Intraocular Biomarker Identification in Uveitis Associated With Juvenile Idiopathic Arthritis

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Purpose. To investigate the presence of biomarkers in aqueous humor (AH) from patients with uveitis associated with juvenile idiopathic arthritis (JIA).

Methods. AH ($n = 73$) and serum ($n = 105$) samples from 116 children were analyzed using surface enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-ToF MS). The samples were divided into the following groups: JIA, silent chronic anterior uveitis (AU), other uveitis entities, and noninflammatory controls. Statistical biomarker identification was performed using the SELDI-ToF Biomarker Analysis Cluster Wizard followed by multivariate statistical analysis. Biochemical identification of biomarkers was performed by polyacrylamide gel protein separation, followed by liquid chromatography tandem mass spectrometry. ELISA was performed in a number of AH samples representing all four study groups.

Results. In the JIA group, one AH protein peak at mass/charge (m/z) 13,762 had qualitative and quantitative differences in expression compared with the other uveitis entities and the controls, but not to the group of silent chronic AU. Its quantitative expression in AH of patients with JIA and other silent chronic AU was positively associated with uveitis activity. The protein at m/z 13,762 in AH was identified as transthyretin (TTR). The TTR concentration in AH differed significantly between the study groups ($P = 0.006$) with considerably higher TTR concentrations in JIA and silent chronic AU samples positive for m/z 13,762 than those of the other uveitis and control groups.

Conclusions. TTR is a potential intraocular biomarker of JIA- associated uveitis. Its role in the pathogenesis of silent chronic AU with and without arthritis needs further investigation.

Keywords: aqueous humor, proteins, uveitis, juvenile idiopathic arthritis, biomarker

Juvenile idiopathic arthritis (JIA) is the main underlying systemic cause of uveitis in children.[1] Uveitis in JIA is characterized by mostly anterior location, bilateral involvement, an insidious onset and an asymptomatic course. Most children with JIA-associated uveitis are being diagnosed after the diagnosis of arthritis has been made. However, in 23% of children, uveitis can be the first sign of JIA with arthritis being diagnosed months to years after the diagnosis of uveitis.[2,3]

Keeping in mind this atypical presentation of JIA and the absence of a definitive diagnostic test, there is a strong rationale for efforts to identify specific biomarkers in the ocular fluid of children with JIA-associated uveitis. Proteomic techniques based on mass spectrometry approaches are nowadays being widely applied in autoimmune diseases for the identification of biomarkers, which could serve for diagnostic, prognostic, or even therapeutic purposes.[4] Although biomarker profiling of aqueous humor (AH) has not frequently been performed, the applicability of surface enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-ToF MS) for this purpose has been demonstrated.[5–8]

In this report, we provide data on proteomic analysis of paired AH and serum samples of pediatric uveitis patients, including JIA, silent chronic noninfectious anterior uveitis (AU), other uveitic entities, and noninflammatory pediatric controls with cataract and/or glaucoma.

Methods

Study Participants

A total of 116 children who visited the Department of Ophthalmology of the University Medical Center Utrecht (UMCU), The Netherlands, between 2004 and 2010, participated in this study. The diagnosis of uveitis was made by an ophthalmologist specialized in childhood uveitis. Uveitis was evaluated according to the criteria of the International Uveitis Study Group.[9] Uveitis was diagnosed if 1+ or more cells in the anterior chamber were seen over at least two eye examinations. All children from the uveitis group were additionally evaluated by a pediatric rheumatologist and/or immunologist to confirm

Table 1. Overview of Available Samples Within the Patient Groups

	Number of Patients				
Material Available	**Total** $N = 116$	**JIA** $N = 17$	**Silent Chronic AU** $N = 10$	**Other Uveitis** $N = 62$	**Controls** $N = 28$
AH, *N* (%)	73 (63)	14 (82)	8 (80)	31 (50)*	20 (71)†
Serum, *N* (%)	105 (91)	16 (94)	9 (90)	59 (95)	21 (82)
Paired AH and serum, *N* (%)	62 (53)	13 (76)	7 (70)	27 (44)	15 (54)

* Including 10 intermediate, 3 infectious, and 18 uveitis of undefined etiology.
† Including 11 congenital cataract, 3 traumatic cataract, and 6 congenital glaucoma.

or to exclude a systemic disorder. The diagnosis of JIA was based on the criteria of the International League Against Rheumatism (ILAR).[10]

For analysis, both AH and serum samples were divided into four groups: JIA-associated uveitis, silent chronic AU, other uveitis entities (including symptomatic anterior, intermediate, posterior and panuveitis of various etiology), and noninflammatory controls (congenital cataract [$n = 11$], traumatic cataract [$n = 3$], and congenital glaucoma [$n = 6$]). Table 1 shows the available ocular fluid and serum samples within these specific groups. Table 2 shows the distribution of age, sex, duration, and activity of uveitis within the groups of available AH samples.

The diagnosis of JIA-associated uveitis was confirmed by a pediatric rheumatologist. Silent chronic AU presented a separate group with bilateral insidious anterior uveitis when other infectious and/or systemic causes were excluded by examination by a pediatric rheumatologist and/or immunologist (10/10 patients), serological tests (10/10 patients), and AH analysis (5/10 patients, 4/5 seronegative for antinuclear autoantibodies [ANA]). Infections, which were ruled out by AH analysis in patients with silent chronic AU, included herpes simplex virus (5/5), varicella zoster virus (5/5), cytomegalovirus (1/5), and *Borrelia burgdorferi* (1/5). In these patients a systemic diagnosis of JIA could not be made, although their clinical features of uveitis were consistent with JIA-associated uveitis. Moreover, ANA was positive in 5 out of 10 children with silent chronic AU. Active uveitis at the time of sampling was defined as at least trace cells in the AH on examination prior to AH puncture in the same eye. Additional signs of intermediate uveitis in patients with chronic anterior uveitis were scored if there was a history of vitreous opacities and/or inflammatory cells in the vitreous, out of proportion for "spillover" from the anterior chamber.

The study was approved by the independent institutional review board (IRB) of the UMCU. The legal guardians of all study subjects from whom samples were obtained during surgery signed a written informed consent document. The research adhered to the tenets of the Declaration of Helsinki.

Sample Collection

AH and serum samples were collected at the UMCU, The Netherlands. From 62 patients paired AH and serum samples were collected. From 11 patients only AH was collected, whereas from 43 patients only serum samples were available (Table 1). AH samples from children with uveitis were obtained for diagnostic purposes ($n = 25$) or during therapeutically necessary intraocular surgery ($n = 48$). In the JIA and silent chronic AU groups samples were obtained during therapeutically required surgery in all but one patient with silent chronic AU. The AH sample from this patient was collected to exclude an infectious etiology of uveitis before initiating immunosuppressive therapy. All samples from control patients without uveitis were collected during elective intraocular surgery. AH samples were extracted by an experienced ocular surgeon using a binocular microscope (OPMI VISU 150 Stativ S7; ZEISS, Jena, Germany). The samples were stored at –80°C within 5 hours of collection.

SELDI-Tof MS Analysis

Normal Phase (NP20) Protein chip arrays (Bio-Rad, Hercules, CA) were used and pretreated according to standard manufacturer's procedures. Each array was conditioned with 1 μL deionized water. Then 1 μL AH or serum was applied onto the array and allowed to mix with the water. After air drying, the arrays were washed twice with 5 μL deionized water to remove salts. When the spots were dry, 1 μL sinapinic acid (SPA), an energy absorbing molecule (EAM), was applied twice on every spot at an interval of 5 to 10 minutes. Each array was analyzed with a standard protocol where 350 shots were fired on each spot using a laser intensity of 6000 nJ, a deflector setting at mass/charge (m/z) 3000, a detector sensitivity of nine, and a molecular mass detection range from m/z 1000 to 200,000. The optimization range was from m/z 3000 to 50,000. The target mass was set at an m/z of 10,000 thomson (Th). Calibration was performed with the All-in-One Protein Standard calibrant (Ciphergen Biosystems Inc., Fremont, CA). The data were clustered in JIA, silent chronic AU, other uveitis, and noninflammatory controls.

Automatic peak detection by Cluster Wizard (Ciphergen Express software 3.0; Ciphergen Biosystems Inc.) was performed using the settings of four times signal-to-noise ratio and a valley depth of three. The cluster width was specified as dynamical adjustment of the mass window based on the width of the peak. In order to be assigned as a peak, it had to be present in 5% of total AH samples and 10% of total serum samples. For automatic peak detection within JIA and silent chronic AU samples only, the peak had to be present in at least 20% of the spectra. To ensure accurate statistical analysis, spectra were manually inspected for mislabeling of peaks and adjusted if necessary. ProteinChip Expression Difference Mapping (Ciphergen Biosystems Inc.) was used for the initial selection of candidate biomarkers. Unsupervised multivariate hierarchical cluster analysis was used for assessment of sample relationships. The proximity of spectra and the branching patterns in the dendrogram reflect sample similarity.

Protein Purification and Identification

Fourteen samples of patients with JIA and silent chronic AU containing the marker at m/z 13,762 (Fig. 1) were pooled yielding a volume of almost 2 mL undiluted AH. Confirmation of the presence of the peak at m/z 13,762 was performed using the same array preparation protocol as described above. The arrays were read on a Proteinchip Enterprise 4000 system (Ciphergen Biosystems Inc.) with target m/z at 13,000, matrix attenuation at m/z 2500, and m/z range from 0 to 40,000. The protein concentration of the pooled sample was determined by

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TABLE 2. General Characteristics of the Patient Groups Based on AH Availability

Characteristics	Number of Patients								
	Total $N = 73$	JIA $N = 14$	Silent Chronic AU $N = 8$	Other Uveitis $N = 31$	Controls $N = 20$	*P* Value*	*P* Value JIA vs. Silent Chronic AU†	*P* Value JIA vs. Other Uveitis†	*P* Value JIA vs. Controls†
Females, *N* (%)	40 (55)	10 (71)	6 (75)	16 (52)	8 (40)	0.199	-	-	-
Age, y (median; range)‡	10.6 (0.1–23.9)	11.5 (4.9–23.9)	11.1 (8.1–16.6)	13.8 (0.1–17.8)	4.2 (0.4–15.9)	<0.001	1.000	0.831	0.003
Duration of uveitis, y (median; range)‡	1.5 (0–22.3)	8.1 (0.9–22.3)	3.5 (0.5–11.3)	0.5§ (0–7.7)	NA	<0.001	0.630	0.003	NA
Active status of uveitis, *N* (%)	37 (70)	7 (50)	4 (50)	26 (84)	NA	0.027	1.000	0.089	NA

NA, not applicable.

* Fischer's exact test was used for analysis of categorical variables. Mann-Whitney *U* test and Kruskal-Wallis test were used to compare medians between the groups.

† Bonferroni correction for multiple testing is applied.

‡ At the time of aqueous puncture.

§ In 13 patients duration of uveitis was shorter than 3 months.

both the bicinchoninic acid (BCA) protein assay and the Bradford protein assay (Bio-Rad, Hercules, CA) to calculate average protein concentration. Protein concentration in the pooled sample was estimated to be 1375 μg/ml. Protein purification was performed by denaturing SDS-Page (12% BisTris precast gels; Life Technologies, Paisley, UK). The gels were fixed and stained with coomassie brilliant blue (Sigma-Aldrich, St. Louis, MO) for 30 minutes. Protein bands resolved in the molecular weight range of interest were excised from the polyacrylamide gel. Proteins were digested with trypsin as previously described.[11] Prior to liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) analysis samples were resuspended in 30 μL 5% acetonitrile (ACN)/0.1% formic acid. Peptides were resolved by reverse phase chromatography (Biobasic column, 180 uM × 15 mm; ThermoScientific, Waltham, MA) over a 30 minutes ACN gradient at a flow rate of 2 μL/min using the Surveyor LC system (ThermoScientific) followed by MS/MS spectra acquisition using an LCQ Deca XP Plus (ThermoScientific).

Raw data files were converted into Mascot generic files using the MassMatrix File Conversion Tool (Version 2.0; MassMatrix Database Search Engine, Cleveland, OH) for input into the Mascot searching algorithm (Matrix Science, Boston, MA). The data files were searched against SwissProt (v. 2012_07; UniProt consortium, Cambridge, UK) with human taxonomy using the following search criteria: tryptic peptides with up to one missed cleavage and carbamidomethylation of cysteines and oxidation of methionines, which were set as variable modifications.

In parallel to protein digestion equivalent gel bands were excised for passive elution and determination of actual protein mass. Excised bands were cut into 1-mm^3 sized pieces and washed with 100 mM ammonium bicarbonate. The washing solution was discarded and gel slices dehydrated with 50% ACN/50% 100 mM ammonium bicarbonate. Solvent was discarded and gel pieces air dried at 42°C to remove all traces of solvent. A minimal volume of protein elution solution (50% formic acid, 25% ACN, 15% isopropanol, 10% water vol/vol/vol/vol) was added to cover the gel pieces. Proteins were extracted for 1 hour with 30 minutes of sonication. The supernatant was collected and analyzed by SELDI-ToF MS directly on a gold array without preconditioning.

Biomarker Confirmation

To confirm the SELDI-Tof LC/MS/MS result AH samples from JIA patients ($n = 4$), patients with silent chronic AU ($n = 4$), patients with other uveitis entities ($n = 5$), and noninflammatory controls ($n = 5$) were analyzed by ELISA (AssayMax Human Prealbumin ELISA Kit; ProAssay, St. Charles, MO). Samples were tested using four serial 10-fold dilutions starting at 1:100. The assay was performed according to the instructions of the manufacturer. Protein concentrations for each dilution were calculated by linear regression curve-fitting of the standard curve followed by dose-response extrapolation using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA). Sample concentration is the mean of the concentrations for each dilution in the linear part of the standard curve.

Statistical Analysis

The SELDI-Tof cluster lists were exported to SPSS version 15.0.1 (SPSS, Inc., Chicago, IL) for further statistical analysis. The Kruskal-Wallis test and Mann-Whitney *U* test were used for nonparametric analysis of differences between the groups. For comparison of more than two groups Bonferroni correction of *P* values was applied: calculated *P* values were multiplied by the number of comparisons. ANOVA and binary logistic

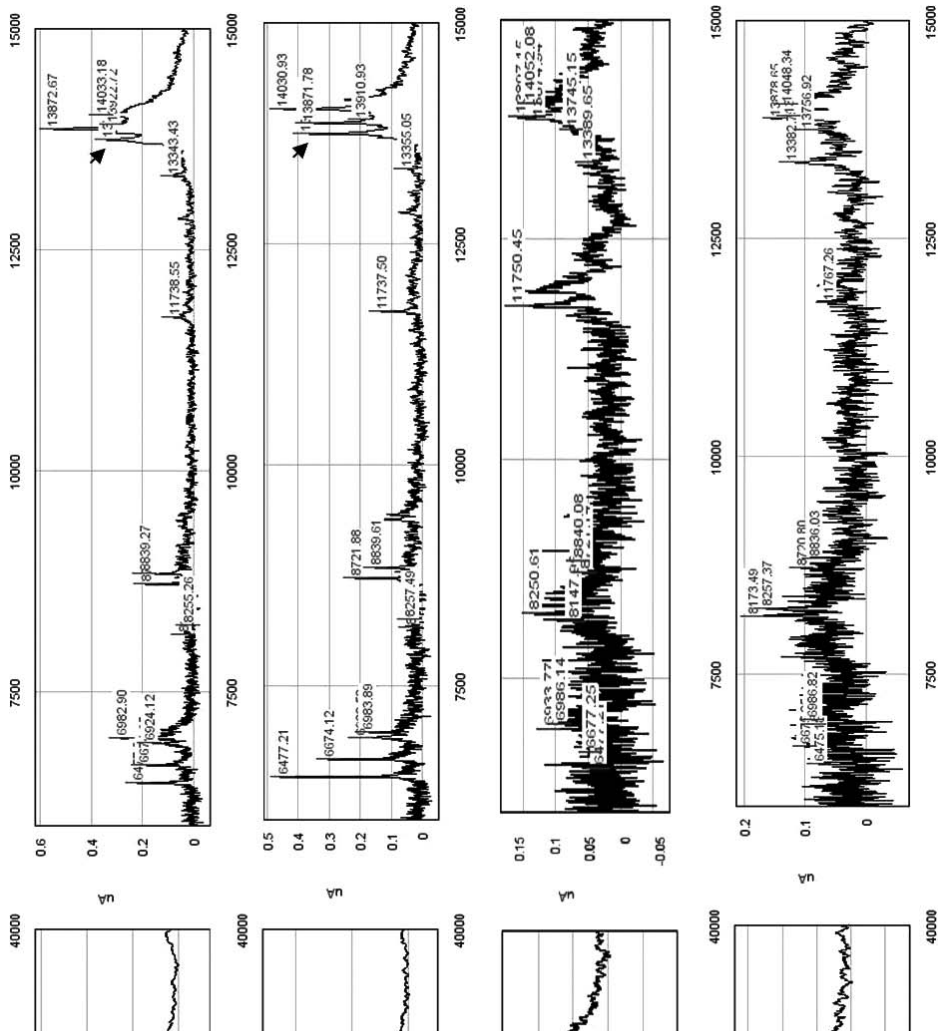

Figure 1. Aqueous humor SELDI-ToF MS spectra representative for the four study groups: (**A**) JIA-associated uveitis; (**B**) silent chronic anterior uveitis; (**C**) other pediatric uveitis; and (**D**) noninflammatory pediatric controls. On the *left hand side* an overview of the peaks in the range 2500 to 40,000 Da are displayed. On the *right hand side* the same spectra, now including peak labels, are shown zoomed in from 6000 to 15,000 Da. The peak at m/z 13,762 is labeled with an *arrow* in (**A**, **B**).

regression with adjustment for covariance of age and duration and activity of uveitis were used for quantitative and qualitative analysis of potential biomarkers. Spearman's rho correlation coefficient was computed to estimate correlations between SELDI-ToF peak intensities and between biomarker ELISA concentration and quantitative and qualitative SELDI-ToF results. The Pearson χ^2 test or the Fisher's exact test was used to compare associations between categorical variables when appropriate. *P* values less than 0.05 were considered significant.

Results

SELDI-Tof MS Cluster Peak Analysis

To investigate the presence of possible biomarkers for JIA-associated uveitis and to help the identification of candidates for specific intraocular biomarkers in JIA-associated uveitis cluster peak analysis of the SELDI-ToF MS data was performed. Figure 1 displays a representative peak profile for each of the four groups within the m/z range of 2500 to 40,000 Da, including a close up in the range of 5000 to 15,000 Da.

When analyzing all AH samples, 26 protein peak clusters were detected. Of these, 17 showed significant differences in expression levels between the four study groups (Supplementary Table S1, Table 3). When analyzing the absence or presence of these 17 protein peaks, six of them at m/z 6475, 6672, 8725, 8840, 13,762, and 27,981 displayed significant differences between the groups (Table 3). In the JIA group both the presence and expression levels of the peak at m/z 13,762 were significantly increased compared with other uveitis entities and controls, but not compared with silent chronic AU (Table 3). Globally JIA and silent chronic AU samples showed similar protein profiles and these groups did not differ in any of the peak cluster intensities or detection frequencies (Supplementary Table S1, Table 3).

Spearman's rho correlation coefficient analysis of the peak expression intensity at m/z 13,762 in JIA-uveitis patients revealed several significant positive and negative correlations with the other peaks, which are presented in Table 4. Positive correlations were found with peaks at m/z 6989, 13,884, 13,914, and 27,981 (Spearman's rho correlation coefficient between 0.754 and 0.837). Negative correlations were observed with the peaks at m/z 8161, 8255, and 16,171 (Spearman's rho correlation coefficient between –0.723 and –0.873; Table 4).

Cluster analysis of serum revealed 21 peak clusters (Supplementary Table S1). Significant differences in expression intensity were only detected between JIA-associated uveitis and the other uveitis entities. The peak at m/z 13,762, which was significantly upregulated in the AH of JIA and silent chronic AU patients, was not detected in serum. Similarly, the AH peaks correlating with the peak at m/z 13,762 (m/z 6928 and 6989, and m/z 8161, 8255, and 16,171) were not detected in serum (Supplementary Table S1).

Using the presence or absence of AH-specific protein peaks a classification tree was made. Based on the presence of the protein at m/z 13,762 in AH, 9 out of 14 JIA patients were clustered as well as three patients with other uveitis entities (two ANA-positive undefined panuveitis and one ANA-positive posterior uveitis with papillitis) were identified incorrectly yielding a sensitivity of 64% and a specificity of 95% (Fig. 2). For the calculation of the specificity silent anterior AU cases were not taken into account. Adding the protein detected at m/z 8255, that had the strongest negative expression correlation with the 13,762 peak (Table 4), into the algorithm one incorrectly identified patient with papillitis could be filtered out, which increased the specificity of the classification tree to 96%. When applying the algorithm to the AH of patients with silent chronic AU, they were clustered together with JIA-associated uveitis patients with a similar sensitivity (5/8, 63% and 9/14, 64%, respectively; Fig. 2). All control patients were clustered separately due to the absence of the peak at m/z 13,762.

To further study the protein profiles in the AH of patients with JIA and silent chronic AU a separate automatic peak detection procedure using the same analytical conditions was performed. In this analysis, 19 of the initial 26 peak clusters were identified specific for these two groups. An unsupervised heat map for these 19 peak clusters revealed distinct protein expression patterns in samples from patients with active and inactive uveitis independently of diagnosis of JIA (Fig. 3). The separation between groups based on the protein expression showed major correlation with uveitis activity (Fig. 3). All but one inactive JIA and silent chronic AU samples clustered together and could be distinguished from samples from patients with active uveitis based on the protein expression pattern. One sample from a JIA patient with clinically inactive uveitis showed a protein profile, which was comparable with active samples (Fig. 3). Six upregulated proteins at m/z 6928, 6989, 13,762, 13,884, 13,914, and 14,040 were most clearly associated with uveitis activity (Fig. 3). Multivariate analysis of the expression of the peak at m/z 13,762 showed it significant association with activity of uveitis independent from the association with distinct study groups ($P < 0.001$), and absence of significant association with age or duration of uveitis. Within the JIA and silent chronic AU samples no significant differences in protein expression were found regarding type of JIA, ANA serological status, immunosuppressive treatment, presence of cataract, secondary glaucoma, cystoid macular edema, or myopia. Patients with detected m/z 13,762 peak more frequently had additional signs of intermediate uveitis (within JIA 67% vs. 33%, $P = 0.031$; within all silent chronic AU including JIA 71% vs. 29%, $P = 0.024$).

Biomarker Identification

As the target at m/z 13,762 is most indicative for JIA and silent chronic AU an attempt was made to purify and identify this protein by LC/MS/MS. Due to the limited volume of AH, all samples of JIA and silent chronic AU containing the m/z 13,762 peak were pooled and verified by SELDI-ToF MS (Fig. 4). As shown in Figure 4, peak clusters at m/z 13,800 and 6900 were most abundant; the ion population at m/z 6900 most likely represents the double charged species of the singly charged population at m/z 13,800. Subsequent SDS-PAGE resolved several bands within the range of 3 to 20 kDa (Fig. 5A). Passive elution of these bands followed by SELDI-ToF MS analysis demonstrated that band 8 contained the target protein at m/z 6900 and 13,800 (Fig. 5B, compared with Fig. 4B). LC/MS/MS analysis of the same band (band 8) revealed TTR (Mr 13.76 kDa) as the most abundant protein present in the AH in JIA and silent chronic AU samples.

Biomarker Confirmation

A selection of samples from the SELI-ToF experiment (four JIA, four silent chronic AU, five other uveitis, and five noninflammatory controls) were tested for TTR concentration by ELISA to confirm the initial results. The TTR concentration in AH differed significantly between the study groups ($P = 0.006$; Fig. 6). All JIA samples had TTR concentrations, which were considerably higher than those of the other uveitis and control groups. The TTR concentration in the silent chronic AU samples, which contained the m/z 13,762 peak by SELDI-ToF

Table 3. Overview of the Expression and Detection by Seldi-ToF MS of the Selected Protein Peaks in AH Compared Between the Study Groups: Uveitis Associated With JIA; Silent Chronic Anterior Uveitis, Other Pediatric Uveitis Entities; and Pediatric Noninflammatory Controls

	Average Peak Intensity in AH, $N = 73$				*P* Values				Detected Peaks in AH, $N = 73$				*P* Values			
Protein Peaks m/z	JIA $N = 14$	Silent Chronic AU $N = 8$	Other Uveitis $N = 31$	Control $N = 20$	*P* Value*	JIA vs. Silent Chronic AU *P* Value†‡	JIA vs. Other Uveitis *P* Value†‡	JIA vs. Control *P* Value*‡	JIA $N = 14$ (%)	Silent Chronic AU $N = 8$ (%)	Other Uveitis $N = 31$ (%)	Control $N = 20$ (%)	*P* Value*	JIA vs. Silent Chronic AU *P* Value†‡	JIA vs. Other Uveitis *P* Value†‡	JIA vs. Control *P* Value*‡
6,475	0.103	0.234	0.066	0.064	**<0.001**	0.061	**0.035**	0.354	9 (64)	4 (50)	4 (13)	1 (5)	**<0.001**	1.000	0.072	**0.036**
6,672	0.106	0.211	0.05	0.064	**<0.001**	0.063	**0.040**	0.670	9 (64)	5 (62)	2 (7)	1 (5)	**<0.001**	1.000	0.087	**0.024**
6,928	0.107	0.109	0.077	0.061	**0.044**	1.000	0.074	0.107	4 (29)	3 (38)	3 (10)	ND	0.159	-	-	-
6,989	0.198	0.125	0.131	0.091	**0.007**	0.294	0.313	**0.008**	9 (64)	3 (38)	12 (39)	4 (20)	0.258	-	-	-
8,161	0.063	0.058	0.1	0.121	**0.019**	1.000	**0.008**	0.070	3 (21)	1 (13)	8 (26)	7 (35)	0.889			
8,255	0.065	0.063	0.093	0.117	**0.005**	1.000	**0.030**	**0.010**	2 (14)	1 (12)	8 (26)	8 (40)	0.643	-	-	-
8,725	0.096	0.107	0.063	0.061	**<0.001**	1.000	**0.011**	**0.011**	7 (50)	3 (38)	3 (10)	ND	**0.008**	1.000	0.177	0.129
8,840	0.096	0.096	0.068	0.068	**0.004**	1.000	0.121	**0.013**	10 (71)	3 (38)	5 (16)	ND	**0.011**	1.000	0.240	**0.018**
13,361	0.109	0.073	0.085	0.112	**0.022**	0.407	1.000	1.000	13 (93)	5 (63)	19 (62)	11 (55)	0.273	-	-	-
13,762§	0.232	0.229	0.12	0.087	**<0.001**	1.000	**0.001**	**0.002**	9 (64)	5 (63)	3 (10)	ND	**<0.001**	1.000	**0.036**	**0.033**
13,884	0.397	0.214	0.243	0.139	**0.027**	0.426	0.533	**0.031**	10 (71)	2 (25)	16 (52)	8 (40)	0.168	-	-	-
13,914	0.311	0.237	0.199	0.135	**0.022**	1.000	1.000	**0.040**	4 (29)	7 (88)	12 (39)	5 (25)	0.089	-	-	-
17,361	0.069	0.078	0.05	0.047	**0.022**	1.000	**0.012**	0.268	8 (57)	4 (50)	9 (29)	2 (10)	0.092	-	-	-
22,313	0.113	0.108	0.09	0.072	**<0.001**	1.000	0.312	**<0.001**	13 (93)	8 (100)	25 (81)	12 (60)	0.057	-	-	-
27,981	0.091	0.117	0.051	0.036	**0.001**	1.000	**0.044**	**0.041**	10 (71)	5 (63)	12 (39)	3 (15)	**0.012**	1.000	0.292	**0.018**
28,122	0.067	0.087	0.049	0.038	**0.005**	0.827	0.065	0.101	2 (14)	4 (50)	12 (39)	6 (30)	0.326			
33,410	0.316	0.302	0.222	0.135	**<0.001**	1.000	0.261	**<0.001**	13 (93)	8 (100)	31 (100)	18 (90)	0.888	-	-	-

Bold numbers indicate significant P values. ND, not detected.

* *P* values are calculated using ANOVA and binary logistic regression with adjustment for covariance of age.

† *P* values are adjusted for covariance of age and duration and activity of uveitis.

‡ Bonferroni correction for multiple testing is applied.

§ Subgroup analysis excluding the 13 cases with uveitis less than 3 months from the "other uveitis" group with additional adjustment for age, activity, duration of uveitis, and Bonferroni correction still showed *P* value 0.001 for the average peak intensity difference between JIA and the other uveitis group. The *P* value for the difference in the detected peaks between JIA and the other uveitis group was 0.015 with the same statistical adjustments.

TABLE 4. Bivariate Correlations Between the Expression of Protein Peaks in the AH of 14 Patients With Uveitis Associated With JIA

JIA-Uveitis AH	
Spearman's Rho Correlation Coefficient*	
Protein Peak, m/z	**13,762**
6,989	0.754
8,161	*-0.745*
8,255	*-0.873*
13,884	0.780
13,914	0.833
16,171	*-0.723*
27,981	0.837

Italic numbers show negative correlations.
* Only statistically significant correlation coefficients are presented ($P \leq 0.05$).

was comparable with that in JIA samples. Silent chronic AU samples that were negative for this peak had TTR concentrations comparable with the other uveitis entities and controls (Fig. 6). ELISA TTR concentration correlated positively with quantitative and qualitative SELDI-ToF results of the m/z 13,762 peak (Spearman's rho correlation coefficient of 0.560 [$P = 0.016$] and 0.583 [$P = 0.011$], respectively).

DISCUSSION

Our study shows distinct differences in AH protein expressions in JIA-associated uveitis compared with other pediatric uveitis and noninflammatory pediatric controls. Quantitative expression of JIA-specific proteins was associated with the activity of uveitis.

Classically, the diagnosis of JIA is based on clinical ILAR criteria based on specific rheumatologic signs; however, a diagnostic test for this disease is lacking. Moreover, the diagnosis of patients presenting with uveitis prior to arthritis is challenging. In our previous work, we have described a relatively high percentage of patients who have an atypical manifestation of JIA: with uveitis onset primary to arthritis.[2,3] The diagnosis of JIA in these patients was, in some cases, made several years after the initial onset of uveitis. Our results demonstrate similar protein compositions in the AH of patients with JIA-associated uveitis and in patients with silent chronic AU, characteristic for JIA-associated uveitis, but lacking the typical rheumatologic signs. We hypothesize that these patients can be considered as suspected for JIA-association, but with a primary clinical manifestation of uveitis. Intriguingly, while this manuscript was in preparation and 2 years after the initial experiment, one of the patients in the silent chronic AU group indeed developed arthritis 15 years after the onset of uveitis.

Further, similar sensitivity for JIA-associated uveitis and silent chronic AU was found when the patients were classified according to the classification tree, based on presence of a peak at m/z 13,762 (Fig. 2). These findings suggest similar intraocular molecular processes in patients with chronic anterior uveitis regardless the presence of arthritis. The relatively low sensitivity of the classification tree could be explained by an inactive status of uveitis in 50% of these samples, which seemed to have negative quantitative association with the expression of peak at m/z 13,762 (Fig. 3). Another explanation might be a potential heterogeneity within the group of silent chronic AU in which only a subgroup shares the molecular processes of JIA-associated uveitis. However, the strong correlation of the expression of the peak with activity of uveitis within both groups, the same activity rate in these two groups, and the same clustering in the classification tree makes the last hypothesis less feasible. On the other hand the specificity of the classification method was relatively high, although its calculation can be seen only as an estimation due to the absence of a golden standard for the diagnosis of JIA. Interestingly, two patients with other uveitis entities that clustered together with the (suspected) JIA patients had an ANA+ panuveitis without vasculitis and/or retinal scars and without infection or systemic association. Panuveits is a rare but a possible clinical manifestation of uveitis in JIA.[3]

In this study, TTR was identified as a potential biomarker for silent chronic AU, characteristic of JIA-associated uveitis. The nascent protein has a molecular weight of 15.88 kDa. However, as the signal peptide is lost during intracellular protein processing the molecular weight of the mature protein is 13.76 kDa, which is within 0.2% of the observed m/z by SELDI-ToF MS. SELDI-ToF MS analysis of unprocessed AH sample showed that the peak detected at m/z 13.8 kDa resolved into three discernible ions (Fig. 4B), which most likely represent different isoforms of the same protein. Indeed, a plentitude of isoforms have been reported for TTR, many of which are causally associated with formation of amyloid fibrils.[12] Well known systemic functions of TTR are the transport of thyroid hormones and retinol-binding protein (RBP), however, increas-

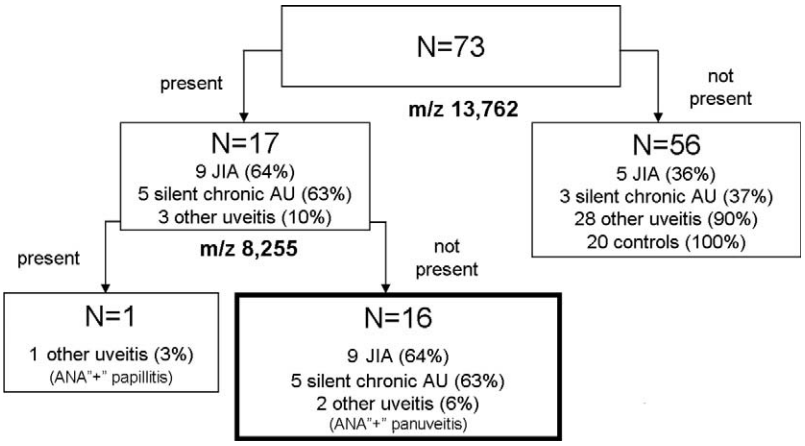

FIGURE 2. Classification tree of patients based on the presence of the peak at m/z 13,762 and the absence of the peak at m/z 8255 in AH. The algorithm's sensitivity per group (in percentages) is indicated in the *bold lined* frame.

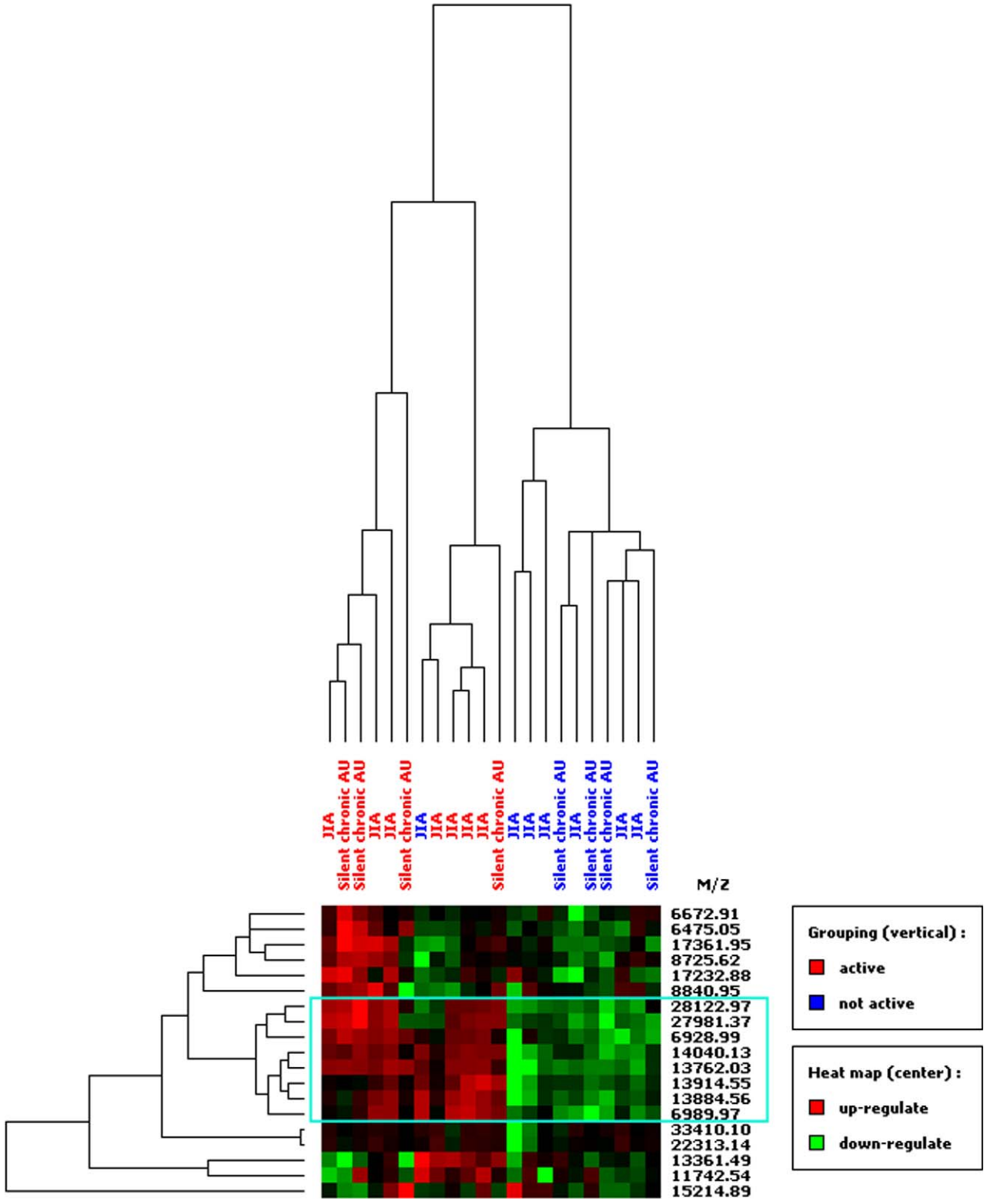

Figure 3. Unsupervised heat map of protein expression in the AH of patients with chronic silent AU with and without diagnosis of JIA based on 19 peak clusters found in these samples. Active samples are displayed in *red*, inactive samples in *blue*. Upregulation or downregulation of proteins is indicated on a scale from *red* to *green*, respectively. The *horizontal axis* displays the individual samples within the JIA and silent chronic AU groups; the *vertical axis* shows the peaks m/z included in the analysis. The proteins most clearly associated with uveitis activity are represented within the *turquoise rectangle*.

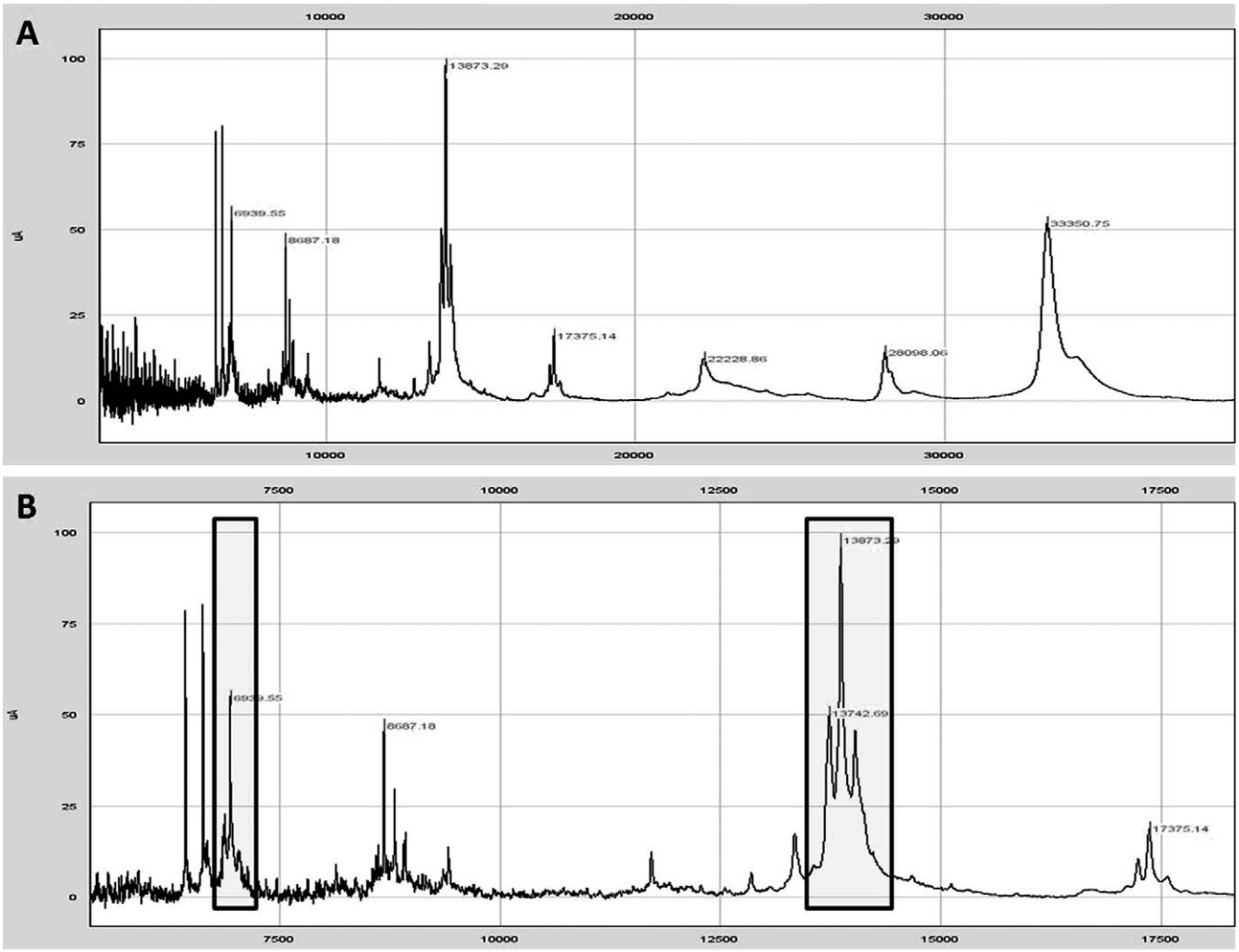

Figure 4. SELDI-ToF MS analysis of pooled AH from patients with JIA and silent chronic anterior uveitis containing the marker at m/z 13,762. (**A**) Spectrum showing ions detected in the m/z range 2500 to 40,000. (**B**) Close up view highlighting the target ions of interest (*black rectangles*). The m/z is represented on the *x*-axis, whereas the peak intensity is shown on the *y*-axis.

ing evidence suggests its involvement in many other biologic processes.[12–14] TTR in the human eye is synthesized by RPE and probably also by ciliary pigment epithelium (CPE), as was recently shown in a rabbit model.[15] TTR is present in almost all ocular tissues in noninflammatory eyes, including AH.[16–18] TTR in the vitreous has been demonstrated not to originate from the blood.[17] Similarly, considering its suggested production by CPE, local production of TTR in AH seems plausible. In our study, the ion at m/z 13,762 was not detected in serum, which suggests its intraocular origin, although direct comparison of serum and AH spectra by SELDI-ToF MS is probably not accurate.

Our results suggest that TTR may play a role in the pathogenesis of JIA-associated uveitis. However, another possibility is that TTR expression is a consequence of this type of uveitis and that it represents a marker for silent chronic noninfectious AU in general. The underlying mechanism is unclear; however; one could speculate that the amyloidogenic properties of TTR may be involved in secondary membrane formation in this type of uveitis. It has been suggested from studies demonstrating TTR production in rabbit CPE cells that TTR expression might contribute to the formation of amyloid fibrils in the pupillary margin and angle chamber.[15] In addition, TTR was reported to be increased in the AH of eyes with myopia and POAG but under expressed in eyes with primary congenital glaucoma.[5,7,19–21] Grus et al. proposed TTR as a biomarker for POAG, based on the upregulation of a 14,132 Da TTR species in the AH of POAG eyes.[7] In our study, no relationship between the expression of our target peak at m/z 13,762 and secondary glaucoma or myopia was found. None of our patients had POAG rendering a role for our TTR isoform in POAG unlikely. In our control group, which consisted for 30% of patients with congenital glaucoma, none of the patients had a detectable peak at m/z 13,762 regardless the glaucoma status. These findings suggest the existence of different isoforms of TTR in AH with potentially distinct functions in different (pathologic) conditions, analogous to numerous variants of TTR in serum and cerebrospinal fluid.[22,23] Further investigations are required to test this hypothesis, however, identification of the specific TTR isoform is a challenge that lies beyond the scope of this study.

TTR produced in the liver is known to be a negative acute-phase reactant, that means that the TTR concentration in the blood decreases following an acute phase response.[14] Moreover, in endotoxin-induced uveitis (EIU) in a rabbit model, intraocular TTR production by CPE was downregulated until 48 hours after the induction of EIU.[15] In our study, however, ocular TTR was overexpressed in patients with JIA-uveitis and

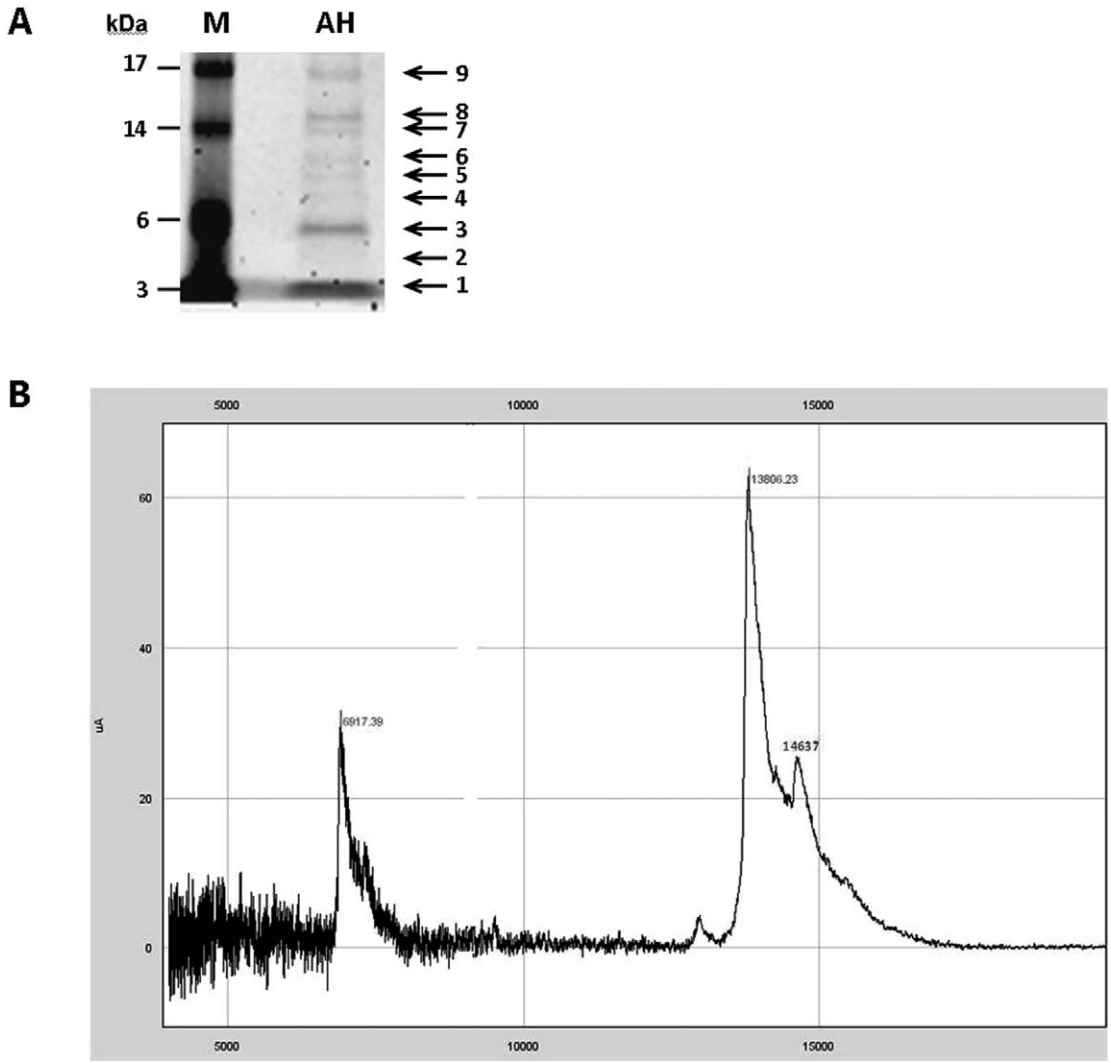

FIGURE 5. (**A**) Coomassie-stained 12% 1D SDS-PAGE containing 31 μg of pooled AH sample (*right lane*). The molecular weight marker (M) is shown in the *left lane*. Molecular weights are indicated on the *left* in kiloDaltons. The proteins bands analyzed in the AH samples are listed on the *right*. (**B**) SELDI-ToF MS spectrum of protein band 8 following passive elution from the excised gel slice. The molecular weight in represented on the *x*-axis, whereas the peak intensity is shown on the *y*-axis.

silent chronic AU with active disease. This discrepancy might be explained by the fact that inflammation in JIA-associated uveitis does not display an acute, but a chronic process with an insidious onset and a silent course. Most patients with JIA and silent chronic AU from this study had this type of uveitis for several years prior to AH collection. In addition, the intensity of inflammation in most JIA samples scored as "active" was mild due to their surgical origin. Differences in TTR expression and structure in chronic and acute intraocular inflammation need to be delineated by further research.

Proteomic analysis of synovial fluid in JIA suggests differential expression of proteins based on disease progression and JIA subtype.[24–26] Unlike synovial fluid, AH protein expression profiles did not seem to correlate with JIA subtype, however, the number of patients is too low for a reliable analysis. TTR was not identified in synovial fluid by other studies or in our SELDI-ToF MS experiments employing synovial fluids from patients with JIA (data not shown). In fact, we could not detect any of the specific peaks detected in the AH of JIA-uveitis patients in synovial fluid in the m/z range 2500 to 40,000. This suggests local upregulation of TTR in the anterior chamber in JIA-uveitis without having a systemic effect.

Under normal conditions, the protein concentration of AH is low (0.1–0.5 mg/mL)[27–29]; however, during inflammation the integrity of the blood–aqueous barrier can be severely compromised, causing remarkably increased protein concentrations.[27–29] This can complicate the interpretation of a comparative proteomic analysis of AH between uveitis and control samples. In our study, we focused on the proteins of low molecular weight, while especially proteins with high molecular weight seem to accumulate in the case of a compromised blood–aqueous barrier.[27] In the past, AH was considered to be a plasma filtrate,[27,28] however, recent studies show the presence of many specific proteins in normal AH.[16,18] Although the functions of the majority of these proteins are not known, their presence suggests active local production by cells and tissues of the anterior segment. Expression levels of AH proteins in our study showed significant correlation with each other, which could imply their interactions in the inflammatory or, alternatively, regulatory molecular mechanisms.

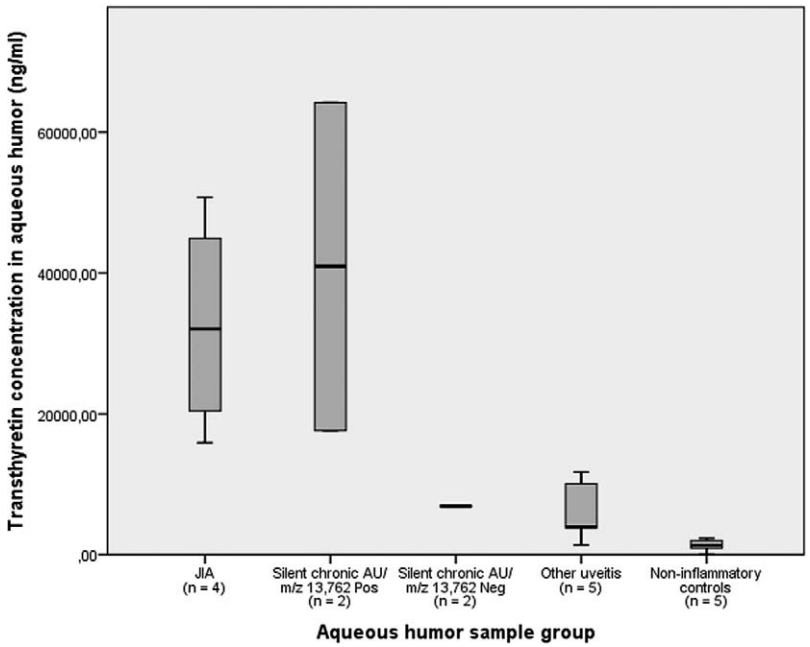

FIGURE 6. Validation results of the discovered biomarker by ELISA. *Boxplot* shows concentration of transthyretin (*y*-axis) in limited number of samples representing different study groups (*x*-axis).

To our knowledge, this is the first proteomics study on AH of children with uveitis. The strong points of our study are the relatively large numbers of inflammatory and noninflammatory pediatric AH samples, which allow for reliable statistical analysis and specific analysis of samples from patients with silent chronic AU, which we consider as suspect for JIA-association with incomplete ILAR criteria. The performed confirmation of the discovered putative biomarker by ELISA adds to the significance of our data. However, a validation using the TTR ELISA on newly acquired samples is warranted in order to truly establish TTR as a biomarker for JIA-associated uveitis and possibly also for silent chronic anterior uveitis without arthritis. Other shortcomings are that not in all cases paired AH and serum samples could be analyzed. Also, despite a relatively large total number of samples, heterogeneity within the sample groups resulted in lack of power to perform some statistical subgroup analyses, such as JIA entity correlation. Lastly, due to the practical and ethical difficulty of collecting noninflammatory ocular fluid in children, it was impossible to match control samples. However, our statistical methods included adjustment for covariance of age and duration and activity of uveitis and demonstrated their insignificance for the main conclusions. A subgroup analysis excluding cases with duration of uveitis of shorter than 3 months (cut off value for chronicity according to the definitions of the International Uveitis Study Group)[9] did not alter the significance of our discovery.

As a variety of conditions have been associated with TTR recently, one should question its general value as a biomarker, although specific isoforms or posttranslationally modified forms of TTR could have specific roles in different (pathologic) conditions. Unfortunately, the resolution of the SELDI-ToF MS technique did not allow for the identification of different TTR isoform relevant to JIA. Therefore, further studies are warranted. Another issue is the relatively low detection sensitivity of SELDI-ToF compared with other shotgun mass spectrometry methods and, hence, poor analytical depth in terms of how many low abundance proteins can be analyzed.

In summary, AH of patients with JIA-associated uveitis has a distinct protein expression profile compared with controls and other uveitis entities. These profiles are comparable with those of silent chronic noninfectious AU patients who had a clinical manifestation of uveitis comparable with JIA-patients, but without clear rheumatologic signs. Expression of these proteins was associated with activity of uveitis. TTR, the protein identified here, could be involved in the pathogenesis of JIA-associated uveitis or in the pathogenesis of chronic noninfectious AU in general; however, its role and the associated mechanism require further investigation.

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References

1. Kanski JJ, Shun-Shin GA. Systemic uveitis syndromes in childhood: an analysis of 340 cases. *Ophthalmology*. 1984; 91:1247–1252.
2. Kalinina Ayuso V, Ten Cate HA, van der DP, et al. Male gender and poor visual outcome in uveitis associated with juvenile idiopathic arthritis. *Am J Ophthalmol*. 2010;149:987–993.
3. Kalinina Ayuso V, Ten Cate HA, van der DP, et al. Male gender as a risk factor for complications in uveitis associated with juvenile idiopathic arthritis. *Am J Ophthalmol*. 2010;149:994–999.
4. Gibson DS, Banha J, Penque D, et al. Diagnostic and prognostic biomarker discovery strategies for autoimmune disorders. *J Proteomics*. 2010;73:1045–1060.
5. Duan X, Lu Q, Xue P, et al. Proteomic analysis of aqueous humor from patients with myopia. *Mol Vis*. 2008;14:370–377.

6. Grus FH, Joachim SC, Pfeiffer N. Proteomics in ocular fluids. *Proteomics Clin Appl*. 2007;1:876–888.
7. Grus FH, Joachim SC, Sandmann S, et al. Transthyretin and complex protein pattern in aqueous humor of patients with primary open-angle glaucoma. *Mol Vis*. 2008;14:1437–1445.
8. Missotten GS, Beijnen JH, Keunen JE, Bonfrer JM. Proteomics in uveal melanoma. *Melanoma Res*. 2003;13:627–629.
9. Bloch-Michel E, Nussenblatt RB. International Uveitis Study Group recommendations for the evaluation of intraocular inflammatory disease. *Am J Ophthalmol*. 1987;103:234–235.
10. Berntson L, Fasth A, Andersson-Gare B, et al. Construct validity of ILAR and EULAR criteria in juvenile idiopathic arthritis: a population based incidence study from the Nordic countries. International League of Associations for Rheumatology. European League Against Rheumatism. *J Rheumatol*. 2001; 28:2737–2743.
11. Standen CL, Perkinton MS, Byers HL, et al. The neuronal adaptor protein Fe65 is phosphorylated by mitogen-activated protein kinase (ERK1/2). *Mol Cell Neurosci*. 2003;24:851–857.
12. Buxbaum JN, Reixach N. Transthyretin: the servant of many masters. *Cell Mol Life Sci*. 2009;66:3095–3101.
13. Fleming CE, Nunes AF, Sousa MM. Transthyretin: more than meets the eye. *Prog Neurobiol*. 2009;89:266–276.
14. Richardson SJ. Evolutionary changes to transthyretin: evolution of transthyretin biosynthesis. *FEBS J*. 2009;276:5342–5356.
15. Kawaji T, Ando Y, Nakamura M, et al. Transthyretin synthesis in rabbit ciliary pigment epithelium. *Exp Eye Res*. 2005;81: 306–312.
16. Chowdhury UR, Madden BJ, Charlesworth MC, Fautsch MP. Proteome analysis of human aqueous humor. *Invest Ophthalmol Vis Sci*. 2010;51:4921–4931.
17. Ramakrishnan S, Sulochana KN, Parikh S, Punitham R. Transthyretin (prealbumin) in eye structures and variation of vitreous-transthyretin in diseases. *Indian J Ophthalmol*. 1999; 47:31–34.
18. Richardson MR, Price MO, Price FW, et al. Proteomic analysis of human aqueous humor using multidimensional protein identification technology. *Mol Vis*. 2009;15:2740–2750.
19. Bouhenni RA, Al SS, Morales J, et al. Identification of differentially expressed proteins in the aqueous humor of primary congenital glaucoma. *Exp Eye Res*. 2011;92:67–75.
20. Duan X, Xue P, Wang N, et al. Proteomic analysis of aqueous humor from patients with primary open angle glaucoma. *Mol Vis*. 2010;16:2839–2846.
21. Shao J, Xin Y, Yao Y. Correlation of misfolded transthyretin in abnormal vitreous and high myopia related ocular pathologies. *Clin Chim Acta*. 2011;412:2117–2121.
22. Ueda M, Misumi Y, Mizuguchi M, et al. SELDI-TOF mass spectrometry evaluation of variant transthyretins for diagnosis and pathogenesis of familial amyloidotic polyneuropathy. *Clin Chem*. 2009;55:1223–1227.
23. Poulsen K, Bahl JM, Tanassi JT, et al. Characterization and stability of transthyretin isoforms in cerebrospinal fluid examined by immunoprecipitation and high-resolution mass spectrometry of intact protein. *Methods*. 2012;56:284–292.
24. Gibson DS, Blelock S, Brockbank S, et al. Proteomic analysis of recurrent joint inflammation in juvenile idiopathic arthritis. *J Proteome Res*. 2006;5:1988–1995.
25. Gibson DS, Finnegan S, Jordan G, et al. Stratification and monitoring of juvenile idiopathic arthritis patients by synovial proteome analysis. *J Proteome Res*. 2009;8:5601–5609.
26. Rosenkranz ME, Wilson DC, Marinov AD, et al. Synovial fluid proteins differentiate between the subtypes of juvenile idiopathic arthritis. *Arthritis Rheum*. 2010;62:1813–1823.
27. Dernouchamps JP. The proteins of the aqueous humour. *Doc Ophthalmol*. 1982;53:193–248.
28. Dias PL. Postinflammatory and malignant protein patterns in aqueous humour. *Br J Ophthalmol*. 1979;63:161–164.
29. Tripathi RC, Millard CB, Tripathi BJ. Protein composition of human aqueous humor: SDS-PAGE analysis of surgical and post-mortem samples. *Exp Eye Res*. 1989;48:117–130.

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