMN	0.002	0.052	<0.001	<0.001
GS × CFH	0.068	<0.001	<0.001	<0.001
GS × C9		0.098		
GS × VTN				
GS × CFI			0.033	0.005
LRGP1 × C1q		0.004	<0.001	<0.001
HABP2 × TGFß		0.031		0.030
HABP2 × FGG	<0.001	0.004	0.001	
VTN × AGT				
VTN × C9				
VTN × CFI				0.019
C9 × CFH		0.008	<0.001	<0.001
CRP × C1q		<0.001	<0.001	0.001
CFH × CFI	0.029	<0.001	<0.001	<0.001
CFH × FGG	0.008	<0.001	<0.001	0.056
CFI × FGG	0.038	0.046	<0.001	
CFI × a1AGP			<0.001	<0.001
CFI × GS			0.033	0.005
FMN × FGG	0.008	0.005	<0.001	
ColXIII × C4BP × HABP2	0.003	0.012	<0.001	<0.001

ADP, adiponectin; AGT, angiotensinogen; BFH, benign familial hematuria; PICP, procollagen type I carboxy-terminal propeptide; TBMN, thin basement membrane nephropathy.
[a]Autosomal Alport syndrome (AS) and histologically determined AS without genetic information.
[b]Genetic characterized AS (set 1, first samples).
The number of patients differ from BM parameter to parameter, because we were unable to measure all parameters in all patients. Insufficient volumes of some samples caused this. n: counts of patient values (min-max) included. $P < 0.05$ is considered significant, $P < 0.01$ highly significant, (Mann-Whitney U-test). Some *P*-values 0.05 to 0.10 are included to show tendencies. All other comparisons yield nonsignificant differences. Bold: favored BMs. Urinary concentrations are normalized by creatinine (c). Healthy controls ($n = 84$–99).

We evaluated the γ-chain of fibrinogen because it was the most elevated in AM (Figure 2). Fibrinogen is an intrinsic constituent of the GBM and acts as a hyaluronan-binding protein. It is intrinsically linked to blood coagulation and tissue fibrosis and is upregulated in focal segmental glomerulosclerosis.[34,67-69] And, urinary fibrinogen γ-chain has a high discriminatory score both alone and, even more clearly, combined with other BMs (Tables 5 and 6, and Supplementary Table S10).

Out of a group of regulatory complement components, C4BP has various roles such as cofactor for CFI-mediated C4b inactivation, inhibiting apoptosis, and inhibiting DNA release from necrotic cells.[70,71] C4BP interacts with various regulatory molecules (including

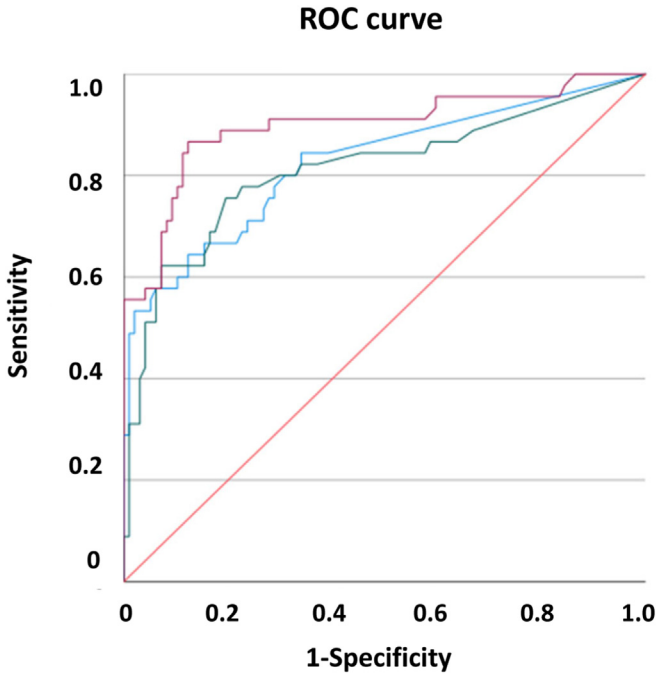

Figure 4. Receiver operating characteristic curves of urinary BMs, comparison of patients with Alport syndrome, thin basement membrane nephropathy, and benign familial hematuria (sample set 2) with controls. Blue: Collagen XIII (ColXIII); green: hyaluronan binding protein 2 (HABP2); magenta: complement C4 binding protein (C4BP); red: reference line. BM, biomarker.

C-reactive protein, C1q, ficolins, CFH, and CFI, which we also identified as BM candidates), modifying the response to cell damage or apoptosis.[72,73] These regulatory proteins are expressed in the podocytes, cells key to AS.[74] CFI, CFH, and C4BP are high in dogs with AS (Figure 2) as well as in urine from children with AS (Tables 4–6, and Supplementary Table S10).

From the family of leucine-rich repeat proteins, we identified LUM and LRGP1 as early BM candidates. These proteins are involved in protein interactions, inflammation, signaling, and cell adhesion.[75,76] In the kidney, LUM belongs to the ECM network, whereas intracellularly LRG1 prevents apoptosis and extracellularly modulates TGF-β1-signaling. LRG1 therefore plays a distinct role in tubular injury and lupus nephritis.[77-82] LUM and LRGP1 are upregulated in AM, as in mice with AS.[13] In urine from children with AS, we were able to verify LRG1 by ELISA, but unable to verify LUM. However, in set 1, LUM declined during disease progression in all severity groups and is therefore a convenient BM of type B (Figure 1) in the same way as PICP and C1q, (Supplementary Figure S9).

In previous studies, reduced levels of GS, an actin-interacting protein,[83,84] are linked to organ injury including CKD, correlating with disease progress and mortality.[85-87] We previously identified GS as a BM in mice with AS.[13] Dogs with early AS

Table 6. Receiver operating characteristic curve analysis, comparison of patients with [a]Alport syndrome, thin basement membrane nephropathy, and benign familial hematuria (set 2) with controls

BM	Cut-off	Sensitivity	1-specificity	AUC	BM×BM	Cut-off	Sensitivity	1-specificity	AUC
Serum									
TGFß	30.82	0.733	0.158	0.830					
CFI	16760	0.769	0.111	0.846					
Urine									
ColXIII	0.98	0.617	0.131	0.817	ColXIII×FMN	13.75	0.725	0.232	0.796
HABP2	0.62	0.596	0.072	0.808	C4BP×ColXIII	5.20	0.723	0.102	0.856
C4BP	4.04	0.894	0.192	0.898	GS×CFH	579.6	0.745	0.253	0.815
CRP	4.29	0.674	0.210	0.765	LRGP1×C1q	0.90	0.667	0.105	0.801
CFH	21.72	0.851	0.153	0.903	C9×CFH	3058	0.617	0.121	0.799
CFI	1.07	0.596	0.224	0.718	CRP×C1q	0.72	0.694	0.221	0.806
FGG	183	0.591	0.1	0.753	CFH×CFI	20.74	0.702	0.182	0.850
					C4BP×ColXIII×HABP2	2.16	0.652	0.052	0.819

AUC, area under the curve; CRP, C-reactive protein; FGG, fibrinogen γ-chain.
Receiver operating characteristic curve analysis by SPSS (cf. Methods section). Only BMs with AUC >0.700 are shown.
[a]Autosomal Alport syndrome (AS) and histologically determined AS without genetic information.
Concentration ranges of BMs: Serum: TGF-ß1 and CFI, ng/mL. Urine: ColXIII, HABP2, C4BP, CFH, CFI, FGG, GS, LRGP1, and C1q, ng/mg creatinine; CRP, C9 and FMN, pg/mg creatinine.

have heterogeneous results after mass spectrometry (Supplementary Figure S6 and Figure 2). However, we were able to observe slightly higher values in serum from affected dogs (Supplementary Table S5) and in urine from children with AS (Tables 4 and 5).

Several other proteins which are crucial for podocytes, such as dynein, myosin IXA, FMN, talin 1, sacsin, and fukutin, were upregulated in AM.[88] However, we found no reliable ELISAs for these dog proteins. Therefore, we only analyzed the human samples with talin and FMN. FMN, validated, belongs to a family of proteins involved in the polymerization of actin[89] but is not known as a BM for AS. FMN is notably higher in urine than blood but only significantly so in children with early stages of intermediate and severe AS and in combination with ColXIII (Tables 5 and 6, and Supplementary Figure S9).

Although increased in our study group vitronectin,[34,49,90-93] complement component C9,[34] and angiotensinogen have insufficient diagnostic power (Tables 4–6).

Several minor subfractions of albumin and transferrin showed increased deamidation rates in AM, probably by transglutaminase activities.[94,95] Transglutaminases also catalyze crosslinking of ECM molecules. Coagulation factor XIII, which also has transglutaminase activity, is increased in AM (Figure 2), and possibly responsible for these modifications.[96] We detected minor parts of albumin carbamoylated already in preclinical dogs with AS confirming[21] although that this nonenzymatic modification was associated with advanced CKD.[97] Their use for diagnostics however requires PTM-specific ELISA.

Our most promising BMs did not correlate with individual and clinical parameters (Supplementary Results). Therefore, our BMs reveal an additional benefit and no special cut-offs must be considered in clinical practice.

CONCLUSIONS

We identified several promising real-world BM candidates for very early stages of AS. We included the requirement that diagnostic BMs must cover a wide window of opportunity because the exact stage of AS in a child depends on multiple factors such as the type and location of the mutation, age, diet, protein and salt intake, and other environmental factors. Preclinical glomerular injury in AS is indicated by BMs of 3 different origins as follows: (i) ColXIII, a membrane protein from glomerular endothelial cells; (ii) HABP2, a constituent from the GBM; and (iii) C4BP from podocytes. Moreover, we were also able to establish several other components usable for early diagnostics of AS, such as FMN, LRGP1, and GS (from injured or destroyed cells), fibrinogen γ-chain (from ECM), C-reactive protein, CFH, CFI (regulatory complement components), as well as complement factors C1q and complement component C9.

Although our results are statistically supported, and promising, we had to obtain them from a limited number of samples. This was because of the rarity of the diseases and the pandemic conditions during our research. Further studies therefore need to include larger numbers of samples and ensure similar sample numbers in the different BM groups. Such further studies should also focus on the time course of our BM candidates during disease progression. For application in the real world, we suggest using a combination of diagnostic BMs rather than single parameters. Furthermore, in the near future, our BMs may also serve as prognostic markers and markers of response to nephroprotective therapy.

DISCLOSURE

The authors have declared no conflicting interest.

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Data Availability

The datasets generated or analyzed during the current study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

HR and OG designed the study and wrote the manuscript. MN conducted animal breeding and analysis, and provided dog samples. HR and BT performed mass spectrometric analysis, major parts of data analysis, ELISAs and statistical analysis. AL analyzed parts of mass spectrometric data. JS, OM, FW, AD, JB, DR, WK, and UJ-K provided clinical samples from children. All authors interpreted the results, critically drafted and revised the manuscript, and approved the final version.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Figure S1. Chromatograms of dog serum proteins after native size exclusion chromatography (1D-SEC).

Figure S2. Global protein distribution after anion exchange chromatography (2D-AEC).

Figure S3. Distribution of 2 examples of plasma proteins from NM after 2D-AEC.

Figure S4. Distribution of serum amyloid after 2D-AEC and mass spectrometry identification of its peptides as example of a uniformly altered protein.

Figure S5. Distribution of α1B-glycoprotein after 2D-AEC and mass spectrometric identification of its peptides as example of a protein with several proteoforms with different degrees and direction of alteration under AS.

Figure S6. Distribution of gelsolin after 2D-AEC and mass spectrometric identification of its peptides as example of a second protein with several proteoforms with different degrees and direction of alteration under AS.

Figure S7. Distribution of biomarker candidates, adiponectin and the complement component C1q after 2D-AEC and mass spectrometric comparison of their peptides in AM to NM.

Figure S8. Distribution of the biomarker candidate ColXIII after 2D-AEC and mass spectrometric comparison of its peptides in AM to NM.

Figure S9. Comparison of the concentrations of biomarker candidates in serum and urine from patients (set 1 and set 2) and controls.

Figure S10. Proteoforms and PTM of albumin.

Figure S11. Proteoforms and PTM of transferrin.

Table S1. Specification of ELISA-Test kits applied to dog and human samples.

Table S2. List of protein chains identified by the Proteome Discoverer in 4 sample pools of blood plasma from dogs.

Table S3. List of protein chains identified by the Sieve in 4 sample pools of blood plasma from dogs.

Table S4. Mass spectrometric identified biomarker candidates.

Table S5. Concentrations of some biomarker candidates determined by ELISA in dog samples.

Table S6. Correlation of biomarker concentrations in urine one with each other's.

Table S7. Correlation of serum and urinary biomarker candidates with urine albumin-to-creatinine ratio and serum creatinine in patients with AS and similar diseases.

Table S8. Correlation of serum and urinary biomarker candidates with eGFR and cGFR in patients with AS and similar diseases.

Table S9. Correlation of serum and urinary biomarker candidates with serum C-reactive protein in patients with AS and similar diseases.

Table S10. Receiver operating characteristic curve analysis, comparison of patients with AS (set1, first visit) with controls.

STARD Checklist.

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